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THE EFFECTS OF HYDROGEN IONS ON THE RESTING MEMBRANE POTENTIAL OF FROG SARTORIUS MUSCLE

By

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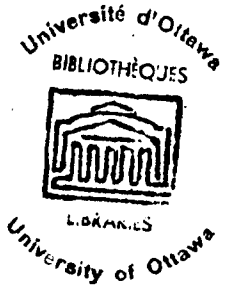

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ABSTRACT

A considerable amount of work has been done on the study of the effect of H^+ ion on the resting membrane potential of frog skeletal muscle fibres and other kinds of tissue as well. The observations, however, are rather contradictory. This may be due to differences in membrane properties of these tissues. The conditions under which the experiments were carried out might also have important effects on the responses as shown in the present paper.

The present study has been carried out in order to establish the effects of H^+ ions on the resting membrane potential of frog sartorius muscle fibres and to investigate the mechanism by which H^+ ions exert their effects.

The results show that in frog sartorius muscle under relatively normal conditions, i.e. in Ringer's solution (Table II), the short term effect of lowering pH was to increase the resting membrane potential, while that of raising pH, to reduce the potential. Under these conditions the change in resting membrane potential is small, usually about 1 - 4 mV. Accompanying the change in resting potential is an equally rapid and reversible change in membrane resistance. Thus, the membrane resistance increases as pH falls. Consideration of the equivalent circuit of cell membrane suggests that the changes in resting membrane potential may result from membrane resistance changes. Since the responses to changes in pH persisted even in the absence of Na^+ the study could be focused on the relative effects of H^+ on K^+ resistance and Cl^- resistance of the membrane.

When the relationship between the electrochemical potentials of the ions inside and outside of the cell is changed, the response to pH could provide some clues in the estimation of the relative effects of H^+ ions on the component resistances of the cell membrane. It has been observed that in muscle fibre with the relation: $E_{Cl} > E_m > E_K$, lowering pH decreased the resting membrane potential. This would occur if H^+ reduced the Cl^- conductance of the membrane, or it decreased Cl^- conductance to a greater extent than K^+ conductance. The interpretation can be fitted into the response of normal cell with the relation: $E_K > E_m > E_{Cl}$, since with a higher Cl^- resistance in acid the influence of E_{Cl} on E_m would decrease. In each case there is a shift of E_m toward E_K. A theoretical consideration using the known interrelation between membrane potential, intracellular Cl^- concentration and the flux of Cl^- and K^+ together with the measured increase in the membrane resistance in lowering the pH confirms that H^+ acts chiefly on Cl^- resistance of the membrane in frog sartorius muscle.

The physiological significance of the response with change in membrane potential to altering extracellular pH is unknown.

CHAPTER I

Introduction

Bioelectrical potentials

Bioelectrical phenomena are characteristic of living organisms. In living tissues, there exists an electrical potential difference between the inside and outside of a cell. In a resting muscle fibre for example, the inside of the cell is electrically negative with respect to the outside. The potential difference between the two sides of the membrane is called the resting potential. In excitable tissues, the magnitude and polarity of the electrical potential alter when the cell becomes activated. The new form of the potential thus attained is known as action potential. The magnitude of either the resting potential or the action potential varies with different tissues or the same tissue of different species as shown in Table I.

1. Generation of resting potential

As to the generation of the resting potential two theories have been proposed. Although they have a point in common, that is, the potential arises from the difference in the ion concentrations on the two sides of the cell (Table I), the two theories differ from each other in how the ion concentration gradients across the cell are caused and maintained. One of the theories stresses the predominant importance of the cell membrane, hence the name membrane theory. The other one, generally known as adsorption theory, suggests that the concentration gradients of the ions across the cell arises from the selective ion-binding ability

TABLE I

Ion contents and electrical potentials of various tissues.

Tissue	Resting Potential mV	Action Potential mV	Spike Height mV	K_o mM/l	K_i mM/l	E_K mV	Na_o mM/l	Na_i mM/l	E_{Na} mV	Cl_o mM/l	Cl_i mM/l	E_{Cl} mV	Ref.
Loligo Axon	-62	+35	104	10	360	-91	460	110	+69	540	83	-54	H
Sepia Axon	-62	+35	120	10	360	-89	460	43	+52	540	-	-	H
Frog sciatic nerve	-71	+40	116	2.5	110	-95	120	37	+30	120	-	-	H
Frog sartorius muscle	-88	+31	119	2.5	115	-98	120	26	+46	120	11	-88	H
Carcinus leg nerve	-71 to -94	-	116	10	230	-85	460	-	-	540	-	-	H
Electrophorus	-80	+50	130	-	-	-	-	-	-	-	-	-	Hoy
Ascaris lumbricoides	-10	-110	120	-	-	-	-	-	-	-	-	-	Cas
Rat Purkinjie fibre	-92	-	-	2.7	140	-105	150	13	+65	160	-	-	H
Skeletal dog muscle	-90	+30	120	2.7	140	-	150	12	-	140	-	-	Hoy
Halicystis (Seaweed)	-70 to -80	-	-	12	6	-	498	557	-	580	603	-	Cas
Hydrodictyon	-	-	-	0.02	763	-	1.3	4.0	-	1.1	553	-	Cas

References: H - Hodgkin (1951)
Hoy - Hoyle (1957)
Cas - Castillo, Del et al (1964)

of the cytoplasm, while the membrane, exhibiting little limitation to ions, is a boundary for the big molecules only. Both of them are based on a considerable body of evidence, but can only be summarized very briefly here.

(a) Membrane theory

The theory originated in 1890 from Ostwald-Bernstein's hypothesis, which proposes that the resting potential, being a form of the diffusion potential, has the characteristic described by the Nernst equation:

$$E_m = \frac{RT}{F} \times \ln \frac{C_i}{C_o} \quad \text{..... (1)}$$

where R represents the gas constant, F, Faraday's constant and T, absolute temperature; C_i and C_o stand for the intra- and extracellular ion concentrations, respectively. E_m designates membrane potential.

In order to explain the maintenance of concentration gradients of ions, Boyle and Conway (1941) suggested that the cell membrane was permeable to K^+ and Cl^+ ions, while impermeable to Na^+ ions. According to them K^+ and Cl^+ ions on both sides of the membrane maintain a Donnan equilibrium as follows:

$$\frac{K_i}{K_o} = \frac{Cl_o}{Cl_i} = \exp (VF/RT) \quad \text{..... (2)}$$

In the case of frog skeletal muscle, the ratio is approximately 50:1. Therefore, the resting potential arises from a Donnan equilibrium. However, it has been found that the impermeability of cell membrane to Na^+ is not absolute. Also, the K^+ and Cl^+ relationship does not follow

strictly the Boyle and Conway equation, but varies to a considerable extent with different conditions, e.g. change in temperature or K^+ concentration etc.

Because of the incompleteness of Boyle and Conway's hypothesis, Hodgkin and Katz (1949) suggested the modified Goldman constant field equation (1943) for the calculation of the resting potential in non-steady cells. The equation takes into consideration the permeability of Na^+ ions.

$$E_m = \frac{RT}{F} \times \ln \frac{P_K K_o + P_{Na} Na_i + P_{Cl} Cl_o}{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i} \quad \ldots\ldots(3)$$

P_K, P_{Cl} and P_{Na} represent the K^+ permeability, Cl^- permeability and Na^+ permeability, respectively. 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 Logigo giant axon it is $P_K : P_{Cl} : P_{Na} = 1 : 0.45 : 0.04$ (Hodgkin 1951).

Under certain conditions P_K becomes sufficiently large as compared with P_{Na} and P_{Cl}, the resting potential will be determined by the ratio of $K_i : K_o$ and the Goldman's constant field equation can be reduced to the form as follows:

$$E_m = \frac{RT}{F} \times \ln \frac{K_i}{K_o} \quad \ldots\ldots\ldots\ldots (4)$$

The theory proposed by Hodgkin and co-workers had been tested by altering extracellular K^+ concentration and comparing the membrane potential measured at each K^+ concentration level with those predicted by the Goldman equation. It has been found that at high K^+ concentration

level the measured membrane potential is in good agreement with that predicted by the equation, while at low extracellular K^+ concentration the membrane potential changes non-linearly. The deviation of the resting potentials from the theoretical values at low K^+ concentration is attributed to the slight permeability to Na^+ ions. Since at low K^+ concentration the contribution of the ion to membrane potential becomes smaller as compared with the total concentration gradient of all participant ions, the Na^+ permeability which has only a negligible effect at normal K^+ concentration, now exerts relatively greater influence upon the membrane potential.

It is clear from equation 3 that, unless all the ions are at equilibrium a change in the permeability to an ion species will result in an alteration in the membrane potential. In the cell in which $E_K > E_m > E_{Cl}$ an increase in K^+ permeability or a decrease in Cl^- permeability, for example, will render the cell more negative inside with respect to the outside.

The membrane theory is based on the assumption that the major monovalent ions, especially K^+ ions, have the same mobility and diffusion coefficient in both cytoplasm and the external fluid. That this is the case has been shown by some workers. Thus, Keynes and Lewis (1951) reported that in crab nerves about 97% of the total K^+ ions were free to exchange with extracellular K^+ and a similar but less exact result was found in frog muscles. Steinbach (1950) observed that muscle protein bound a small amount of Na^+, but almost all K^+ ions in homogenates of the tissue were free to exchange. Similar results have been

found in red blood cells by others, e.g. Raker et al (1950).

Thus, the cell membrane functions as a sieve which allows selective penetration of K^+ and Cl^- ions and a lesser penetration of Na^+ ions. However, this selective permeability itself does not explain the fact that normally the intracellular concentration of K^+ is high, while that of Na^+ is low. In order to account for this fact an active Na^+ transport mechanism has been put forward. This means that the process is energy consuming and the ions are transported against their electrochemical gradients. A considerable body of evidence regarding the mechanism has been obtained, but only some is quoted here.

It has been found that in Sepia giant axon the ratio between the measured passive influx and efflux of Na^+ ions is 61 : 31 (Keynes 1951), while the ratio calculated by assuming that all Na^+ ion movements are passive is approximately 50 : 1. Similarly, in frog sartorius muscle, the measured influx and efflux of Na^+ ions is 13 and 16, respectively (Harris and Burn, 1949). Thus, the ratio of influx to efflux of the ion is about 0.8 :1 but the calculated ratio is about 160 : 1 (Hodgkin, 1951). On the other hand, in both tissues, the observed ratio between influx and efflux of K^+ ions is not far from unity, and therefore, is in good agreement with the calculated ratio. This discrepancy is explainable on the basis of an active Na^+ transport, although Ussing (1947) suggested that it might be due to an exchange diffusion of Na^+ ions.

The resting potential cannot be maintained indefinitely in the absence of metabolism. Gerard (1932) reported the resting potential in frog nerve was decreased by anoxia. A similar effect of anoxia on the resting potential in crustacean nerve was claimed by Cowan (1934). Ling (1952) reported that in frog skeletal muscle the resting potential decreased to zero in 2 or 3 hours upon application of anoxia and iodoacetic acid, while either of these factors applied alone failed to do so.

In the experiment by Hodgkin and Keynes (1955) it was observed that in squid axon about 95% of Na^+ efflux and 90% of K^+ influx were reversibly abolished in the presence of metabolic inhibitors. Furthermore, a drop of temperature from 18° to 0.5° C immediately reduced Na^+ efflux to nearly zero and raising the temperature restored the efflux of the ion rapidly. They also observed that Na^+ efflux was reduced by $^2/_3$ when K^+ ions were removed from the medium.

It has been widely accepted that active Na^+ transport does exist in those tissues which have been studied. A problem which arises from the fact of an active Na^+-pump would be how Na^+ and K^+ ion transports are related to each other and how intimate is the relation between the active process and resting potential in a cell. According to some workers, the active Na^+ efflux and K^+ influx are closely coupled. Thus, one Na^+ ion is extruded in exchange for a K^+ ion, hence the term neutral Na^+-K^+ pump. However, from the above mentioned finding and many others, it seems the link between Na^+ efflux and K^+ influx is not equally rigid

in all tissues. Recently, an electrogenic Na^+ pump in mammalian muscle and nerve has been proposed (Page and Storm, 1965; Adrian and Glayman, 1966; Ritchie, 1967). According to them it is the Na^+ ions which are extruded by the active process while K^+ ions move in accordance with the potential gradient produced by the active Na^+ transport.

Although the exact mechanism of the active transport of ions is far from completely understood, it is becoming clear that enzymatic processes are involved in the active ion transport. Probably the ion carrier itself in the active transport is an enzyme system of phosphoprotein in nature (Skou, 1965) which catalyzes the chemical processes. In fact, there are a number of features which are common to both the enzyme system extracted from cell membrane homogenates and active Na^+ transport in intact cells. First, both require the presence of Na^+ and K^+ ions. Secondly, competitive inhibition of Na^+ activation by K^+ and of K^+ activation by Na^+ have been observed in both cases. Thirdly, both are sensitive to some cardiac glycosides, e.g. ouabain. In addition, it has been found that in the enzyme system Mg^{++} is essential for its activity, while Ca^{++} has inhibitory effects on the enzyme. Now it is accepted by most that ATP is the direct source of the energy utilized in the process. The energy-rich substance is also the substrate of the enzyme system. The failure of application of metabolic inhibitors to bring about an immediate change in ion concentrations and a drastic drop in resting potential, may be due to the stored ATP. On the other hand, the relatively rapid effect of the change in

temperature of application of ouabain on the Na^+ pump may be attributed to the direct action of these factors upon the enzyme system.

To summarize, according to the membrane theory, both the active transport and selective permeability of the cell membrane are responsible for the generation of the resting potential. Since the active transport is carried on in a definite direction, that is, Na^+ ions are extruded from, while K^+ ions are drawn into the cell, this will result in a development of the concentration gradients and provide potential energy for the passive movements of these ions. However, the active transport process itself does not separate charges (if the process is in the strict sense of neutral Na^+-K^+ exchange). It is the selective permeability of the cell membrane which controls the passive fluxes of these ions and helps separate the charges on both sides of the cell. If the membrane were equally permeable to Na^+ and K^+, the passive fluxes of these ions down their concentration gradients would be the same, and no separation of charges would be possible. As to the Cl^- ions, although an active Cl^- transport in squid axon has been suggested (Keynes, 1963), generally Cl^- ions move across the membrane passively and in accord with the potential generated by the separation of charges.

If one imagines the concentration gradients of K^+, Na^+ and Cl^- across the cell membrane to be batteries, each having the e.m.f. equal to their equilibrium potential and being connected in series with a resistor representing the resistance offered by the membrane to each ion species and that the major portion of the membrane acts as a

capacitor, then the characteristics of the membrane can be described by applying Kirchoffs Laws. An equivalent circuit representing a resting membrane is illustrated in Fig. 1. (Hodgkin and Huxley, 1952). The values on the diagram apply to frog skeletal muscle.

Also one can imagine that the batteries are being charged continuously through the active transport process at the expense of metabolic energy. In doing so, the voltages of these batteries are maintained constant.

Perhaps it is appropriate here to discuss briefly several points which are closely associated with the membrane theory:

(i) Nature of cell membrane -

Since the bimolecular layer model of the cell membrane was suggested by Danielli and Davson (1935), more and more evidence has been obtained in favour of it by means of direct methods. According to the study with electromicroscope, 3 or sometimes 5 layers of different density in electronmicrograph can be identified.

It is generally accepted that cell membrane consists of a bimolecular layer of lipid which is sandwiched on both sides by non-lipid layers, most probably protein or polysaccharide in nature. The molecules of the lipid layer are arranged in such a way that the polar groups are placed near the outer and inner surfaces of the membrane, while the long water-repulsive hydrocarbon chains are located in the middle. It is the lipid layer from which the resistance to the passage of inorganic ions arises.

Fig. 1

The equivalent circuit of resting membrane of frog skeletal muscle.

R_K, R_{Cl} and R_{Na} represent the membrane resistances to K^+, Cl^- and Na^+ ions, respectively. E_K, E_{Cl} and E_{Na} are equilibrium potentials of K^+, Cl^- and Na^+, respectively. C_m stands for the membrane capacitance. R_i represents the cytoplasmic resistance, while R_o, the resistance of the external medium.

References:

(a) Shanes (1958)

(b) Hodgkin and Horowicz (1959)

(c) Horowicz (1960)

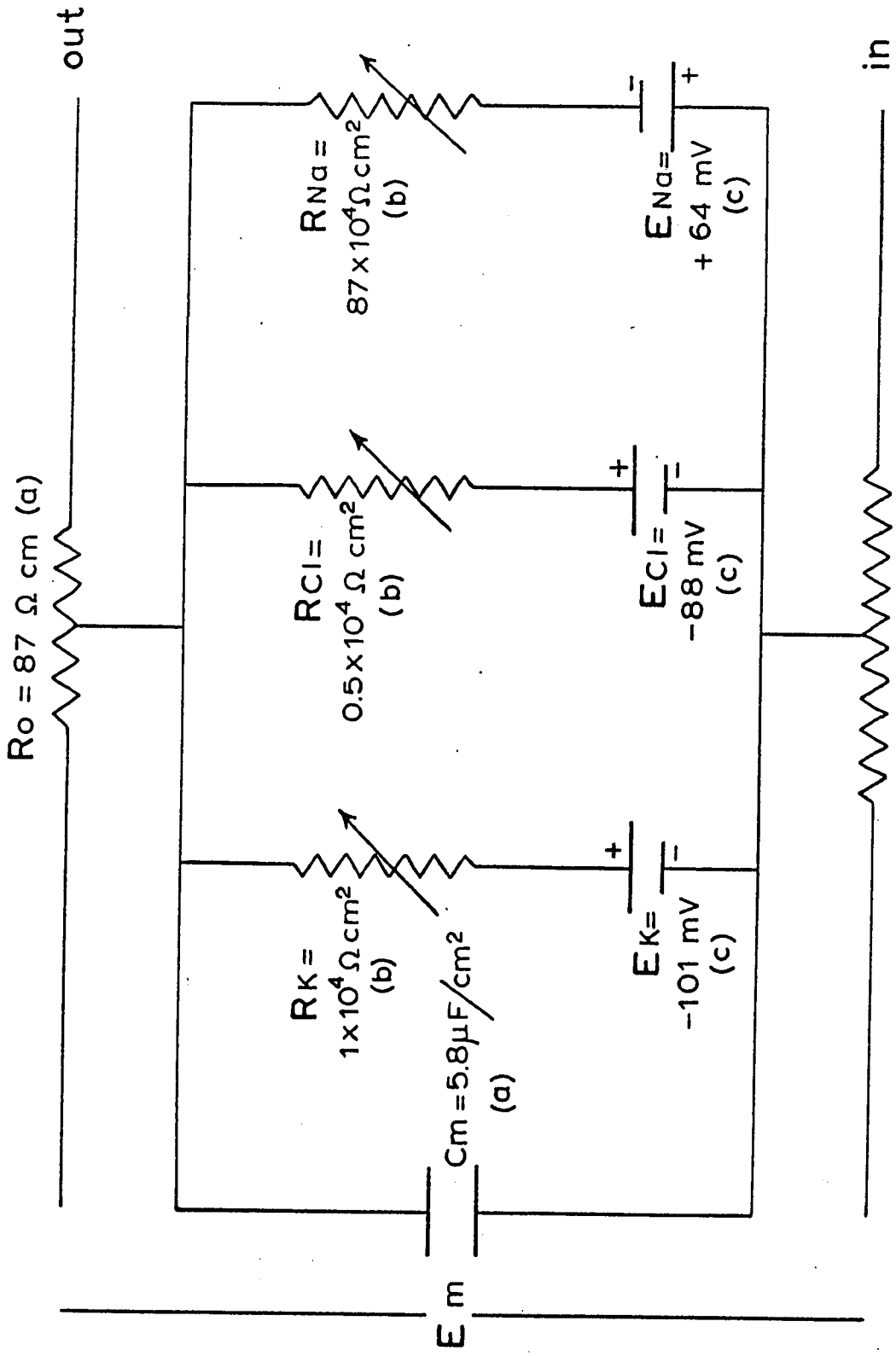

out
in
Ro = 87 Ω cm (a)
Ri = 250 Ω cm (a)
RNa = 87 x 10⁴ Ω cm² (b)
ENa = + 64 mV (c)
RCl = 0.5 x 10⁴ Ω cm² (b)
ECl = -88 mV (c)
RK = 1 x 10⁴ Ω cm² (b)
EK = -101 mV (c)
Cm = 5.8 μF/cm² (a)
Em

fig. 1

In order to explain the passage of inorganic ions through cell membrane, it has been suggested that the membrane is perforated by channels. Whether the channels represent permanent or temporary openings or simply the intermolecular spaces is to be further clarified. However, it is thought that thermal-dynamic agitation, e.g. Brownian movement, may happen from time to time in the lipid layer, of which the molecules are otherwise tightly bound. During the agitation the lipid molecules may oscillate in two directions, i.e. either move in the plane of the membrane or from one layer to the other. As a result of this thermal agitation, temporary pores extending through the membrane may form (Widdas, 1963).

By calculation, in squid axon about $2 \times 10^{-6}\%$ of the total surface area of the cell membrane is occupied by the channels. This area increases to $8 \times 10^{-5}\%$ during excitation of the tissue (Shanes, 1958). The membrane resistance is attributed to the portion of the cell membrane which is occupied by the channels, while membrane capacitance depends on the interchannel spaces of the membrane.

It has been suggested by Mullin (1956) that the pore radius in muscle fibre is of the order of 4.05 Å. In red blood cells the pore is about 4.2 Å in radius. By calculation Boyle and Conway (1941) proposed the pore radius to be about 20% greater than the radius of the hydrated K^+ ion but smaller than that of the hydrated Na^+ ion.

It has been suggested by some workers (Michaelis, 1925; Teorell, 1935, 1953) that there are charges on the walls along the channels, some being negative, others positive. Cations pass through the membrane along the negatively charged channels, while anions, along the positively charged channels.

(ii) Passive movement of ions across the cell membrane -

It is generally accepted that the small inorganic ions pass across the membrane through the water-filled channels. It seems to be the case that cations entering the channel depends on the hydration energy as well as the crystal ionic radius. But in the case of anions of which the crystal ionic radii are larger than those of cations, the penetration across the membrane depends on their naked radii.

That the ions which enter the membrane pores interact with the membrane component has been suggested by Mullin (1956). According to this solvation hypothesis, if the pore size is smaller or slightly larger than the hydrated ion, penetration of the ion would be equally difficult. In the case of smaller pore size and larger hydrated ion radius, the reason is clear, while in the reverse situation the difficulty arises because the requirement of the ion for the solvation beyond a specific hydration level would not be compensated by the solvation between the ion and the wall. Also implied by the solvation hypothesis is that changes in permeability may result from alteration in the size of the channels and/or from the development of the solvation which thereby, reduces the energy barrier to entry. In addition

to the limitation set by the sizes of the channels and hydrated ions, alteration in the fixed charges on the membrane may also influence ion permeability (Meyer and Sievers, 1936; Manery, 1966). If the membrane takes on positive charges it may become more permeable to certain anions. However, how membrane permeability changes under various conditions is still an unsolved mystery.

(b) Adsorption theory

In contrast to the membrane theory the adsorption theory suggests that the cytoplasm plays the major role in the K^+ ion accumulation in living cells. In principal, the theory suggests that the selective retention of K^+ ions is due to the reversible binding of the ions with protein or other macromolecules. Troshin (1960) claims that all water in cytoplasm is in bound form and the solubility in it is much lower than in extracellular solutions. Consequently, only a small fraction of the cellular K^+ ions is free, while the majority are bound. According to Ling's (1952) earlier suggestion, the chance of an ion to be bound with a fixed charge depends on the hydration energy of the ion. K^+ ions have greater tendencies to be bound than Na^+ ions because hydrated K^+ ions are smaller than hydrated Na^+ ions.

On the other hand, the readiness of the binding depends on the physicochemical properties of the macromolecules at a given moment. 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^+. Similar findings

have been obtained by Ungar and co-worker (1960). They found that in the extract of rat cerebral cortex selective retention of K^+ and Na^+ occurred in the complete absence of cell membrane and that ion retention ability of the membrane free extracts varied with the states of the tissue. Thus, Na^+ diffused at a faster rate from resting brain extracts than from active brain and K^+ left the excited brain extracts faster than the resting brain.

By using radioactive isotopes in the study of ion exchanges between both sides of the cell, some workers (Simon et al, 1959; Simon 1959) have come to the conclusion that there are two phases inside the cell. One is called free intracellular phase, the other, the ordered phase. In the free intracellular phase, tentatively, the endoplasmic reticulum, K^+ and Na^+ concentrations are about the same as in the extracellular solution and free exchange of ions between this phase and the outside is possible. But in the ordered phase the macromolecular electrolytes are at high concentration levels. It is the ordered phase which plays the major role in the selective retention of certain ions under a given condition.

Recently, modified forms of adsorption theory have been proposed. First, Ling (1960) claims that instead of the bulk concentration of K^+ ions, the concentration of surface-fixed anionic charges and the affinity of the anionic groups for the cations are the determining factors of the resting potential. Secondly, Kurella (1960) confirms the pre-existing potential in intact cells. He claims that in cytoplasm

ions are bound to the polyelectrolytes by the ionic bond, but not by covalent bonds. As a result the ions remain electrochemically active, i.e. they behave nearly as in aqueous solutions. The concentration gradients of ions across the membrane, therefore, set up potential difference on both sides of the cell. On this point Kurella's suggestion is similar to the membrane theory but differs from the original form of the adsorption theory which argues against the pre-existing potential and claims that resting potential is nothing but an artifact arising from the method of measurement.

2. Measurement of membrane potential

A tremendous amount of work has been done in the measurement of the bioelectrical potentials. Because of the difficulties inherent in the study itself, the values of the bioelectrical potentials can only be considered approximate to those in the intact organism. Among the difficulties, some arise from the living tissues themselves, while the others are associated with the methods. The errors thus produced can only be eliminated to a certain extent.

The methods which are employed in measuring cell potential can be put into two groups as follows:

(a) Extracellular electrode methods -

The idea of this method is to measure the potential difference between two regions of the surface of a cell or tissue. Regions under different conditions or receiving different treatments show a difference in potential. The electrical potentials measured by the extracellular

electrode method fall into two groups:

(i) Injury potential - It is the potential difference between an intact region and a cut end of the same tissue.

(ii) Demarcation potential - The term designates the potential of a treated intact region relative to another intact region which remains untreated and serves as a reference. For instance, the potential difference between the regions of a nerve fibre soaked in K^+ rich solution and in normal Ringer's solution.

One of the most serious drawbacks of this method is the reduction of potential due to the short-circuiting in the extracellular electrolyte solution. This, however, can be overcome by greatly increasing the extracellular resistance. Since both potentials are actually a function of the extracellular resistance and current flowing longitudinally outside the cell, and an intact cell can be considered as a circuit of three resistors in series, i.e. extracellular, membrane and protoplasmic resistors, the membrane potential actually measured depends on the ratio of the extracellular resistance to the total resistance. It is clear that any change in the extracellular or intracellular resistance will result in an alteration in the magnitude of the potential change.

(b) Intracellular electrode method -

In order to obtain a direct measure of the resting potential or other electrical events in a cell, intracellular electrode method has proved to be more satisfactory than the first one. In brief, the

intracellular electrode method consists of the insertion of a tiny glass pippet filled with electrolyte solution, while another electrode is placed in the outside of the cell as reference. This method requires that the intracellular electrode be so tiny that the damage of the membrane due to the insertion of the electrode must be negligible and that the insertion must not affect the normal electrical activities of the cell. Besides, there are other limitations in the application of the method. Usually errors may arise from the following sources:

(i) Error due to the diffusion potential of the electrode inserted in the cell -

It has been found that KCl or NaCl may diffuse from the insertion electrode. If this is the case and the cation and anion diffuse at different rates a diffusion potential is developed. The potential thus produced may reduce the measured potential by several mV, even if highly concentrated electrolyte solutions are used. It has been claimed that theoretically 3M KCl may give a diffusion potential of 5 mV, while 0.1 m KCl gives 22 mV. The diffusion potentials may cause error in the direction of reducing the observed membrane potential (Ling and Gerard, 1949; Shanes, 1958).

(ii) Error may arise from charges developed on the glass or from the blocking of the tip when the electrode is in the cell. These exaggerated the difference between K^+ ion and Cl^- ion mobilities and the diffusion potential thus produced.

3. Intracellular pH

Since in the experiments presented in this paper extracellular pH was changed over a considerable range, it is useful to discuss briefly the information regarding the intracellular pH in muscle fibres.

Several methods had been used in the measurement of the intracellular pH (Caldwell, 1956). Of these methods two are still employed by the recent workers. They include:

(a) The method in which a weak acid or base is used. The intracellular pH is calculated from both the amount of the acid or base in the cell or tissue extract and that of its corresponding ions. The calculation makes use of the Henderson equation:

$$pH = pK_a + \log_{10} (A^-/HA) \quad \ldots\ldots\ldots\ldots\ldots (5)$$

Fenn (1928), Stella (1929) and Conway and Fearon (1944) etc, used CO_2 - HCO_3^- in the study of intracellular pH in muscles of different species. NH_3 - NH_4OH was used by Netter (1934). Recently Waddel and Butler (1959) described the method in which DMO (5,5-dimethyl-2,4,-exazolidinedione), a weak acid, is used. More recently this method was employed in the study of the intracellular pH in mammalian skeletal muscle (Adler, et al, 1965).

(b) Use of H^+ ion sensitive glass microelectrode. The technique consists of the impalement of a H^+ ion sensitive glass microelectrode into a cell and a reference electrode in the bathing fluid. It is the part of the microelectrode, usually a few μ in length, exposed to the surrounding fluid, while the rest is insulated. One of the drawbacks in

the application of this technique is the uncertainty that all the unsealed portions of the electrode is in the inside of the cell. If part of the unsealed electrode is in the outside, the observed pH changes would not represent those in the cell. However, with this technique the response with changes in intracellular pH to the changes in the external the medium can be followed continuously. (Caldwell, 1954, 1956, 1958; Kostyuk and Sorokina, 1960; Carter et al, 1967).

The values of intracellular pH in muscle of various species observed by most of the workers fall in a rather narrow range, i.e. between 6 and 7. Thus, using microelectrode method Caldwell (1958), reported that intracellular pH in crab muscle was very close to 7. By similar methods Kostyuk and Sorokina (1960) observed the intracellular pH in frog sartorius muscle to be 7.1. Employing DMO method Adler, et al (1965) got similar intracellular pH values in rat diaphragm, i.e. 6.9. These values differ considerably from that obtained by Conway and Fearon (1949). The latter workers reported the intracellular pH in rabbit skeletal muscle to be 6.

Again, differing from those old data, recent studies show that intracellular pH is relatively constant despite a rather big change in extracellular pH. Thus, in frog sartorii, changes in extracellular in the range of 5 - 10 cause only a slight change in intracellular pH, i.e. less than 0.5 pH unit. However, when CO_2 is used as acidifying agent there is rapid drop in the intracellular pH. In the application of one atmosphere of CO_2, for example, the intracellular pH decreases

from the initial value 7 to 6.4 at equilibrium, even though the extracellular pH is maintained at 7.3. Most of the recent studies have come to the conclusion that the intracellular pH changes in the same direction as the extracellular pH; the change in pH inside the cell is reversible and is much slower (except in the case of CO_2) and to a much lesser extent as compared with that in the outside. These behaviours of the changes in intracellular pH are the same in various muscles.

The mechanism which underlies the relation between intra- and extracellular pH values is not fully understood. Conway and co-workers, considering their measured intracellular value of 6 in rabbit muscle, had suggested that the relation between the intracellular and extracellular pH values and the resting potential might be expressed by the equation as follows:

$$pH_i - pH_o = \log \frac{H_o^+ \text{ activity}}{H_i^+ \text{ activity}}$$

or

$$= \frac{\text{Resting potential}}{58} \qquad \text{.....(6)}$$

However, in the experiments by others (Caldwell, 1958; Kowtyuk and Sorokina, 1960; Adler, et al, 1965) a remarkable discrepancy has been observed between the measured intracellular pH levels and the theoretical values predicted by the Conway equation. The discrepancy became more obvious as the extracellular pH levels were made much higher or lower than normal. These results seem to lend a strong support to the hypothesis of the active H^+ ion transport proposed by Fenn

and Cobb (1934), Fenn and Mauerer (1935).

However, that metabolic inhibitors failed to change the intracellular pH appears to Kostyuk and Sorokina (1960) to be the argument against the active H^+ ion transport mechanism in muscle. According to them the intracellular buffering systems play the major part in the maintenance of the relatively constant intracellular pH in muscle.

4. Hydrogen ion concentration and cell activity

The study on the action of H^+ ion and CO_2 upon the activities of the living tissues has been closely studied for a long time. This is not surprising since CO_2 is one of the end products in the metabolic processes which takes place in all living things and the rate of production of the gas or the ability to eliminate this end product may result in alteration of the H^+ ion concentration. Because of the intimate relationship between CO_2 and H^+ ion concentration, at least in the living tissues, the following questions arise: What effects to CO_2 and H^+ ions have on the living tissues? Is CO_2 or H^+ ion alone responsible for the observed phenomena? Do these factors exert their effects by the same mechanism, or does each have its own mode of action?

(a) Effects of H^+ and CO_2 on the excitable tissues

The earliest recorded observations on the effects of CO_2 on nerves were made by Waller (1895) and the effects of the gas on voluntary and cardiac muscle by Waller and Sowton (1896). In the cutaneous pectoris of frog, Haywood (1927) found that CO_2 produced

inexcitability which could not be imitated by non-penetrating acid, e.g. HCl. But according to Horton (1929), the difference between the effect of CO_2 and that of the H^+ concentration was the time required for these factors to exert their action; there was no specific chemical action of the gas. Gasser's (1933) experimental results showed that the action spike of the axon was strongly pH sensitive. Similar findings were reported by Lehmann (1937). He showed that in mammalian A nerve fibres, the threshold of excitation could be altered by changing the pH of the medium. Using rabbit atrium, Vaughan Williams (1955) found the conduction velocity was affected by the changes in PCO_2, that is, the higher the PCO_2 the faster the conduction velocity. Since the conduction velocity is closely associated with the changes in the electrical properties of the cell membrane of excitable tissues, it is reasonable to suspect that PCO_2 affects the electrical property of the cell membrane of atrial muscle fibres. But on the other hand, McElroy et al (1958) claimed that the changes in H^+ ion concentration rather than the specific action of CO_2 or HCO_3^- concentration was responsible for the changes in cardiac activities : heart rate, contractility etc., at least in the isolated guinea pig heart.

(b) Effect of H^+ ions and CO_2 on the electrical activities of the excitable tissues

Even before the use of the microelectrode technique in the study of electro-physiology, studies had been made on the effects of CO_2 and the H^+ ions on the electrical activities of nerves and muscles.

The experiments by Necheles and Gerard (1930) demonstrated that exposure to CO_2 caused a sharp and brief rise in resting potential in the frog sciatic nerve. This was followed by a great and maintained fall in the potential. On replacing CO_2 by O_2 there was a further brief drop and then a gradual rise to a new level, which was usually lower than the original. The response was observed for CO_2 concentration from 10 to 100% and was reversible except in extreme concentration, e.g. 100%. Necheles and Gerard's findings were confirmed by Shanes (1948). He found that 5% CO_2 produced a rapid initial rise which was followed by a slow fall in the demarcation potential in frog sciatic nerves. He also observed that the response was modified by the K^+ concentration in the medium. In the absence of K^+ from the medium there was little response to the CO_2.

Since the introduction of the microelectrode technique to the study of biology and later the improvement in the application of this technique, workers in cell physiology have been able to investigate the electrical activities in the individual cells. This has provided a better insight into the action of their factors.

Using improved glass microelectrodes Ling and Gerard (1949) measured directly the changes in membrane potential of frog sartorius fibres in response to a rise or fall of PCO_2 or to the changes in H^+ concentration. Their results showed that 5% CO_2 caused a fall in resting potential by 10 - 15 mV and higher CO_2%, e.g. 20 - 100% depolarized the muscle fibres by about 25 mV in 30 minutes. The responses were reversible

when the application of CO_2 was terminated. On the other hand, the resting potential was found relatively unaffected by the changes in pH of medium between 5 - 10 by using buffer systems other than CO_2 - HCO_3^-. There was a slight rise with time when pH was increased up to or above 10, and a rapid fall in resting potential at pH 4.5. The resting potential became zero at pH 3.0. Even at this pH level the depolarization was still partly reversible if pH was raised.

Coraboeuf and Boistel (1957) showed that in the ganglionic chains of insect CO_2 (100%) caused an immediate fall of the resting potential by about 7 mV, that is, from -68 mV to -55 mV, in 25 seconds. Action potential in this tissue was also decreased by the gas. They also noted that the effect of CO_2 varied with the K^+ concentration in the bathing fluid.

The experimental study by Meves and Völkner (1958) showed that CO_2 had both depolarizing and hyperpolarizing action in skeletal muscles, depending on the original level of the resting potential. In the case of high resting potential, i.e. greater than 90 mV, CO_2 depolarized the muscle fibre, while at low resting potentials, CO_2 had the opposite effect. They also found that changes in pH by other buffers had similar effects to those of CO_2.

Crab muscle fibres were found sensitive to changes in CO_2% (Caldwell, 1958). The resting potential in this tissue decreased from -51 mV to -27 mV and from -55 mV to -32 mV in 7 - 15 minutes when the bathing fluid (sea water) was saturated with CO_2 (pH 4.98). On the

other hand, changes in pH with other acids had only little effects on the resting potential.

In the experiment by Kostyuk and Sorokina (1960) similar effects of CO_2 and H^+ ions on frog sartorii were also observed. Thus, when the bathing solution was saturated with CO_2 at a pressure of one atmosphere there was a drop of resting potential from -83 mV to -52 mV in about 25 minutes. A decrease in pH with other acidifying agents had a definite although small depolarizing action, but an increase in pH to 9 - 10 caused slight hyperpolarization. In their work much attention was paid to the relation between the changes in resting potential and in the intracellular K^+ concentration as pH of the bathing solutions or PCO_2 was altered.

Hecht and Hutter (1963) reported a small depolarization of 2 - 3 mV in sheep cardiac Purkinje fibres on raising the extracellular pH, while a lowered pH caused a slight hyperpolarization in Na - Ringer's solution. In their work as well as in those experiments by Brooks and Hutter (1962, 1964), studies on the effects of H^+ ion concentration on the membrane conductance were carried out.

When exposed to an increased PCO_2, the viseral ganglia of Aphysia fasciata were depolarized rapidly (Chalazonitis, 1963). A depolarization of about 4 - 5 mV resulted in spontaneous firing. But a longer exposure had narcotic effects, i.e. excessive depolarization, decrease in the height and rate of the rising and falling phases of the spikes and finally the activity being abolished. As in other

tissues, the response to CO_2 was found to be reversible.

The usual hyperpolarizing action of CO_2 on the nervous tissues was also observed in cat cortical neurons. Krnjevic et al (1965) showed that the resting potential in this tissue was increased by 1 - 5 mV with a rise of PCO_2 from 4% to 20% or 30%. The response could be repeated several times.

In Harris' experiments (1963, 1965), it was found that the resting potential in frog sartorii soaked in K^+ rich Ringer's solution was depolarized from -36 mV (pH 7.3) to -24 mV (pH 6.3) when acidified solution (by CO_2) was admitted.

Using $KHCO_3$ or $NaHCO_3$ buffered isotonic sucrose solution, Mainwood (1965) found that the resting potential in frog sartorii decreased by 7.1 - 16.5 mV when the PCO_2 was raised to 20% (pH 6) and by 9.5 - 34.5 mV when PCO_2 was raised to 50% (pH 5.8). Usually these responses were not obvious at the beginning of the sucrose washing period. Perfusing the muscles with phosphate buffer Ringer's solutions of the corresponding pH values only caused slight changes in the resting potential. Furthermore, the K^+ content of the muscle soaked in K-phosphate buffered sucrose solution was found relatively constant in the first 20 minutes and then decreased very slightly. The intracellular Cl^- concentration decreased very rapidly in the first 20 minutes then remained relatively constant.

Because of the fact that H^+ ions and CO_2 have effects on various tissues and that cells respond in different ways under different

conditions, a number of hypotheses have been put forward to account for the actions of the two agents. Some of the proposed mechanisms will be discussed in the last part of the paper.

To summarize, it is apparent that although much work has been done the results are still rather contradictory. It seems that both H^+ ions and PCO_2 have effects on the membrane potential of muscle fibre, or other excitable tissues and that several monovalent ion species may be involved in the response. The present study has been carried out in order to clarify the problems.

The response with changes in E_m to changes in pH or PCO_2 seems to be chiefly the phenomena associated with the cell membrane. Since it has been suggested that the membrane permeability to ions is changed by these factors, one of the aims of the present study is to investigate quantitatively to what extent each ion species and its permeability would contribute to the observed E_m alteration.

5. <u>The relationship between the effects of H^+ on the membrane potential and mechanical activity</u>

According to Sandow and co-workers (1965), it is the position of action potential in between the mechanical threshold and saturation potential levels which affects the Ca^+ release and excitation-contraction coupling in skeletal muscle. In other words, the effect of any factor on muscle contraction depends on its action upon the height and duration of the mechanical threshold potential and the saturation potential level. The suggestion has been pushed a little further. Thus, it

has been claimed that the substances so far investigated exert their influence on the mechanical activity of muscle in two possible ways. One is the general effect on the surface membrane, that is, by actig on the membrane resistance and altering the duration of negative after-potential. The other is a specific effect on the E-C coupling or Ca^{++}-binding capacity of the relaxing factor in the membrane of transverse tubular system. In some cases the two effects are well separated while in others it is rather difficult to distinguish between the two.

In the substitution of NO_3^-, Br^-, I^-, $CH_3SO_4^-$ or $SO_4^=$ etc, for Cl^-, for example, the membrane resistance of frog skeletal muscle is increased, action potential prolonged and twitch tension potentiated (Hutter and Padsha, 1959; Hutter and Noble, 1960; Mashima and Washio, 1965). Here the general effect is due to the hindrance of the passage of Cl^- through the membrane and the consequent increase in membrane resistance. The specific effect is indicated by the fact that the twitch potentiation produced is more than expected from the prolongation of action potential, or from the increase in membrane resistance, especially in the case of SCN substitution.

The separation of the general and specific effects can also be observed in the substitution of some cations for Na^+ in Ringer's solutions. In the case of TEA (tetraethylammonium) substitution, for example, there is a slight change in membrane resistance. The spike height decreases with the concentration of TEA and action potential prolongs. However, the twitch potentiation is not as much as might be expected from

the prolongation of action potential. The separate effects can also be shown in NH_4^+ substitution. Ten minutes after soaking in 50% NH_4^+ solution the membrane resistance attains a steady value which is slightly lower than the normal level, while the twitch tension decreases continuously with lengthening in time of soaking in the solution (Mashima and Washio, 1965).

It seems that in addition to the surface effect on Cl^- permeability, NO_3^-, Br^-, I^-, SCN^- or $SO_4^=$ etc. anions inhibit the Ca-binding capacity of the relaxing factor after they get into the tubular system of muscle fibre. On the other hand, since the membrane of transverse tubular system of frog skeletal muscle is permeable to these cations, they may act directly on the E-C coupling, once they pass across the membrane of TTS and enter the sarcoplasm. Probably, NH_4^+ depresses, while TEA augments the E-C coupling in frog skeletal muscle.

As will be discussed in detail later, H^+ ions increase the membrane resistance, most probably the Cl^- resistance, in muscles. This may account for the prolongation of action potential in cardiac muscle fibre soaked in acid. However, the change in the duration of action potential in muscle soaked in acid is in the direction opposite to what would be expected from the working model suggested by Sandow et al. Therefore, it is suggestive that other mechanisms may be involved.

6. Objectives and significance of the study

Studies on the effect of H^+ on membrane potential have been carried out by other workers. However, the results are rather contradictory.

It seems that the inconsistance might have arisen from differences in the membrane properties of the cell types investigated. Also, the condition under which the experiments were performed might have a profound influence upon the response. Therefore, the present study was designed to clarify the following points:

(i) The effects H^+ ions have on the membrane potential and membrane resistance in frog sartorius muscle.

(ii) The dependence of the response to pH alteration on the electrochemical gradients of ions across the cell membrane.

(iii) The mechanism by which the H^+ exerts its effect.

Since it has been suggested that the charged groups on cell membranes may play an important part in affecting the electrical property of cell membrane, the study of the effect of H^+ on the resting potential may throw some light on the nature of the charged groups and the structure of the membrane. Also, it may lead to a better insight into the mechanism by which ions pass across the cell membrane.

CHAPTER II

Methods

1. Preparation of muscle

Sartorius muscles of Rana pipiens were used in all experiments. The unanaesthetized frog was killed by cutting off the head; the spinal cord was destroyed and the muscles were disected, each being tied by cotton thread onto a Plexiglas frame as shown in Fig. 2a. The muscle and frame were placed in a bath made of Plexiglas filled with Ringer's solution. The muscle was stretched over rods in the bath to keep the muscle steady in the flowing solution. Connective tissue on the inner surface of the muscle was removed under a dissecting microscope.

The muscle thus prepared was used in the experiment immediately or kept at low temperature (5^{o} - 7^{o} C) overnight.

2. Preparation of solutions

All solutions used in the experiments were isotonic. They were freshly made of deionized, distilled water. In making solutions $CaCl_2$ was added very slowly and the solutions were shaken continuously in order to prevent the precipitation of Ca^{++} ions. The composition of the solutions is shown in Table II.

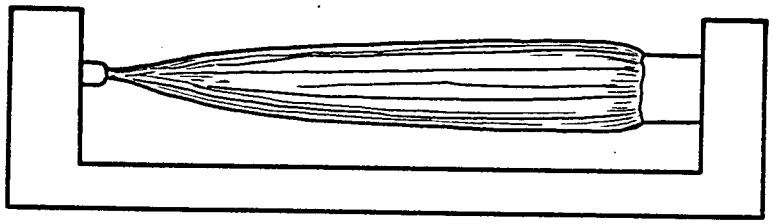
In the case of bicarbonate buffered Ringer's solutions, 2%, 20% and 50% CO_2 in O_2 was used to obtain the desired pH levels, i.e. 7, 6 and 5.8, respectively. All the phosphate buffered Ringer's solutions were gassed by pure O_2 only. In both cases, 1 - $1^{1}/2$ hours were allowed for gassing.

Fig. 2a.

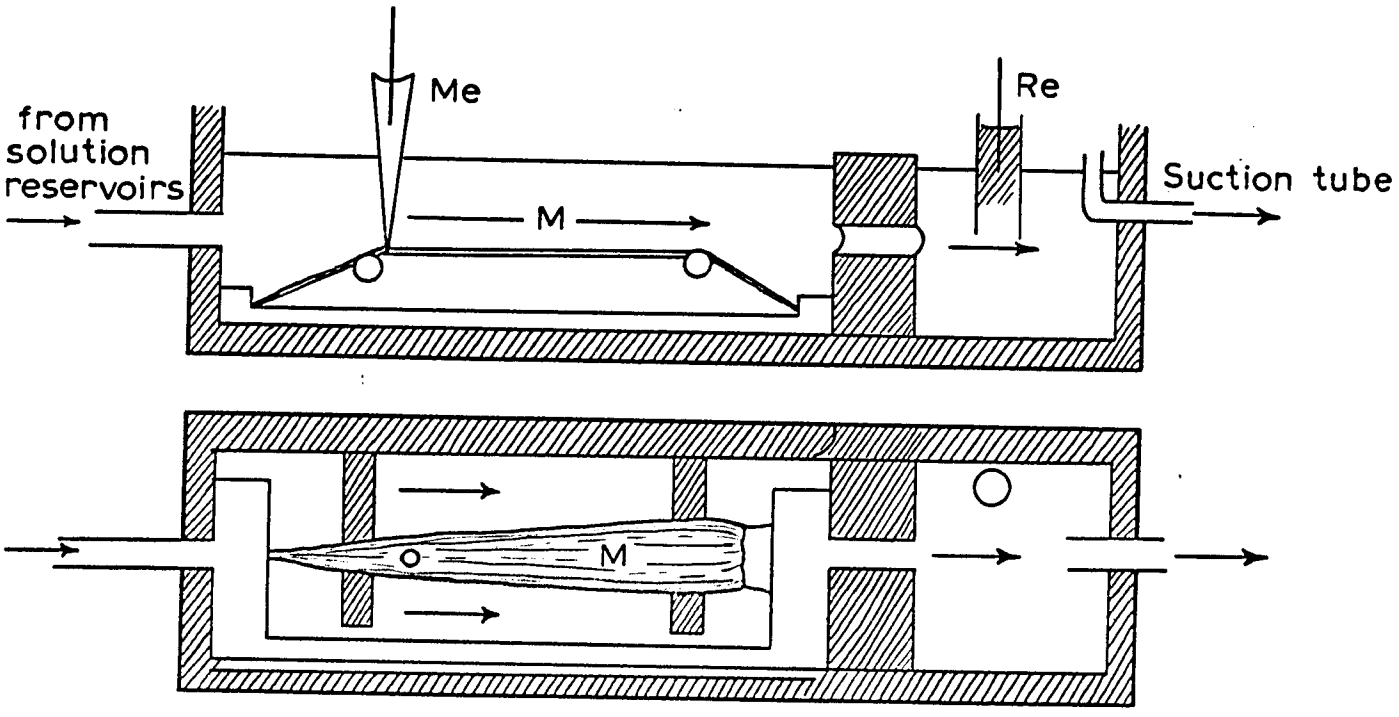
Muscle mounted on a Plexiglas frame.

Fig. 2b.

Muscle in perfusion bath. M - muscle mounted on a frame. Me - microelectrode. Re - reference electrode. The arrows indicate the direction in which solution flows.



a



b

fig. 2

TABLE II

Composition of Solutions (mM)

Solution	K^+	Na^+	Ca^{++}	Choline	Cl^-	HCO_3^-	HPO=	H_2PO_4	$CH_3SO_4^-$	pH
A	2.5	120	1.25	-	120	5	-	-	-	7, 6 or 5.8
B	2.5	120.2	1.25	-	120	-	2.16	0.84	-	7
C	2.5	119.9	1.25	-	120	-	0.76	3.4	-	6
D	2.5	-	1.25	120	120	5	-	-	-	7, 6 or 5.8
E	10	112.7	1.25	-	120	-	2.16	0.84	-	7
F	10	112.4	1.25	-	120	-	0.76	3.4	-	6
G	2.5	5.2	1.25	115	120	-	2.16	0.84	-	7
H	2.5	4.9	1.25	115	120	-	0.76	3.4	-	6
I	10	5.2	1.25	107.5	120	-	2.16	0.84	-	7
J	10	4.9	1.25	107.5	120	-	0.76	3.4	-	6
K	2.5	120.2	1.25	-	60	-	2.16	0.84	60	7
L	2.5	119.9	1.25	-	60	-	0.76	3.4	60	6

The pH values of solutions were measured with a Radiometer pH meter. This was done either right after gassing or at the end of experiments. Sometimes they were checked twice, i.e. before and at the end of the experiment. It has been found that the changes in pH value after a $1^1/2$ hour experiment were less than 0.2 unit. This was true even in the case of bicarbonate buffered solutions. The temperature of the solutions used in perfusion was between 24^o - 27^o C.

3. Preparation of electrodes

Glass capillary tubing (Series No. 12-141, supplied by Fisher Scientific Co. Canada) of 1 mm diameter was pulled into microelectrodes by using a microelectrode puller (two kinds of microelectrode pullers were used . One was similar to that described by Winsbury; the other was produced by Narishige Scientific Instrument Laboratory, Tokyo, Japan). The tip of the electrodes was checked from time to time under a light microscope. This inspection indicated that the tip of the electrode was always smaller than 1 μ in diameter.

The electrodes were first filled with dust-free methylalcohol by letting them boil in the alcohol under vacuum for about 15 minutes. They were then transferred to dust-free deionized, distilled water and left for 30 minutes, the distilled water being changed several times. The water-filled electrodes were now transferred to 3 M KCl or K-citrate solution and were left there for 24 to 48 hours.

The KCl or K-citrate filled microelectrodes were checked by testing their resistance and tip potential. In all the KCl electrodes

selected the resistance fell in the range of 5 - 10 MΩ, while the tip potentials were below 5 mV. The K-citrate microelectrodes all had rather higher resistances, usually between 10 - 15 MΩ and the tip potential was about 20 - 30 mV, or even higher.

It was found that pH of the solutions had practically no effect on the tip potential and resistance of the microelectrodes used in all the experiments presented.

A floating microelectrode technique was employed in some of the experiments. The procedure of preparation of electrodes was almost the same as that described above, except that the water-filled micro-capillary was cut at the level of about 2 mm above the shoulder. The small portion thus cut, including the tip, narrow shank and about 2 mm of the stem was filled with electrolyte solution by soaking in 3 M KCl or K-citrate solution as described previously.

Freshly prepared Ag-AgCl wire was immersed in the filling solution of each glass microelectrode. In the floating electrode the Ag-AgCl wire had a dual function, that is, for electrical conduction and suspension of the microelectrode to the manipulator.

The reference electrode consisted of a small Plexiglas bottle filled with 3 M KCl or K-citrate solution and an agar bridge. The agar jelly was made by heating a mixture of 6% agar powder in normal Ringer's solution. The reference electrode was connected to the whole circuit by an Ag-AgCl wire dipped in the electrolyte solution in the Plexiglas bottle at one end, and at the other end, by the agar-filled U-tube dipped in the bath.

4. Electrical systems

(i) Circuit for potential recording - The electrical system was the same as described by Mainwood (1965) and is shown in Fig. 3.

(ii) Circuit for current delivery - This is shown by the schematic diagram in Fig. 4.

5. Experimental procedure

The bath in which the muscle was perfused with flowing solution is shown in Fig. 2b. It was made of Plexiglas and divided into two compartments of different sizes by a block of Plexiglas with a hole at the centre. The muscle mounted on a frame was placed in the large compartment with its inner surface facing upward, while the agar bridge was dipped into the solution in the small compartment. The solution flowed at a rate of 20 to 30 ml/min. into the muscle chamber, then through the hole in the block into the small compartment from where the solution was removed by a suction tube. Because the opening of the suction tube was near the top of the bath the water level of the bath was maintained constant. Solution reservoirs were connected to the bath at the muscle compartment end through a common tubing of 15 cm long and 0.4 cm in diameter which constituted a dead space of about 2 ml. Switching from one solution to another could be accomplished in a few seconds. At the above mentioned flow rate a desired pH value could be attained in approximately $2\frac{1}{2}$ minutes after switching to the solution of the desired pH from solutions at different pH levels. Therefore about 85 - 95% exchange of the solution in the bath took place in

Fig. 3

Schematic diagram of the circuit for membrane potential recording. A represents a preamplifier having an input resistance of about $10^{10}\ \Omega$ and a gain of 2.3. Cal represents a calibration source which gives -200 to +200 mV in I mV steps. By adjusting the calibration source the tip potential can be balanced to zero. Membrane potential is measured by backing off the deflexion of the meter (M) or on the chart of the pen recorder, with the calibration source. M represents a voltmeter, measuring the tip potential when the switch (S) is in position 3. It also gives readings which are proportional to the resistance of electrode when the switch is in position 2. RI - resistor of 20 MΩ. R2 - 10.5 KΩ resistor. R3 - 3 KΩ resistor. Me - microelectrode. Re - reference electrode. S - switch. When it is in position I the circuit is open. B - a 1.35 volt battery.

Fig. 4

Schematic diagram of the current dilivery circuit. A - a differential amplifier with gain of 90. By measuring the voltage across the 100 KΩ resistor with the amplifier the current passing through the electrode is estimated. B - represents a pulse generator and a wave form generator, from which a single square pulse of constant voltage is produced each time when the generator is switched on. C- stimulation isolation unit. Os - represents oscilloscope. Photographs of the tracing on the screen of oscilloscope are taken by a Grass camera; the camera shutter is opened or closed synchronously when the pulse generator is switched on or off.

Fig. 5

Experiment to test the effect of the difference in pH levels between the electrodes used. Af represents the agar thread; Me, microelectrode; Re, reference electrode; R, the electrical system for recording.

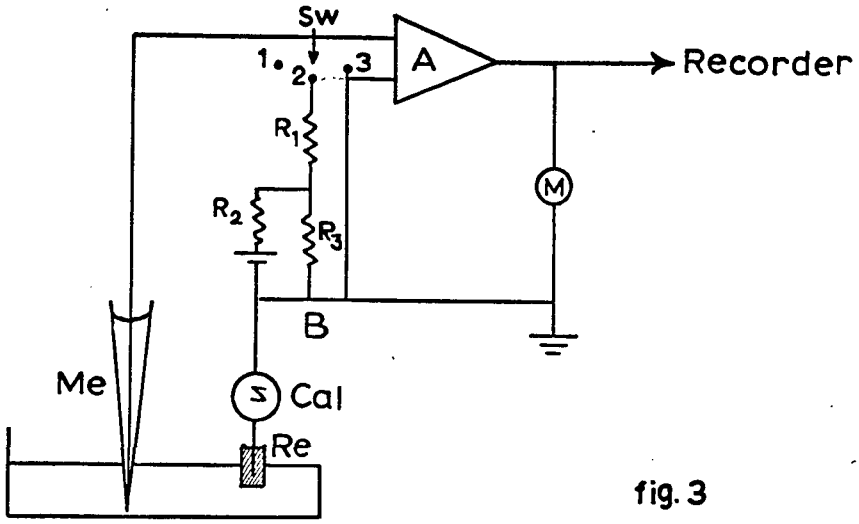


fig. 3

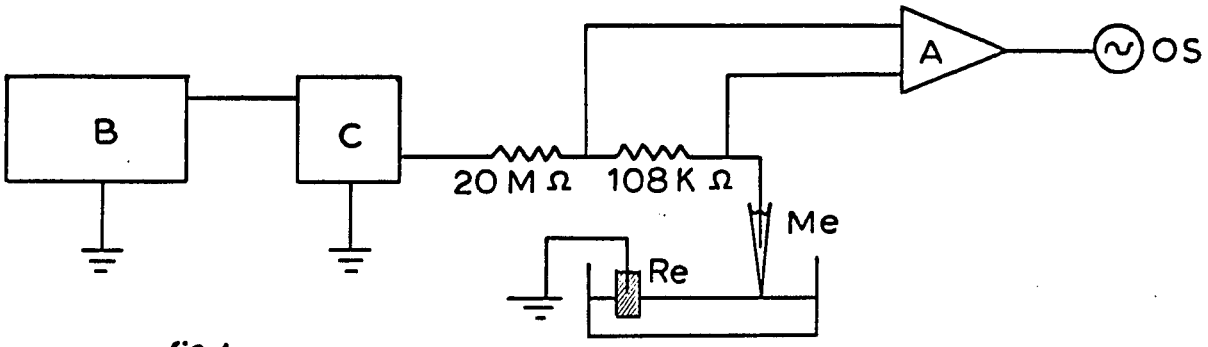


fig.4

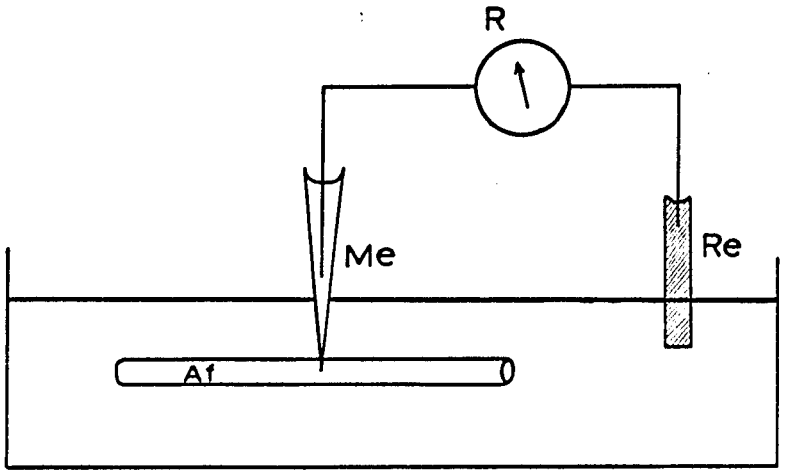


fig. 5

$2^1/2$ minutes after each switching.

At the onset of the experiment the muscle was always soaked in Ringer's solution of pH 7 for about 15 minutes. During this period of time the electrode was checked for tip potential, resistance and sensitivity to pH; tip potential was balanced off, then the impalement started. The electrode was inserted into the fibre near the end into which the solution flowed as shown in Fig. 2b. Potential was balanced off and recorded immediately by a Westronics pen recorder. Ten to 15 minutes later switching to another solution began. This was repeated every 5 minutes until the end of the experiment. Upon finishing, the electrode was taken out of the fibre and tested again for the resistance and tip potential.

When membrane resistance was measured, one fibre was impaled with two intracellular microelectrodes as close to each other as possible. One of the two inserted electrodes measured the electrotonic potential, while the other was a current electrode through which a square pulse of constant current was injected into the fibre. Successful insertion of the two electrodes into the same fibre was indicated by the wave form of the electrotonic potential.

6. Control experiment

The control experiments serve to clarify two points. (1) to see if the changes in membrane potential of the present experiments are artifacts arising from the insertion of the electrode. (2) to test whether the differences between the pH level in the small

regions surrounding the tips of the microelectrode and the reference electrode might be responsible for the observed responses.

Three possible artifacts may arise due to changes in junction potentials in the two electrodes. In any one of the following cases there would be an apparent increase in the measured membrane potential as a result of lowering the pH:

(i) The junction potential of the reference electrode decreases upon lowering the pH.

(ii) The junction potential of the microelectrode increases with a low pH.

(iii) The junction potentials in both electrodes may decrease on lowering the extracellular pH. However, only the change in junction potential in the reference electrode occurs, while that in the inserted microelectrode does not, because of the buffering effect of the cytoplasm.

The first two possible artifacts clearly do not contribute significantly to the results since tests before impalement show no significant potential change in response to pH alterations. The possibility of (iii) was checked by the control experiments carried out as follows:

Agar threads of about 1 mm in diameter were made from a mixture of 6% agar powder in normal Ringer's solution. They were mounted and perfused in a way similar to that described for sartorius muscle. Fig. 5 is a schematic diagram showing the set-up of the control experiment.

CHAPTER III

Results

1. Changes in resting potential (E_m) at various PCO_2

The results of two experiments are shown in Fig. 6 and 12. In each case an increase in PCO_2 results in a fall in pH and an increase in the membrane potential. When the PCO_2 is returned to its previous level, the membrane potential also returns. Although the increase and decrease were in most cases less than 5 mV, they were definite and could be repeated several times in the same muscle fibre. It is interesting to note that usually the response was rather small in the first 10 - 15 minutes after impalement of the fibre, but it became more evident thereafter. In most cases, once the maximum response developed it persisted until the end of the experiment of 45 - 75 minutes duration.

The mean and standard errors of the initial resting membrane potentials and changes in membrane potential of nine muscles at different PCO_2 levels are shown in Fig. 8. The initial membrane potentials of the muscles varied between -83 and -95 mV. Since at the end of the experiments the membrane potentials were in the range of -75 to -92 mV, it seems that in all these cases the effects due to the damage caused by the impalement were small.

In order to remove the overshadowing effect arising from the difference in membrane potential between different muscle fibres the changes in membrane potential in each muscle fibre upon lowering the

Fig. 6

Effect of changes in pH on membrane potential. The muscle was perfused with solution A, equilibrated with 2%, 20% and 50% CO_2 in O_2, to give pH 7.2, 6.2 and 5.8, respectively. The KCL filled electrode was inserted at 0 min.

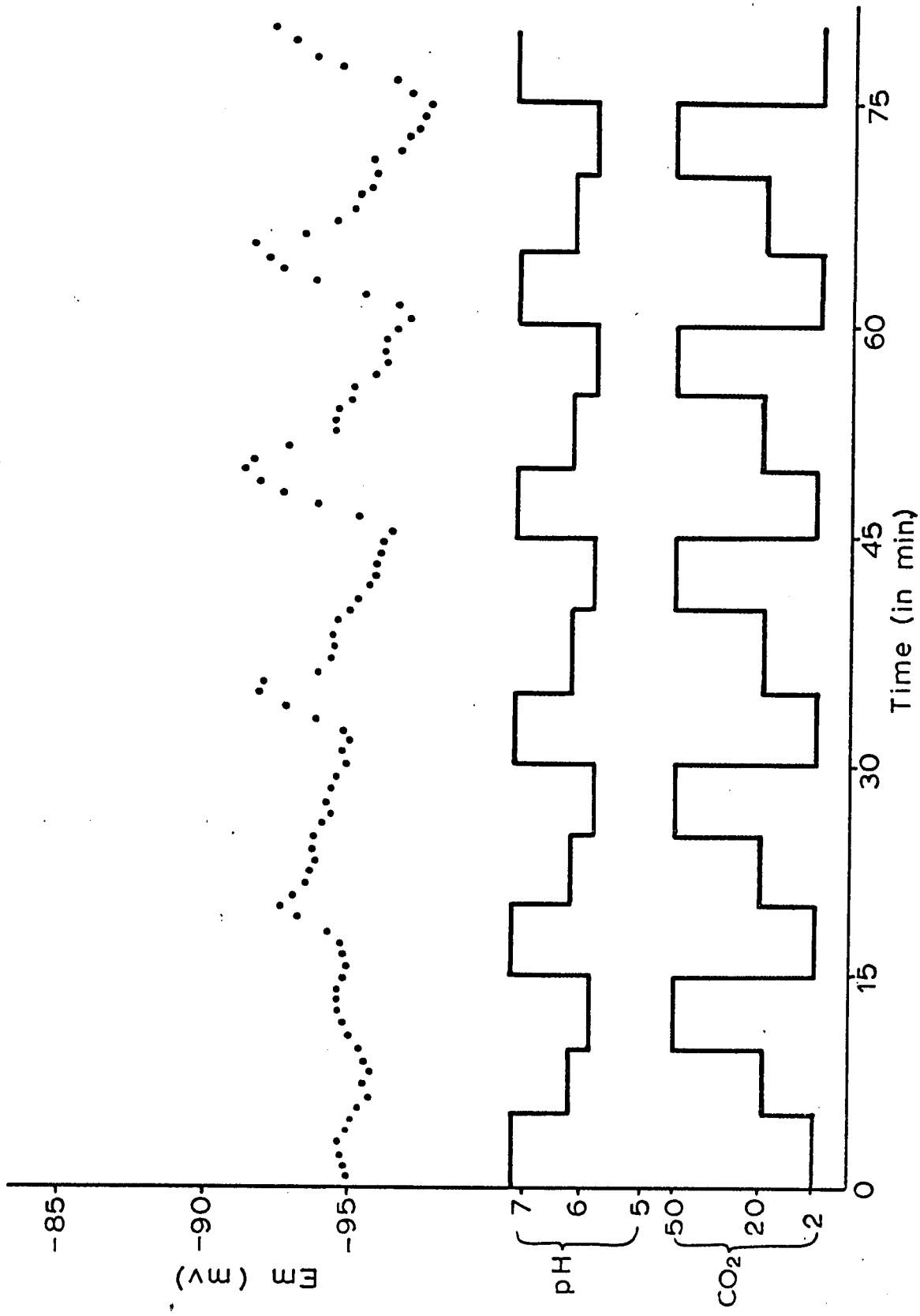


fig.6

Fig. 7

Effect of changes in pH on membrane potential.

The experiment is similar to that of Fig. 6.

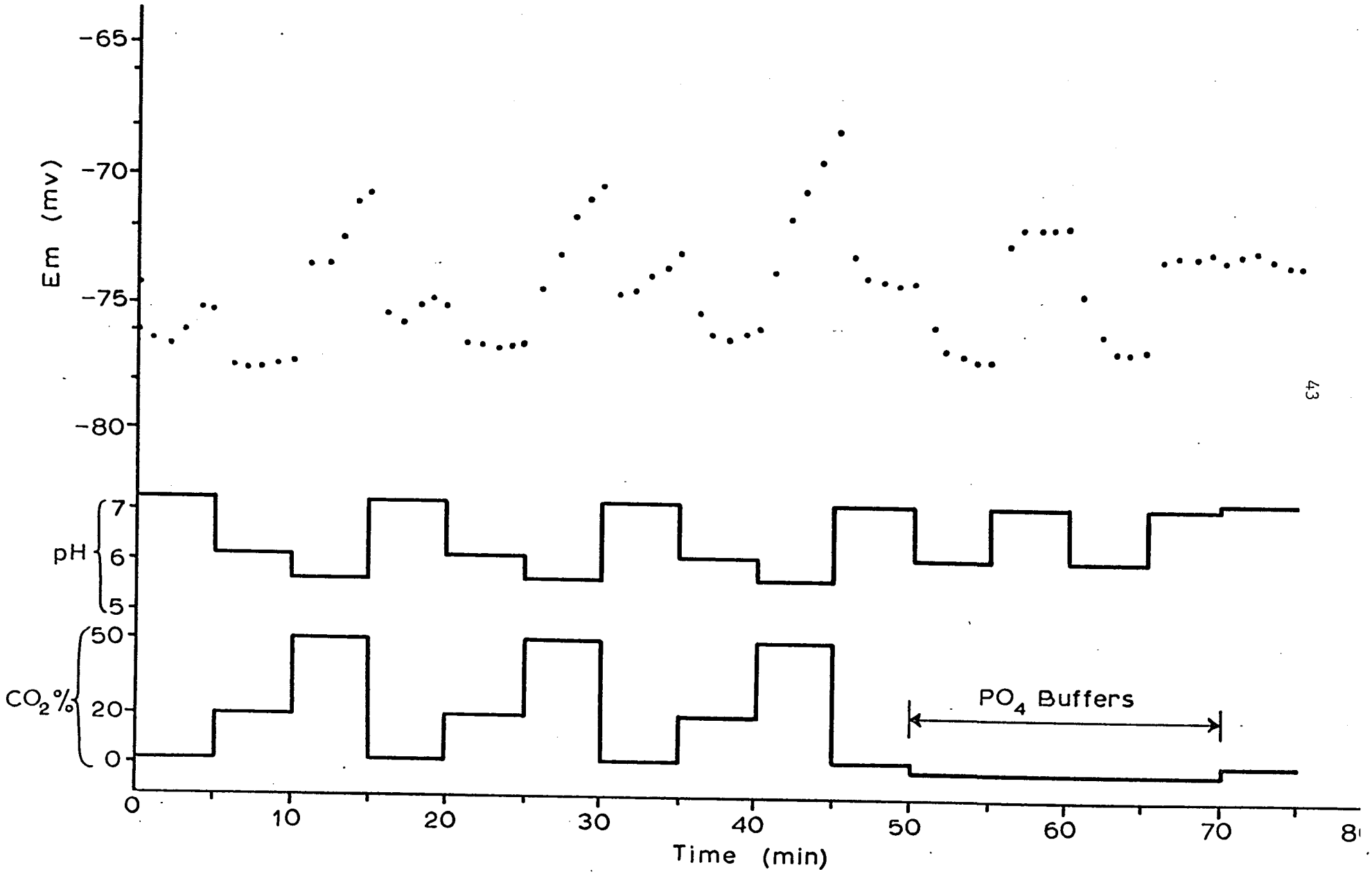

-65
-70
-75
-80
Em (mv)
pH
7
6
5
CO_2%
50
20
0
PO_4 Buffers
0
10
20
30
40
50
60
70
Time (min)

fig. 7

Fig. 8

The mean and standard errors of the results of nine experiments similar to those described in Fig. 6.

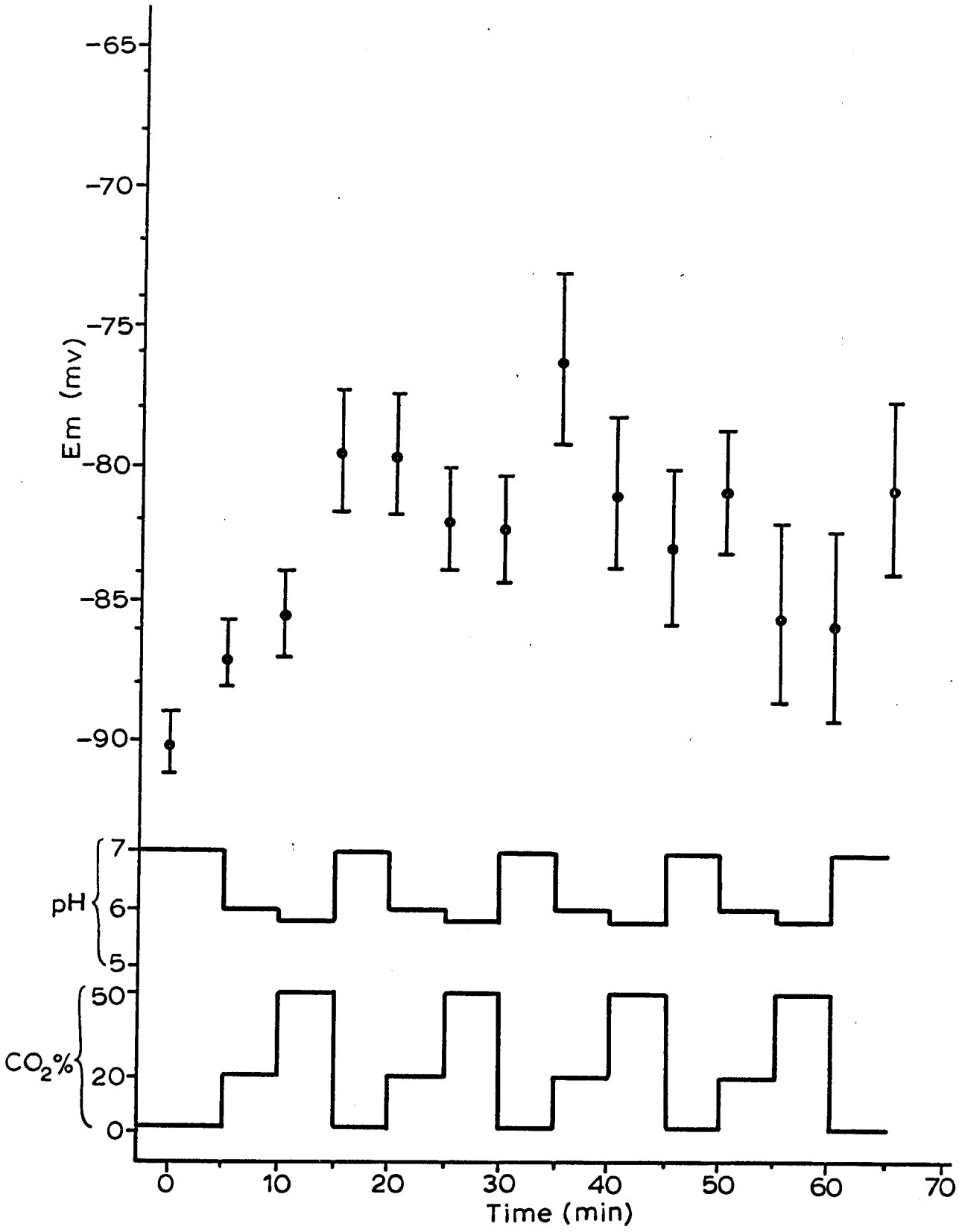

-65
-70
-75
-80
-85
-90
Em (mv)
7
6
5
pH
50
20
0
CO_2%
0
10
20
30
40
50
60
70
Time (min)

fig.8

pH level from 7 to 6 or 7 to 5.8 have been worked out and are shown in Fig. 9.

In a few experiments which were carried out in a similar way to that of Fig. 6 or 12, high PCO_2 depolarized while low PCO_2 hyperpolarized the fibres. In some cases this depolarizing response only occurred in the first 15 - 20 minutes. Responses of this type to changes in PCO_2 or pH are exemplified by Fig. 7.

2. Comparison of the effects of phosphate and bicarbonate buffered Ringer's solutions on E_m

In order to test whether it was the changes in PCO_2 or the changes in H^+ ion concentration which were responsible for the effects described in 1., phosphate buffered solutions of different pH were used instead of the bicarbonate buffer systems. The results are illustrated by two experiments plotted in Fig. 10 and 11. It is clear that the effects with phosphate buffers are comparable with those obtained in the experiments in which bicarbonate buffered solutions were used. In some experiments one buffer was replaced by another while a muscle fibre was impaled with an electrode so that the effect of changing pH with two different buffer systems could be studied in the same fibre. The results of one of the experiments are shown in Fig. 12. For a given pH change, the effects on membrane potential appear to be identical, whichever buffer system is used.

Fig. 9

Analysis of the results of the nine experiments of Fig. 6. Plotted are the means and S.E. of the differences between the membrane potentials measured at pH 7 and 6 (•) or 7 and 5.8 (o). KCl filled microelectrodes were used in most of the experiments included in the graph while K-citrate filled microelectrodes were used in the rest.

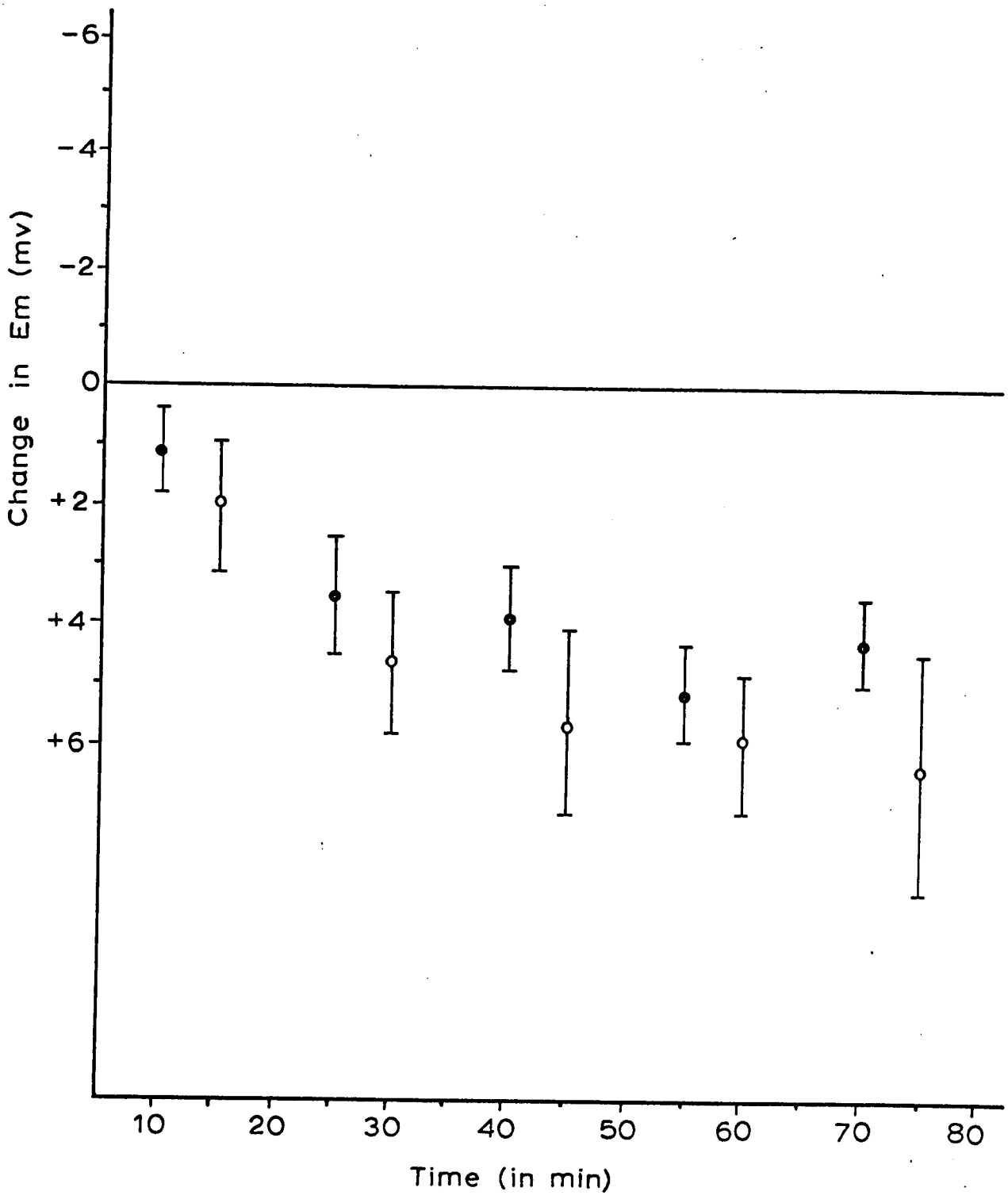


fig.9

Fig. 10

Effects of changes in pH values on membrane potential. Phosphate buffered solutions (solution B and solution C) gassed with pure O_2 were used in perfusion. The microelectrode used was filled with 3M KCl.

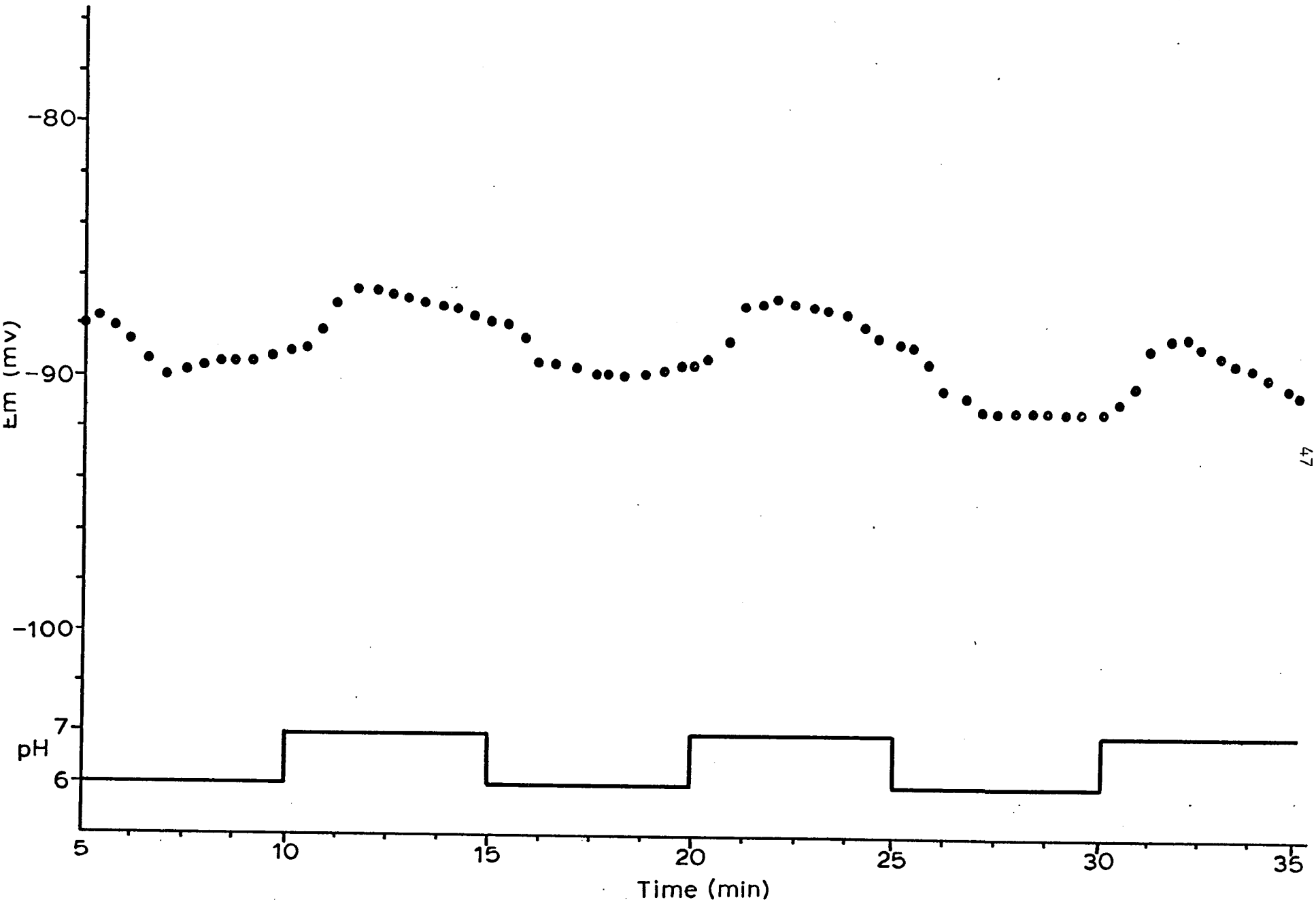


fig.10

Fig. 11

Results of an experiment similar to that described in Fig. 10, except that K-citrate electrode was used instead.

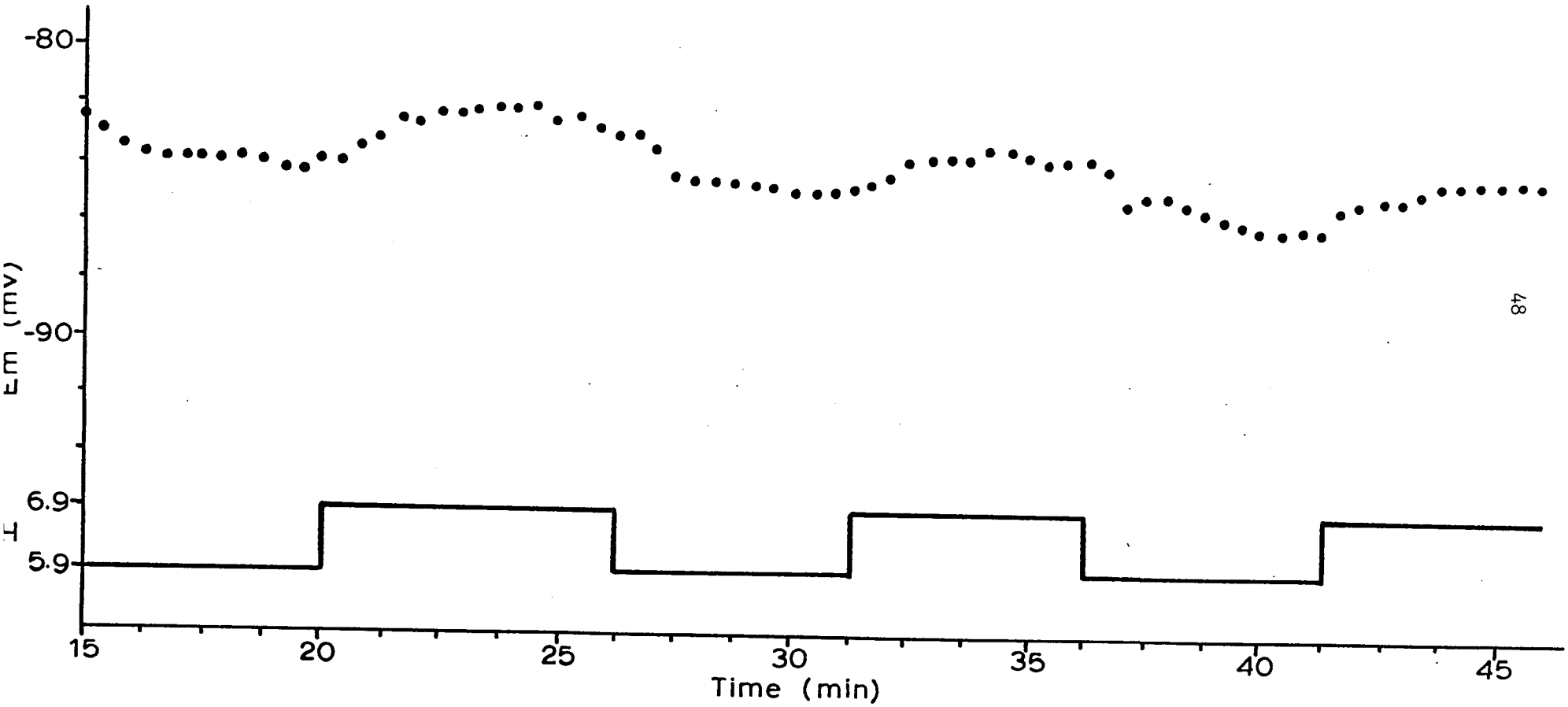


fig. 11

Fig. 12.

Comparision of the effects of changes in pH level and in PCO_2 on membrane potential. Phosphate buffered Ringer's solutions and bicarbonate buffered Ringer's solutions were used in the same muscle. KCl microelectrode.

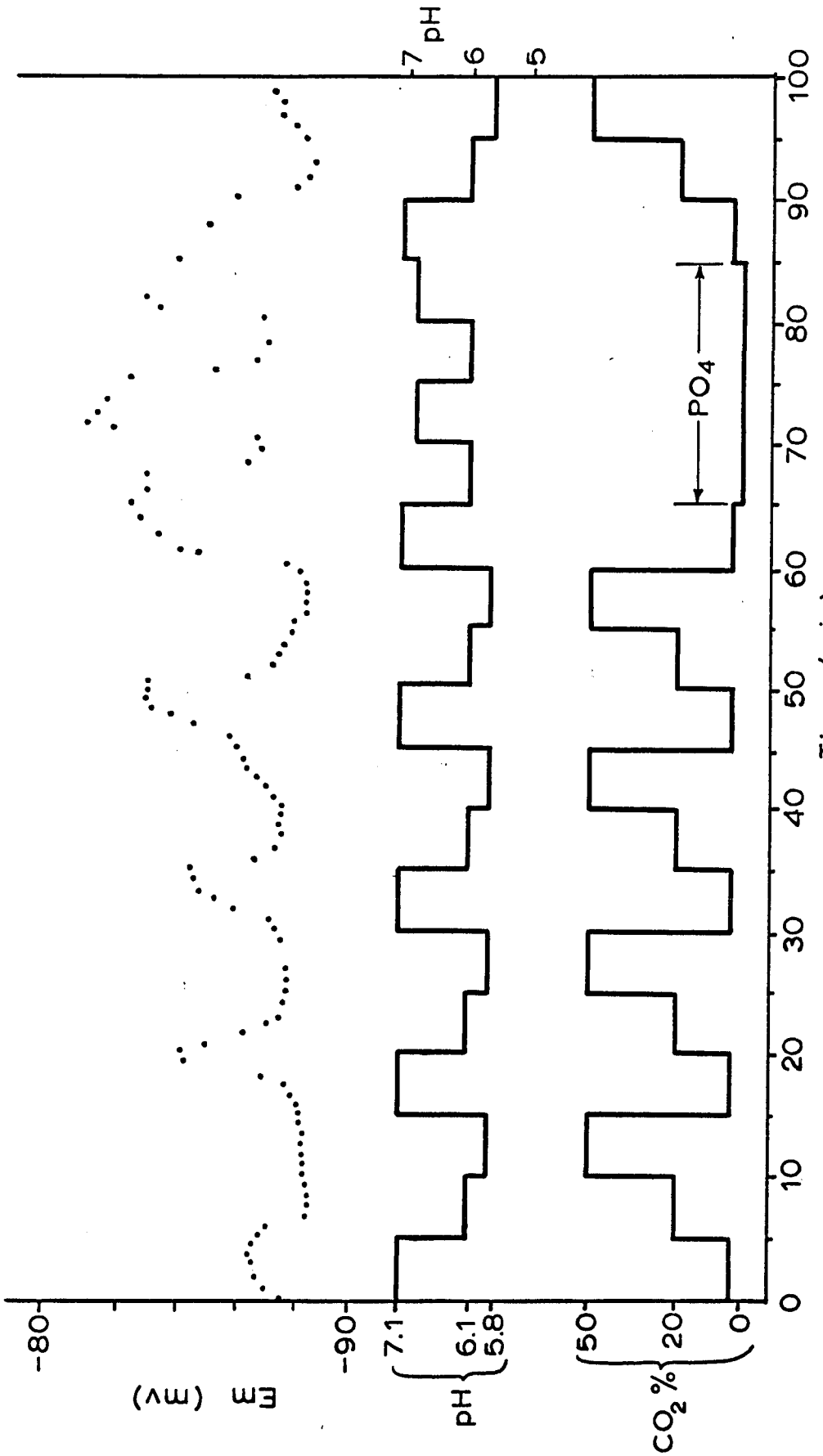

pH
7
6
5
PO_4
Time (min)
0
10
20
30
40
50
60
70
80
90
100
CO_2 %
0
20
50
pH
7.1
6.1
5.8
Em (mv)
-80
-90

fig.12

3. The role of Na^+ ions in the response of resting potential to pH change.

Some workers have suggested that in myelinated nerve, cortical neuron and crab muscles, H^+ ions might affect the Na^+ permeability. This could account for the decrease in membrane potential at high pH and increase at lowered pH. In order to study the possibility of such a mechanism in frog sartorius muscle in response to pH, the tissue was perfused in choline-Cl solution (Solution D) equilibrated with 2%, 20% and 50% CO_2 at pH 7, 6 and 5.8, respectively. The changes in membrane potential in response to raising or lowering pH levels of the Na-free solutions are shown in Fig. 13. Fig. 14 shows the means and standard errors of the membrane potentials in eight experiments with choline-Cl solutions. Because the individual membrane potentials varied to some extent, the changes in membrane potential upon lowering pH from 7 to 6, or from 7 to 5.8 in the same fibres have been studied, and the means and standard errors of the changes in eight muscles are shown in Fig. 15. A comparison between the changes in membrane potential in the presence and absence of Na^+ ions is shown in Table III. The results suggest that the response to changes in pH is not significantly altered if Na^+ is omitted from the extracellular medium. The similarity between the results of this group of experiments and those described in 1. can be interpreted as evidence that in frog sartorius muscle Na^+ ions play little part in the response of cell membrane to changes in pH.

Fig. 13

Effect of changes in pH on membrane potential in the absence of Na^+ ions from the external medium. The muscle was soaked and perfused with choline Ringer's solution (solution D) equilibrated with 2%, 20% and 50% CO_2 in O_2. KCl electrode was used.

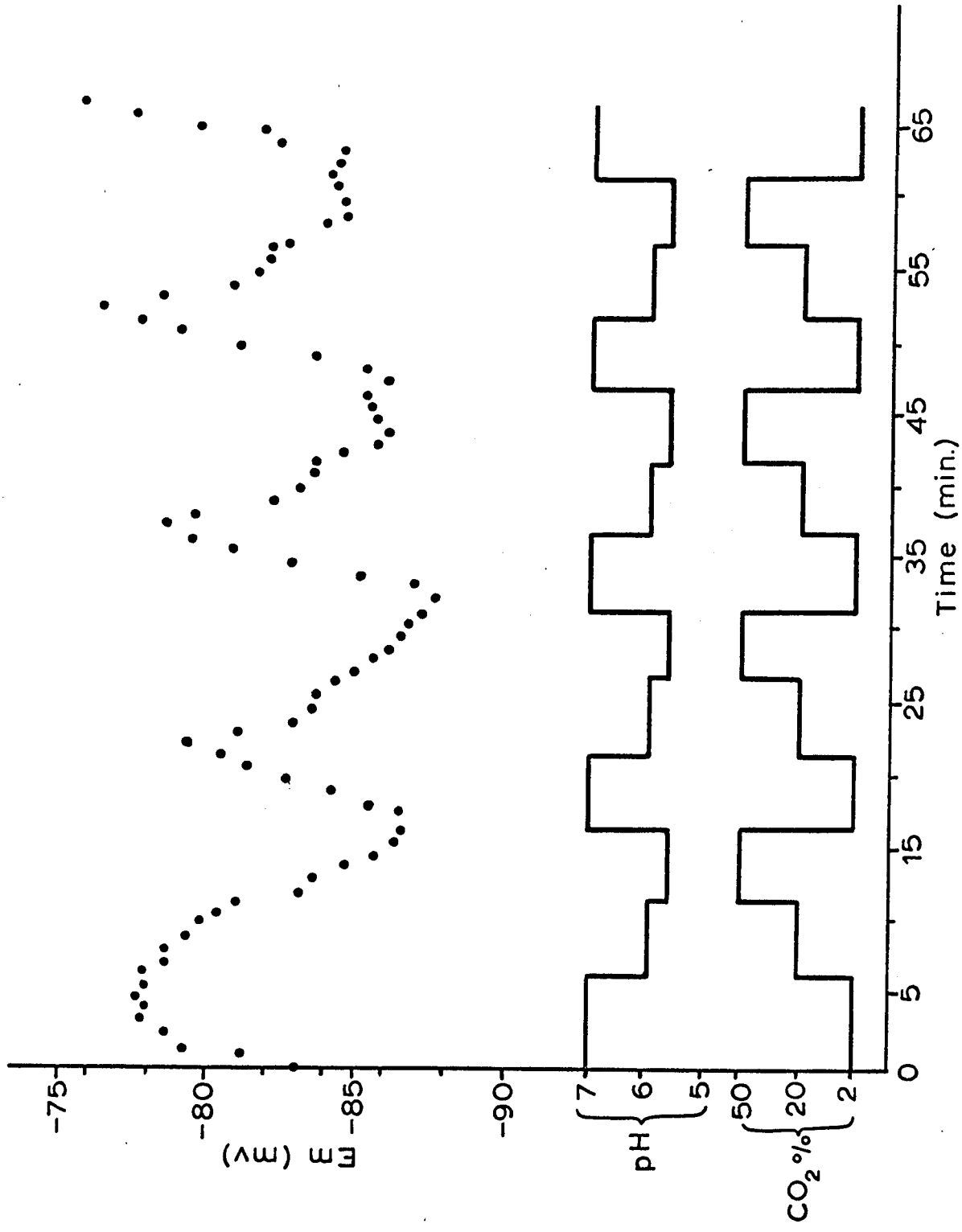


fig.13

Fig. 14

Means and S.E. of eight experiments similar to that described in Fig. 13.

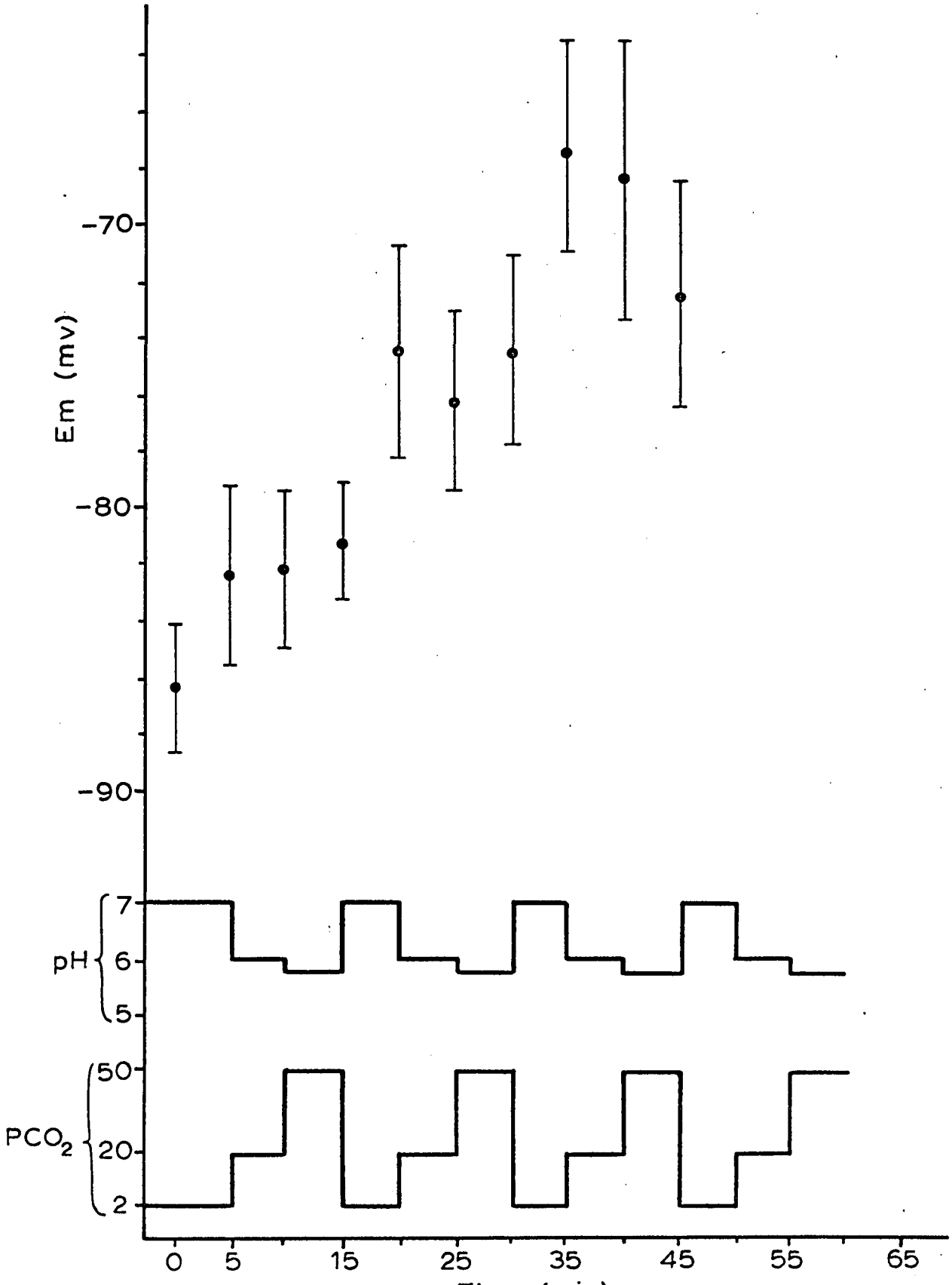


fig.14

Fig. 15

Analysis of results of the eight experiments of Fig. 14. Plotted are the means and S.E. of the differences between the membrane potentials at pH 7 and 6 (•) or 7 and 5.8 (o).

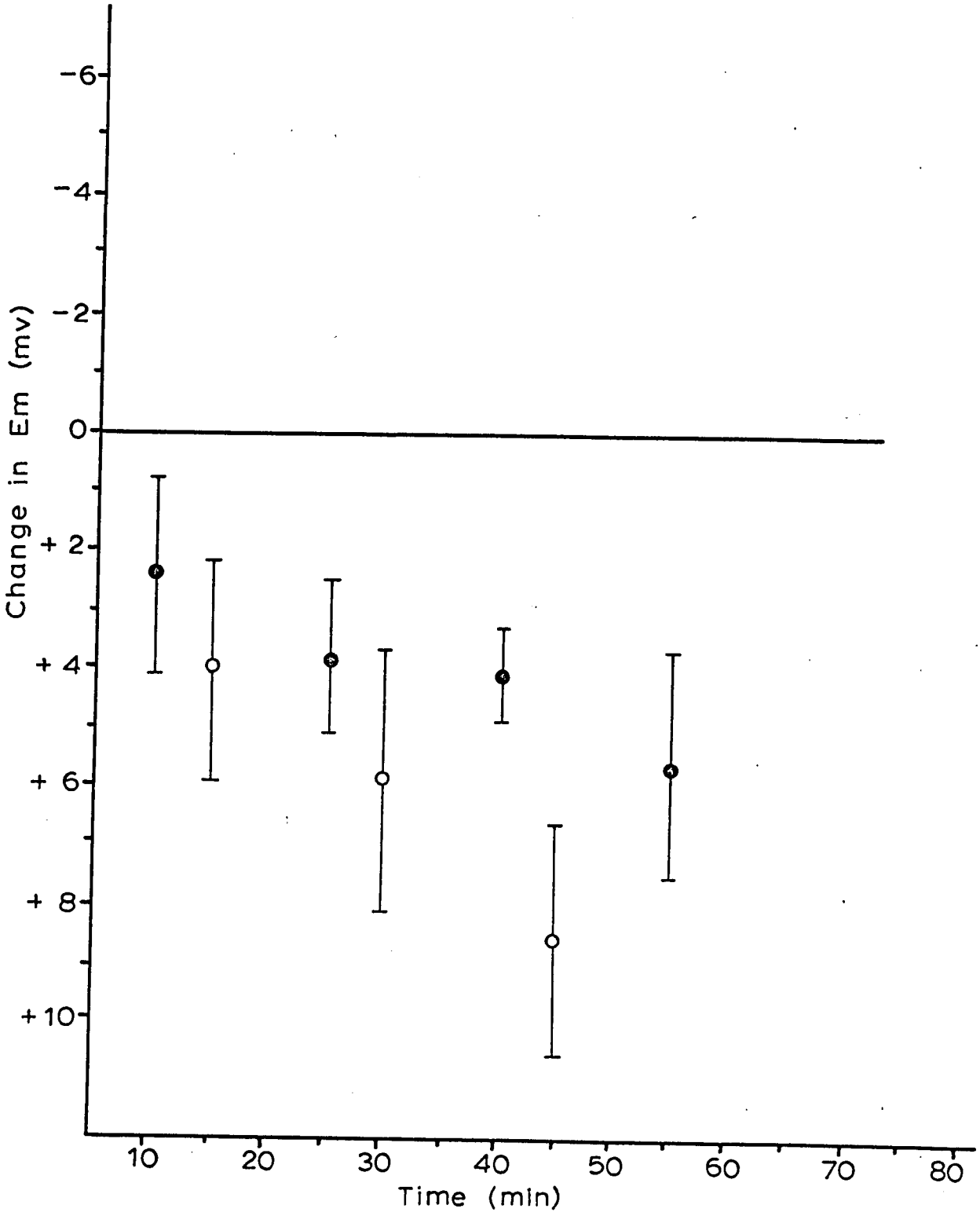


fig. 15

TABLE III

The comparison has been made between the results shown in Fig. 9 and Fig. 15. NR represents the normal Ringer's solution (solution A) at different pH levels, CH, the choline-Cl Ringer's (solution D) at different pH levels. DF is short for degree of freedom. The values on lines from a to i are the changes in E_m when the pH was lowered from 7 to 6, or from 7 to 5.8. The changes in E_m on a horizontal line under same heading, e.g. NR or CH, were obtained in same muscle fibre. The negative sign indicates the decrease in E_m, while the figures without prefixing sign are the increases in E_m. The statistic method employed is the non-paired t test.

TABLE III

Comparison of the effects of changes in H^+ ion concentration in the presence and absence of Na^+ ions on the membrane potential.

Time	10th min.		15th min.		25th min.		30th min.		40th min.		45th min.		55th min.	
pH	7 - 6		7 - 5.8		7 - 6		7 - 5.8		7 - 6		7 - 5.8		7 - 6	
Solutions	NR	CH	NR	CH	NR	CH	NR	CH	NR	CH	NR	CH	NR	CH
Line a	1.0	1.5	1.0	2.5	3.0	2.5	4.0	-	2.5	-	-	-	8.0	-
b	0	3.5	0.3	7.0	1.5	4.5	2.0	8.0	2.5	4.6	3.2	7.5	2.3	5.4
c	2.7	1.7	2.0	0	2.5	-1.5	3.1	1.3	4.5	1.7	-	-	-	-
d	-1.3	3.3	-1.0	0.5	0.4	9.0	3.2	11.5	1.7	-	4.0	10.5	3.0	8.5
e	0.4	0.5	1.0	-2.5	5.0	1.7	7.0	-2.7	5.0	-	6.0	-	8.0	-
f	-0.6	5.7	0	13.0	3.3	7.2	4.5	12.9	3.5	5.5	4.6	13.5	3.3	-
g	2.3	4.5	3.4	11.0	4.3	6.5	2.6	10.0	3.1	5.5	3.0	10.0	5.0	-
h	1.0	-0.8	1.0	1.0	1.2	0.8	2.1	0.7	2.6	3.0	4.0	1.5	4.2	3.0
i	5.4	-	10.5	-	10.5	-	13.0	-	10.0	-	14.5	-	7.0	-
x	1.1	2.5	2.0	4.1	3.5	3.8	4.6	5.9	3.9	4.1	5.6	8.6	5.1	5.6
Σx^2	47.6	81.9	129.9	352.8	183.7	207.4	289.3	462.0	189.3	93.6	318.7	451.0	244.8	110.4
S.D.	2.75		5.32		4.79		6.83		4.50		8.51		5.66	
D.F.	15		15		15		14		12		10		9	
t	1.03		0.78		0.14		0.35		0.05		0.60		0.13	
P	>0.1		>0.1		>0.1		>0.1		>0.1		>0.1		>0.1	

While there may be some slow depolarizing effects due to choline as reported by some workers (Ochs, 1966) this does not seem to significantly affect the response to pH changes.

4. Effects of pH on resting potential following changes in the equilibrium potential of K^+ (E_K) or Cl^- (E_{Cl})

Experiments were carried out to investigate the effects of changes in pH on membrane potential when the relations between the resting membrane potential and equilibrium potentials of K^+, Cl^- and Na^+ were quite different from those under normal conditions. If the membrane permeability to any one of these ions is changed by hydrogen ions then the effect of pH on membrane potential would be altered in a predictable way when the equilibrium potential of the ions involved is suddenly changed. Therefore, the response of muscle fibres following changes in equilibrium potential should throw some light upon the postulated H^+ ion effect on membrane permeability in frog sartorius muscles.

(a) The effect of decreasing E_K

The results of one experiment are shown in Fig. 16. Upon switching from 2.5 to 10 mM K^+ solution the membrane potential decreased by about 16 - 24 mV. The values are less than predicted by the Nernst equation. During the depolarization, a fall in pH decreases the membrane potential. This response was opposite to that observed when the muscle was perfused with normal Ringer's solutions containing 2.5 mM/l of K^+. However, the response did not last long, but became smaller with time. The gradual decrease in the response is probably due to the redistribution

Fig. 16

Effects of changes in pH on resting membrane potential which was at normal level in normal K^+ Ringer's solutions, depolarized in K^+-rich solutions and finally repolarized upon readmission of normal K^+ Ringer's. K-citrate microelectrode was used.

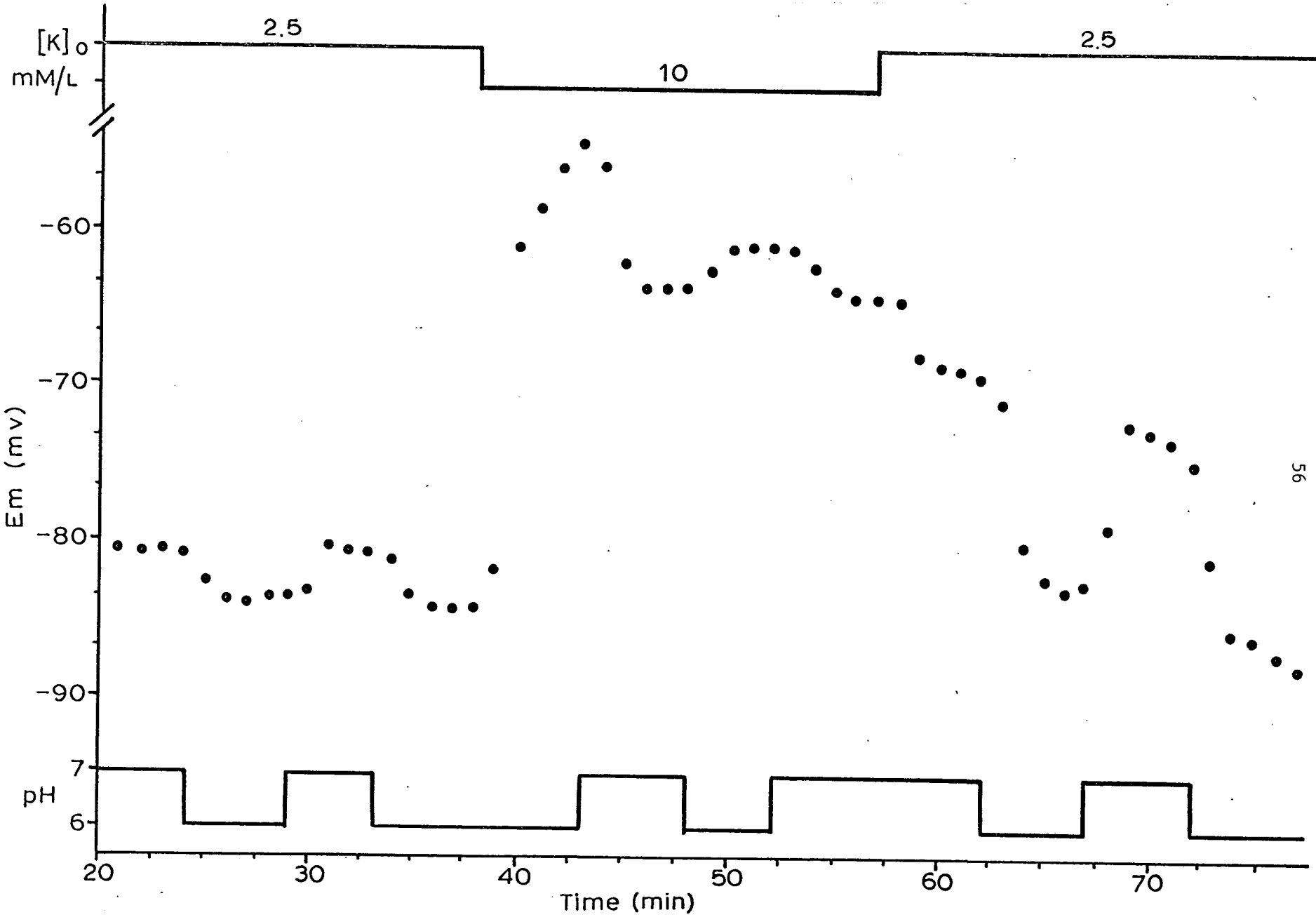


fig.16

of ions. The reason for this will be discussed later.

Upon readmission of normal Ringer's solutions, the membrane potential increased slowly towards the original level and the response to changes in pH reversed again. Thus, membrane potential responded to the changes in pH in the same way as it did before K^+depolarization, except that the magnitude of the changes was much greater than before. A change of 10 - 15 mV was commonly observed at this stage when pH was changed by one unit. This was seen in the first 10 - 15 minutes after switching back to normal Ringer's solutions. Again, the increased response to pH changes upon switching from K^+-rich to normal K^+ solutions did not persist but faded away gradually. Likewise, the results may be explained on the basis of ion redistribution.

Four experiments of this type were carried out and the means and standard errors of the changes in membrane potential following the decrease in pH from 7 to 6, at different K^+ concentration levels are plotted in Fig. 17.

(b) The effects of decreasing E_K in choline Ringer's solution

When muscles were perfused with choline solutions, everything else being the same as those described in (a), the results were practically the same as in the presence of Na^+ ions. One of the three experiments is shown in Fig. 18. The means and standard errors of the changes in membrane potential upon lowering the pH from 7 to 6, at different K^+ concentrations are plotted in Fig. 19.

Fig. 17

Four experiments were carried out in very much the same way as the one described in Fig. 16 and the results are analysed. Plotted are the means and S.E. of the changes in membrane potential when pH was lowered from 7 to 6 and extracellular K^+ concentration was altered as indicated by the horizontal bars on the top of the graph. Decrease in membrane potential is designated by ' - ' and plotted above the zero line, while increase is designated by ' + ' and plotted below the zero line.

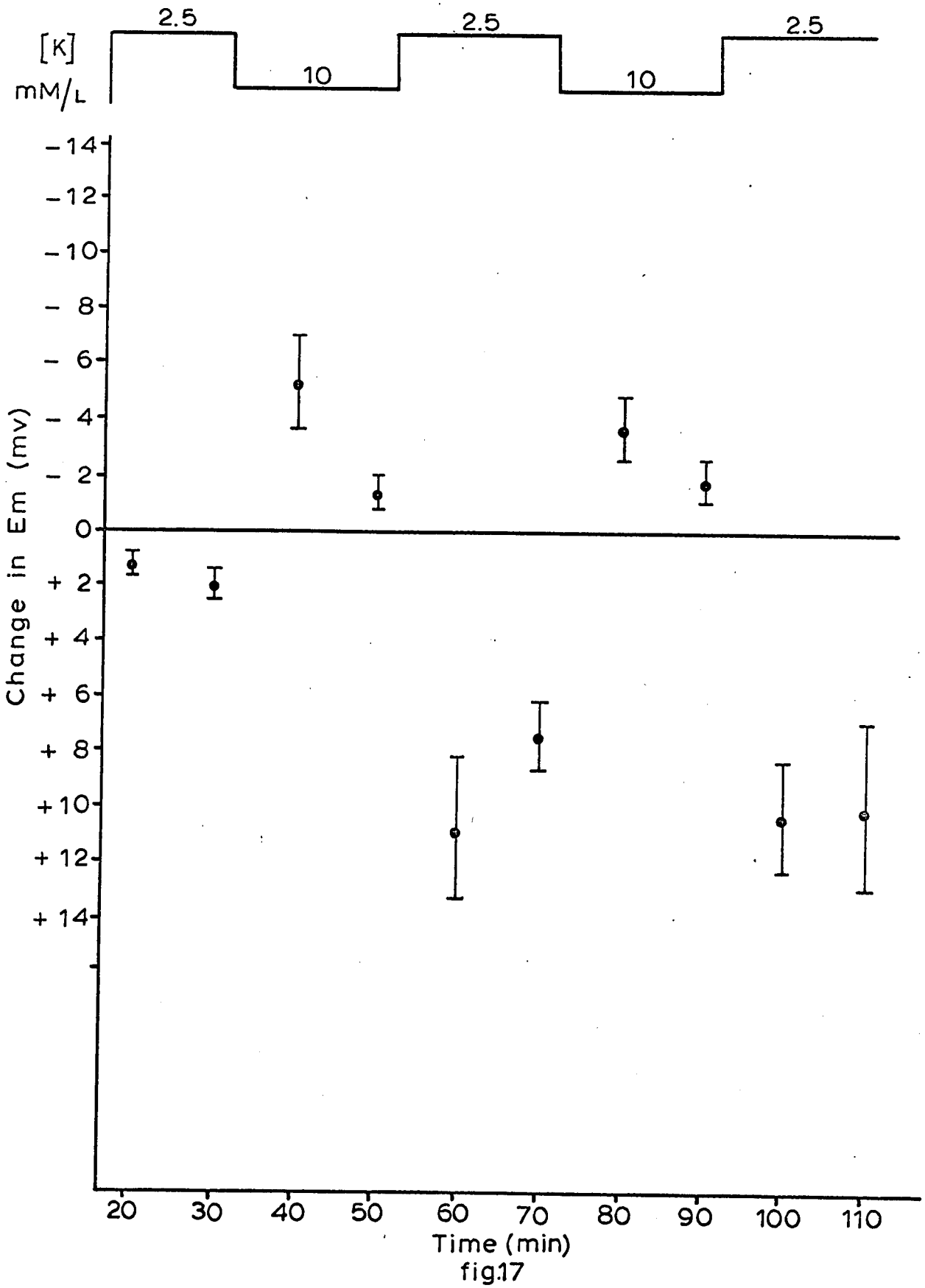

[K]
mM/L
2.5
10
2.5
10
2.5
Change in Em (mv)
-14
-12
-10
- 8
- 6
- 4
- 2
0
+ 2
+ 4
+ 6
+ 8
+10
+12
+14
20
30
40
50
60
70
80
90
100
110
Time (min)

fig.17

Fig. 18

Experiment similar to that described in Fig. 16, except that Na^+ ions were absent from all solutions. K-citrate microelectrode.

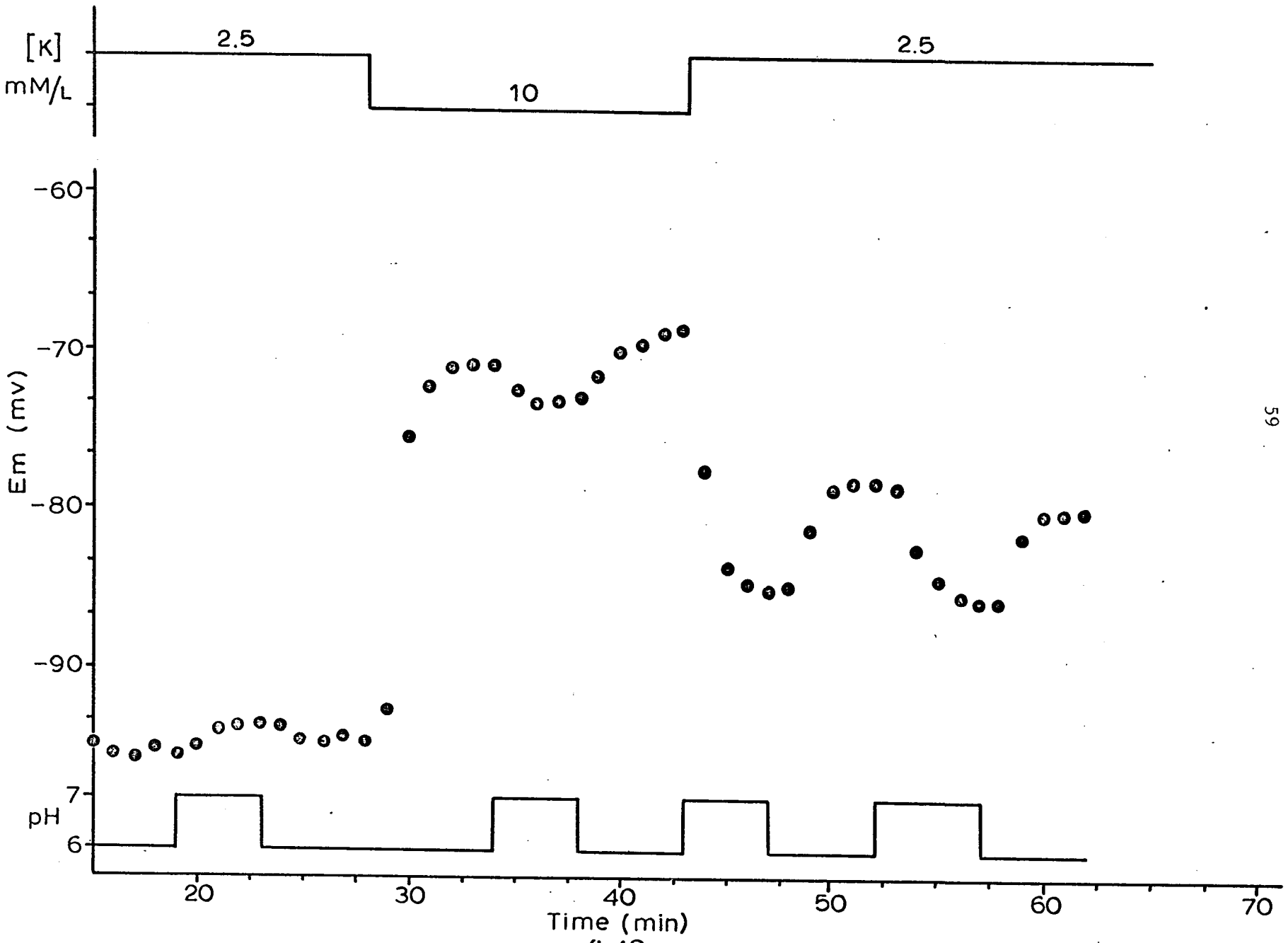


fig.18

Fig. 19

Effects of changes in pH on membrane potential after Na^+ ions were omitted from the perfusing fluid. Three experiments were carried out in the manner similar to the one described in Fig. 18. Plotted are the means and S.E. of the changes in membrane potential when pH level was lowered from 7 to 6 and the extracellular K^+ concentrations were altered.

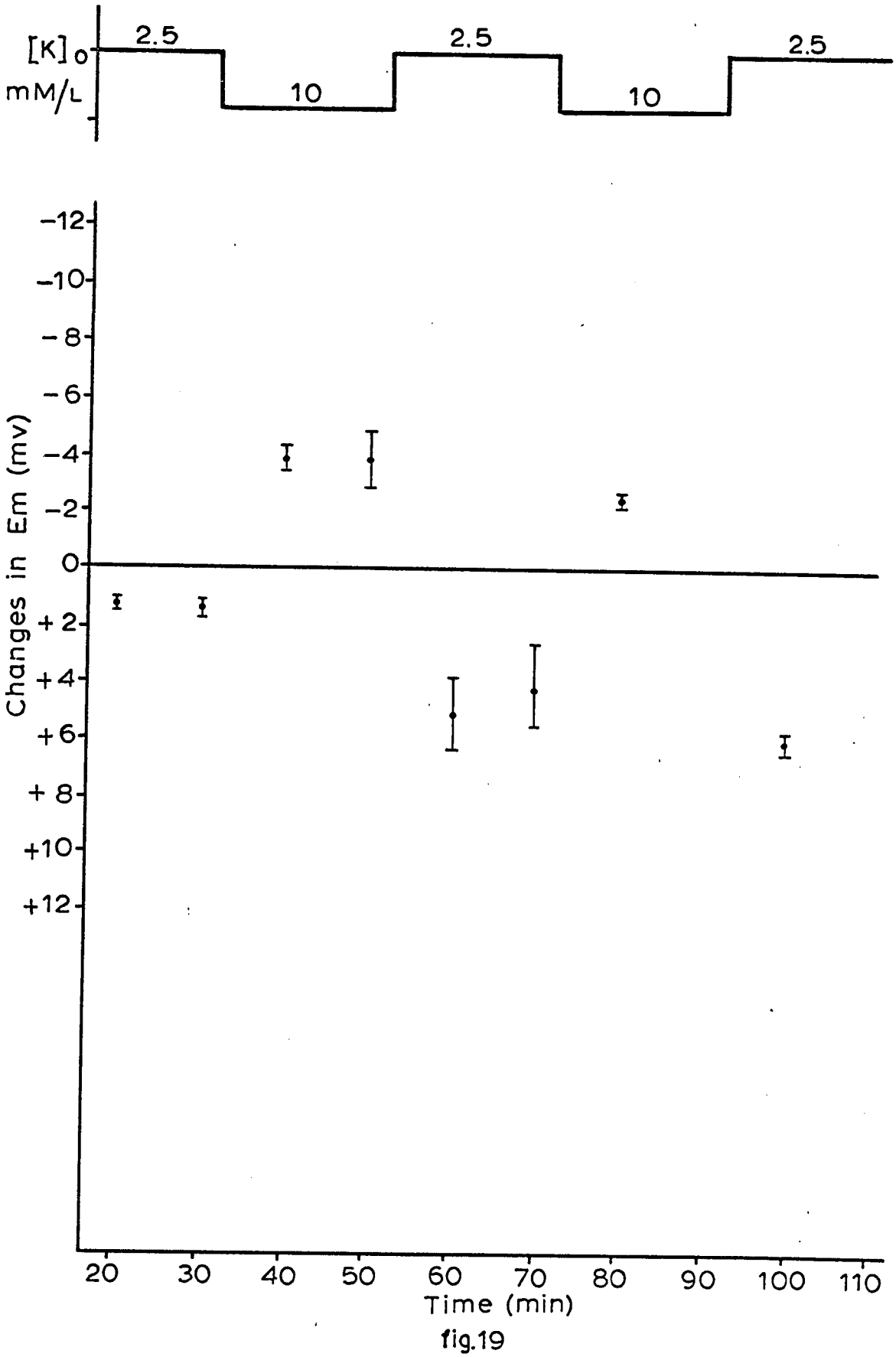

[K]o
mM/L
2.5
10
2.5
10
2.5
Changes in Em (mv)
-12
-10
-8
-6
-4
-2
0
+2
+4
+6
+8
+10
+12
20
30
40
50
60
70
80
90
100
110
Time (min)

fig.19

(c) The effects of decreasing E_{Cl}

Admission of low Cl^- solutions caused a transient depolarization as described by Hodgkin and Horowicz (1959). This is a consequence of the decrease in E_{Cl} and the outflow of Cl^- ions. During this period, membrane potential is particularly responsive to pH changes and the response became greater though in the same direction as that seen in normal Ringer's solution. One of these experiments is shown in Fig. 20. As can be seen in the figure, readmission of normal Cl^- solutions reversed the response to changes in pH, that is, the membrane potential decreased when the pH fell. As in (a) and (b), upon switching from normal to low Cl^- solutions, or from low Cl^- back to normal Cl^- solutions, there was a transient increase in responsiveness to the alteration of pH. Fig. 21 shows the changes in membrane potential in response to the fall of pH from 7 to 6 at different extracellular Cl^- concentration levels.

5. Effects of H^+ ions on membrane resistance

Figure 22 shows photographs of the electrotonic potentials produced by square pulses of constant current. The changes in the magnitude of the potential resulting from changes in pH were consistent in either bicarbonate or phosphate buffer systems.

The total input resistance (R_c) of the muscle fibre is calculated by dividing the current applied (I) into the highest value of the measured electrotonic potential (V) (Fatt and Katz, 1951). Thus,

$$R_c = \frac{V}{I} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Fig. 20

Effects of changes in pH on resting membrane potential which was at normal level during the perfusion with normal Cl^- Ringer's solutions, transiently depolarized in low-Cl solutions and finally repolarized upon the readmission of normal Cl^- Ringer's solutions. K-citrate microelectrode.

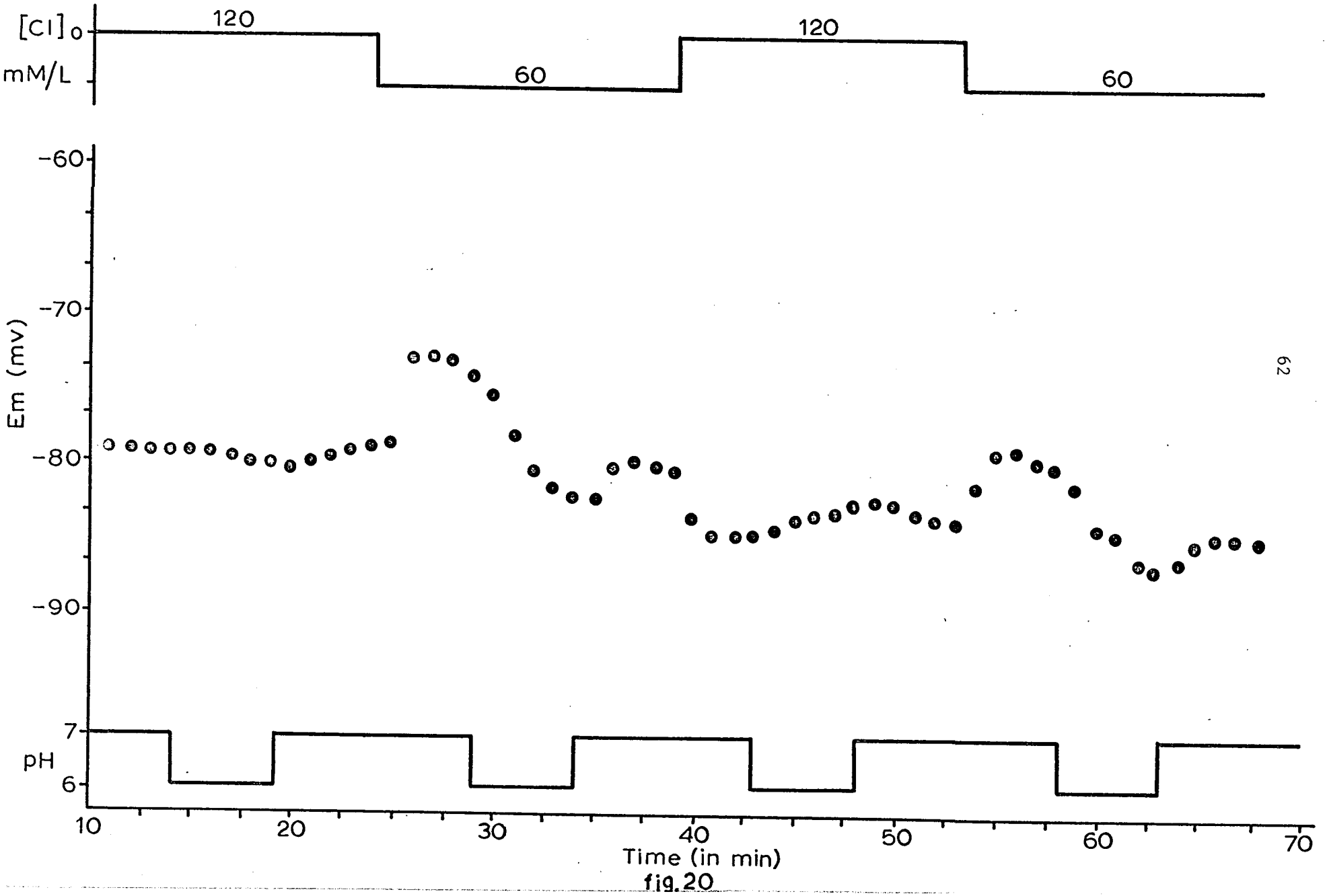

[Cl]o
mM/L
120
60
120
60
-60
-70
-80
-90
Em (mv)
7
pH
6
10
20
30
40
50
60
70
Time (in min)

fig.20

Fig. 21

Results of two experiments similar to the one described in Fig. 20 are analysed. Plotted are the changes in membrane potential when pH was lowered from 7 to 6 and the extracellular Cl^- concentration levels were changed as indicated by the horizontal lines on the top of the graph.

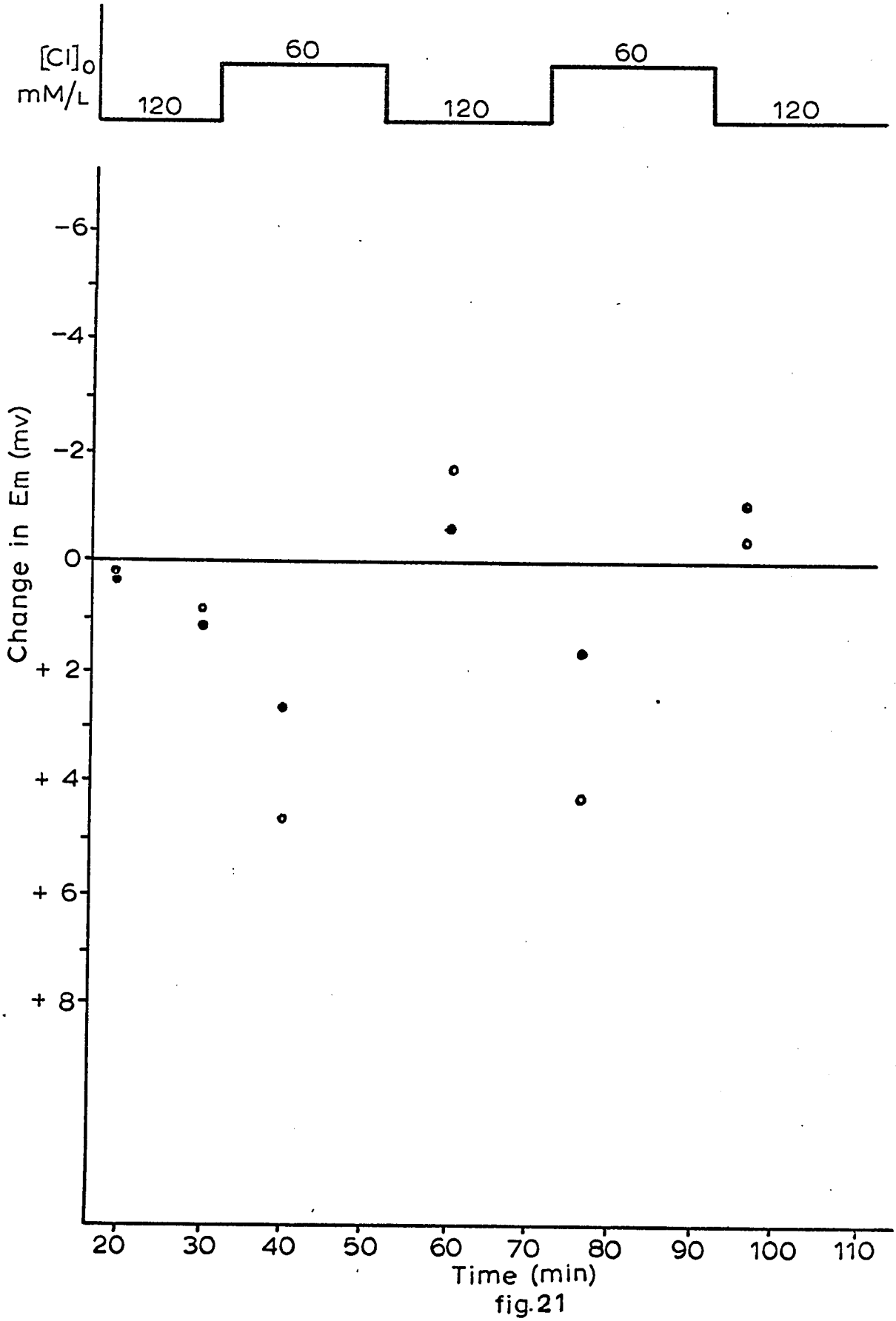

[Cl]o
mM/L
120
60
120
60
120
Change in Em (mv)
-6
-4
-2
0
+ 2
+ 4
+ 6
+ 8
20
30
40
50
60
70
80
90
100
110
Time (min)

fig.21

Fig. 22

Effects of changes in pH on electrotonic potential of muscle fibres produced by applying constant current pulse. The letters a, b, c, d and e refer to different muscles. a, b and c were perfused with phosphate buffered solutions (solutions B and C), while d and e, with bicarbonate buffered solution (solution A). The pH values of the solutions used are shown on the top of each column and the sequence of solutions used is indicated by the arrows. In each muscle the time interval between the two electrotonic potentials shown is 5 minutes. Outward current only was applied in b and inward current only was applied in a and c. In d and e both inward and outward currents were used one after the other with time intervals of less than 1/2 minute. The duration of pulse was 500 msec in a, b and c, while 1,000 msec in d and e. The potential scale (Ve) applies to all muscles. The scale of applied current (Ia) is common to all except e.

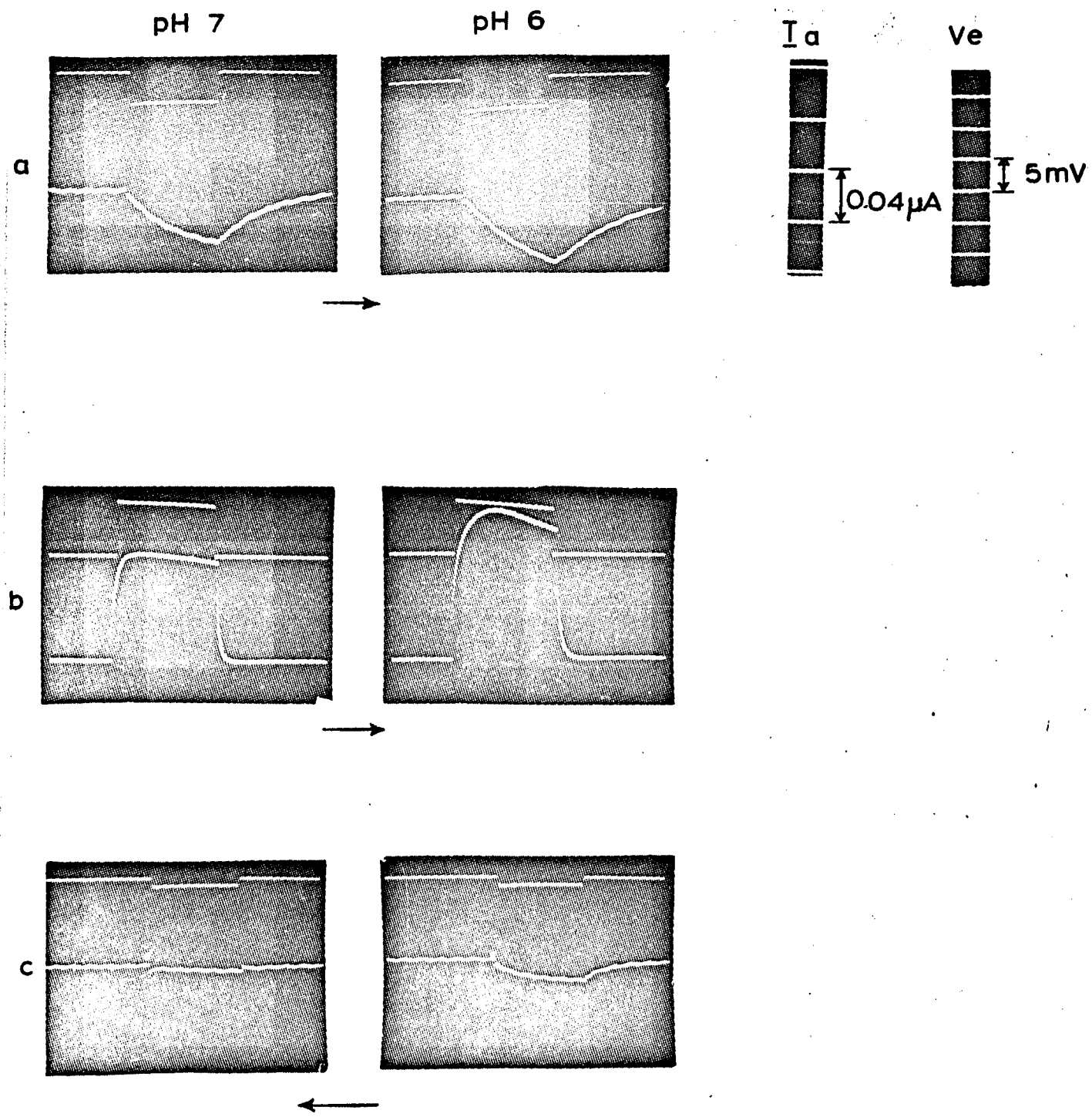


fig. 22

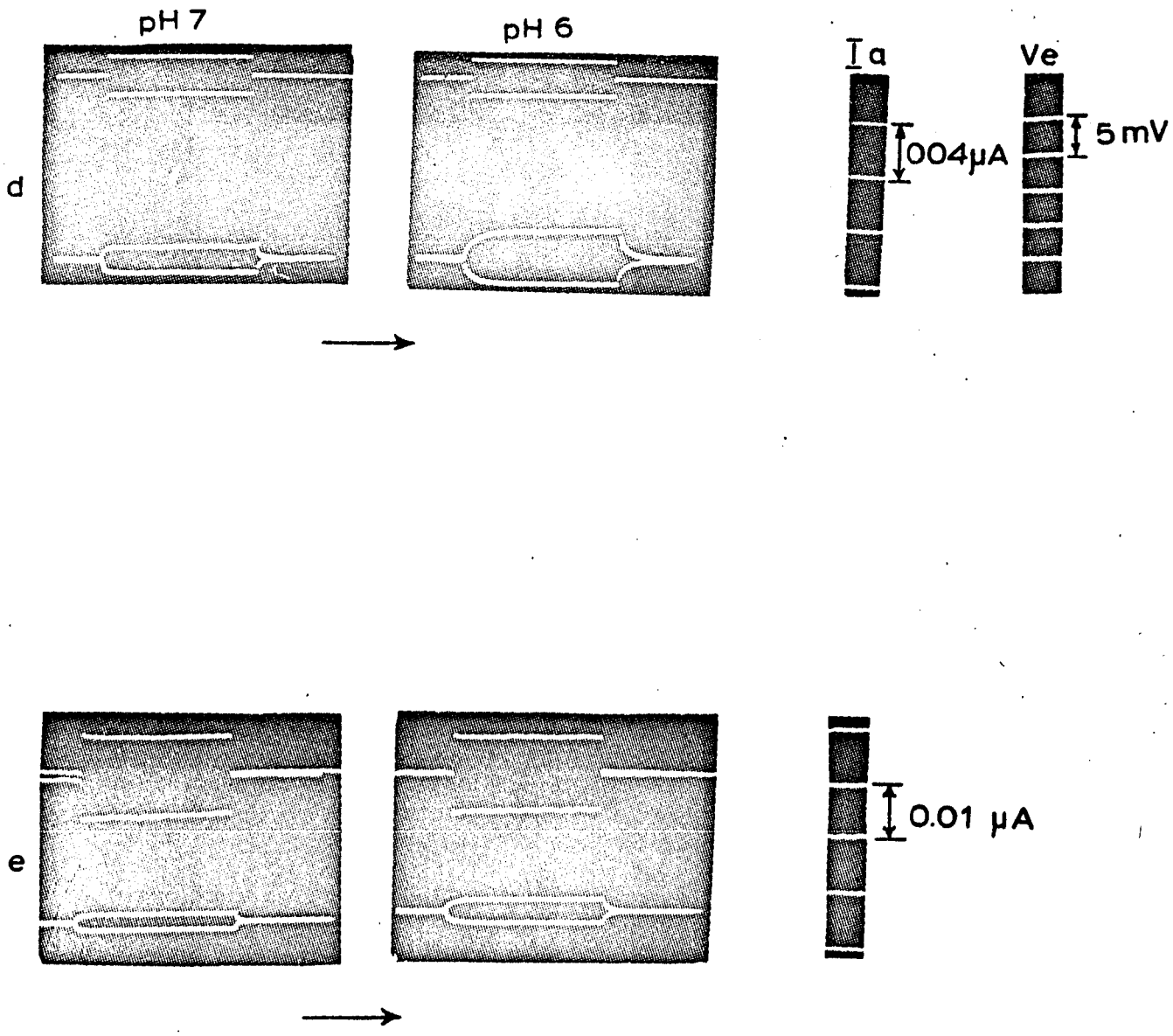


fig. 22

The increase in the total input resistance as the pH of the perfusing solution was lowered was reversible as can be seen in Fig. 23 and 24. It is also clear that the results of the experiments were basically the same, being independent of the buffer systems used. The consistently repeatable effect of changes in H^+ ion concentration on the total resistance of muscle fibre is also shown in Fig. 25, in which the total input resistance values are plotted against pH. With membrane potentials between -55 and -93 mV the total resistance of muscle fibres increased in acid and decreased in alkaline solutions, whether inward or outward current was employed.

The resistance shown here is the sum of the membrane resistance together with the intra- and extracellular resistance. The extracellular resistance was negligible under the conditions described. Furthermore, according to Meves and Volkner (1958), the intracellular resistance in frog skeletal muscle did not change as extracellular pH changed. If these observations are accepted then the present experiments show that a fall in pH results in an increase in membrane resistance.

According to Fatt and Katz (1951), the total input resistance (R_c), membrane resistance (R_m) and the resistance of cytoplasm (R_i) are related to each other as follows:

$$R_c = 1/2 \sqrt{R_m \times R_i} \quad \text{..... (2)}$$

or

$$R_m = 4 \times \frac{R_c^2}{R_i}$$

Fig. 23

Effects of changes in pH on total input resistance of frog sartorius muscle. The muscle was perfused with solution A equilibrated with 2%, 20% and 50% CO_2 to give pH 7.2, 6 I and 58, respectively. Electrotonic potential was produced by hyperpolarizing current of duration 1,000 msec. Both microelectrodes used were filled with 3 M K-citrate.

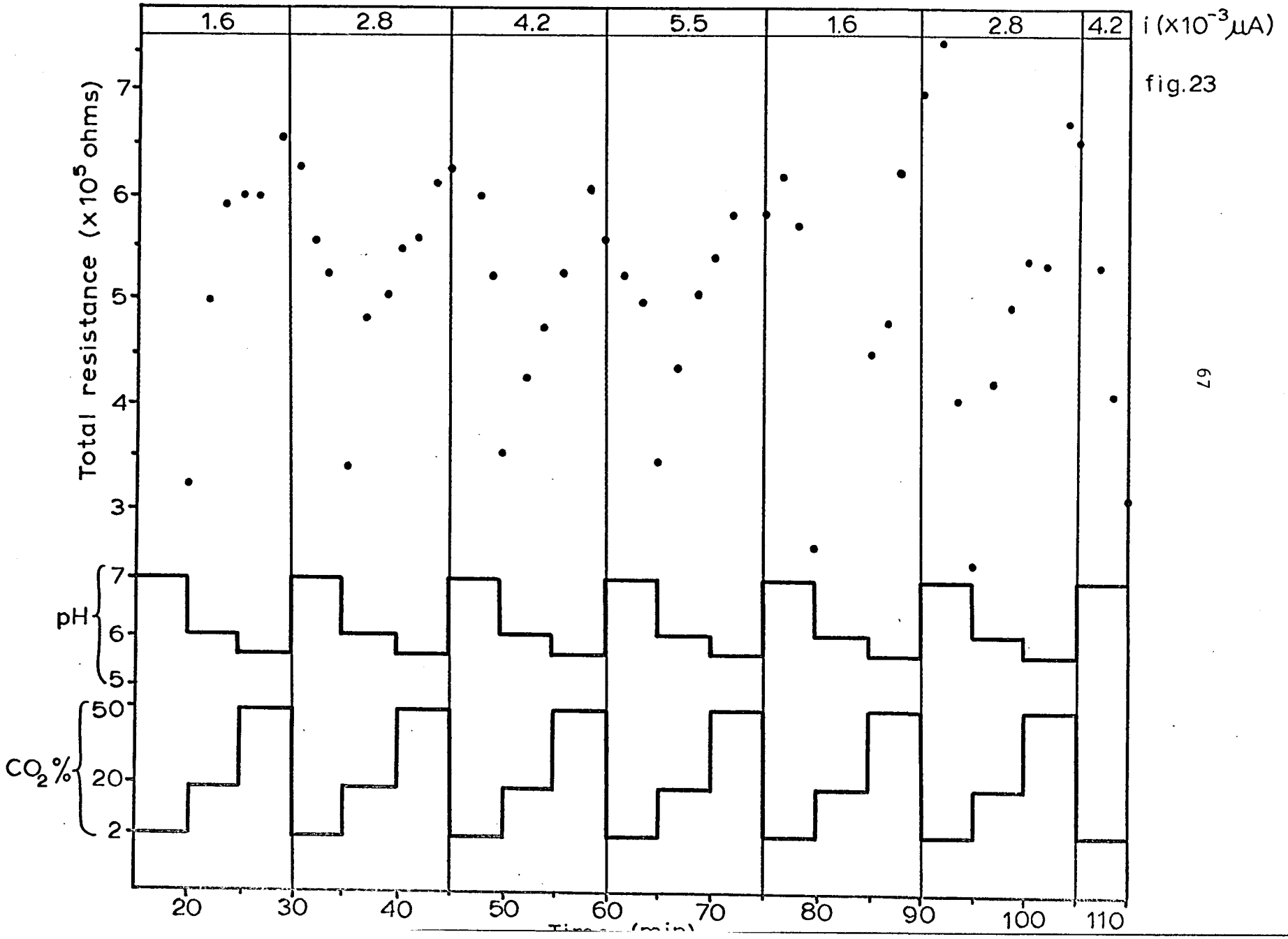

1.6
2.8
4.2
5.5
1.6
2.8
4.2
i (x10⁻³ μA)
Total resistance (x10⁵ ohms)
7
6
5
4
3
pH
7
6
5
CO₂%
50
20
2
20
30
40
50
60
70
80
90
100
110

fig.23

Fig. 24

Experiment similar to that in Fig. 23, except that the muscle was perfused with phosphate buffered solutions (solutions B and C). Hyperpolarizing current of duration 1,000 msec. was applied. K-citrate microelectrodes.

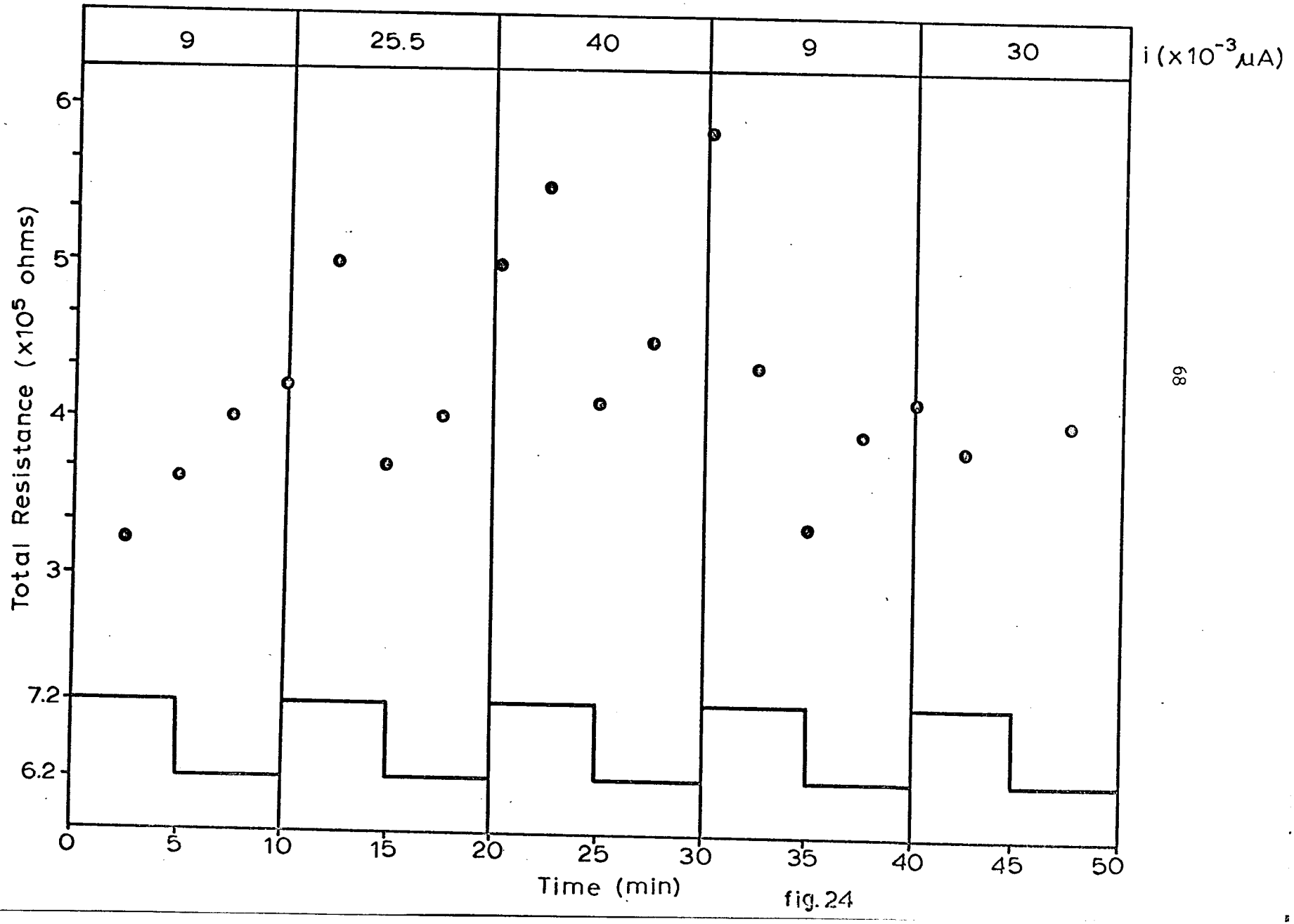

9
25.5
40
9
30
i (x10⁻³ μA)
Total Resistance (x10⁵ ohms)
6
5
4
3
7.2
6.2
0
5
10
15
20
25
30
35
40
45
50
Time (min)

fig. 24

Fig. 25

Distribution of total input resistance at different pH levels. The means and S.E of the measured total resistances of different muscle fibres are represented by different symbols. In most of the muscle fibres resistance was tested 4 to 7 times. Δ, O, Δ were perfused with bicarbonate buffered Ringer's solutions, while the rest with phosphate Ringer's.

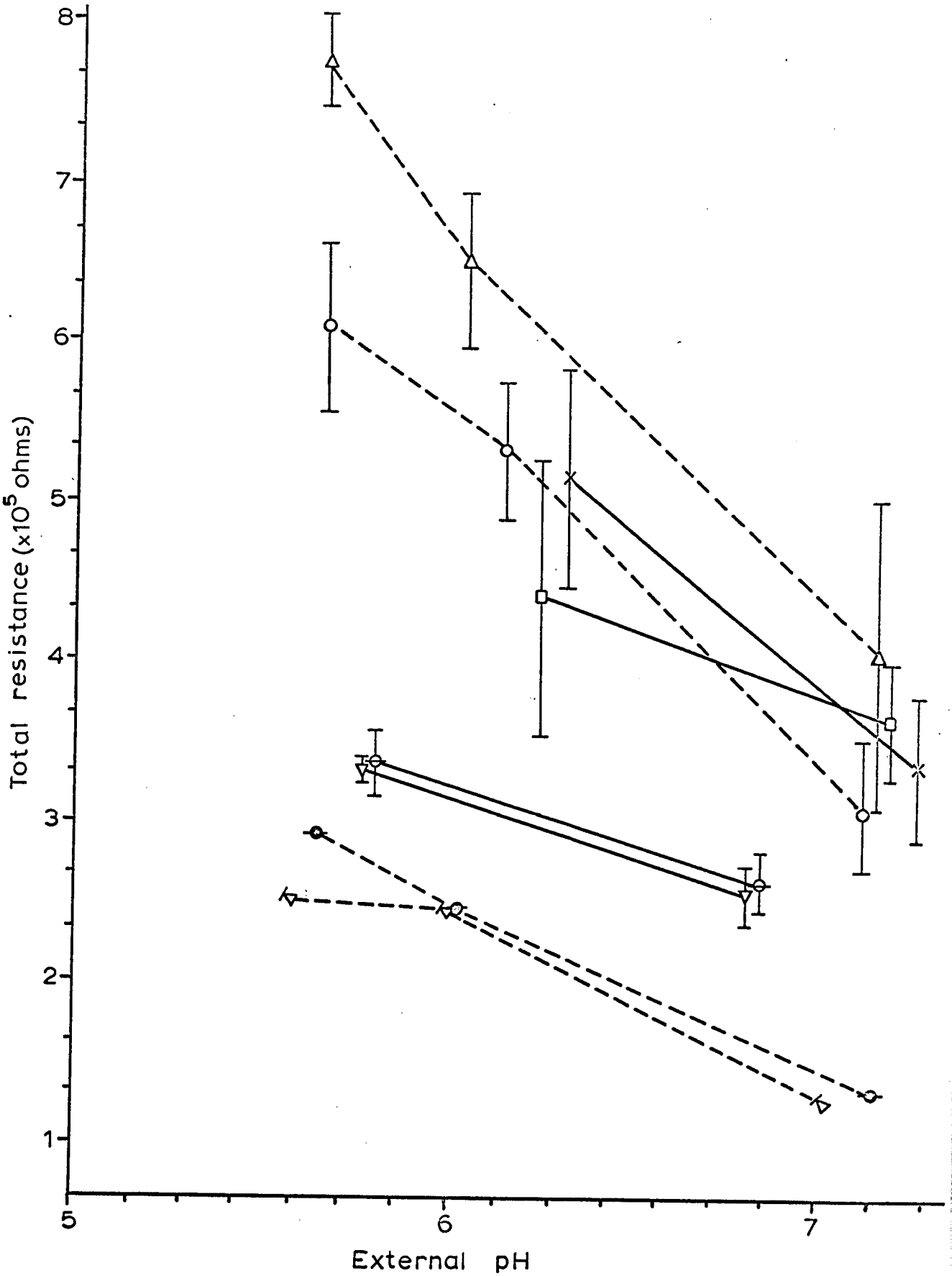


fig.25

If R_i remains constant, it follows that the ratio between the Rm's at two different pH levels should equal the ratio between the squares of R_c at each corresponding pH level. Thus,

$$\frac{R_{m_1}}{R_{m_2}} = \frac{{R_{c_1}}^2}{{R_{c_2}}^2} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where R_{c_1} and R_{c_2} represent the total input resistances at two different pH levels; R_{m_1} and R_{m_2}, the membrane resistance at the corresponding levels.

Now, if R_c increases 40% (Table IV C_{5-6}) in lowering the extracellular pH from 7 to 6, the membrane resistance at pH 6 would be twice as big as that at pH 7, that is,

$$R_{m_6} = 2 \times R_{m_7} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

where R_{m_6} and R_{m_7} represent the membrane resistances at pH 6 and 7, respectively.

As shown in Table IV, when muscles were perfused with phosphate buffer the mean increase in total resistance as a result of lowering pH from 7 to 6 is 54%, while in the case of bicarbonate buffered solutions the mean increase is 77%. While it is clear that lowering pH causes an increase in resistance, there is a large variation in the extent of the increase from one experiment to another and it is not possible to say whether or not the increase is significantly greater in the experiments with bicarbonate buffered solutions.

The large differences observed between resistance measurements are to some extent inevitable in this type of experiments and they are due to a number of factors as follows:

TABLE IV

Some of the results of total input resistance measurements are tabulated to show the change in the resistance as consequence of pH alteration. The letters in the first column represent different muscles tested; the numbers, for example, 1 - 2 or 3 - 4, describe the sequence of the wash-out cycles. The time interval between two resistances compared is 5 minutes. The buffer systems and their pH values are shown in the second column. Ia designates the constant current applied. R_e and R_e' represent total input resistances at different pH levels. ΔR_e is equal to $R_e' - R_e$. Increases (+ %) or decreases (- %) in the total input resistance are shown in the last column. The change, either increase or decrease, is considered with respect to the R_e. Thus $\pm \% = \frac{\Delta R_e}{R_e} \times 100\%$.

TABLE IV

Total input resistance changes in frog sartorius muscle fibres.

Muscle	Solutions and pH	E_m (mV)	I_a (μA)	Direction of I_a	R_e ($\times 10^5$ ohm)	Solutions and pH	E_m (mV)	I_a (μA)	Direction of I_a	R_e' ($\times 10^5$ ohm)	ΔR_e ($\times 10^5$ ohm)	$\pm$ %
A 1 - 2	PO_4 6.8	-65	5.4×10^{-2}	In	2.1	PO_4 5.8	-64	5.4×10^{-2}	In	2.8	0.7	+ 33.3
A 3 - 4	PO_4 6.8	-63	2.8×10^{-2}	In	2.0	PO_4 5.8	-63	5.4×10^{-2}	In	2.7	0.7	+ 35
B 1 - 2	PO_4 7.2	-84	8.1×10^{-3}	Out	4.1	PO_4 6.3	-73	8.1×10^{-3}	Out	2.9	-1.2	- 29.2
B 3 - 4	PO_4 7.2	-76	2.1×10^{-2}	Out	0.8	PO_4 6.3	-73	2.1×10^{-2}	Out	3.0	+2.3	+287.5
C 1 - 2	PO_6 7.2	-89	1.0×10^{-2}	In	3.2	PO_4 6.3	-88	1.0×10^{-2}	In	3.6	+0.5	+ 15.6
C 3 - 4	PO_6 7.2	-88	2.5×10^{-2}	In	3.4	PO_4 6.3	-91	2.5×10^{-2}	In	4.0	+0.6	+ 17.6
C 5 - 6	PO_6 7.2	-90	4×10^{-2}	Out	4.6	PO_4 6.3	-93	4×10^{-2}	Out	6.4	+1.8	+ 40.2
C 7 - 8	PO_6 7.2	-92	9.6×10^{-3}	Out	3.4	PO_4 6.3	-93	9.6×10^{-3}	Out	4.6	+1.1	+ 32.3
D 1 - 2	PO_4 6.3	-61	1.6×10^{-2}	In	6	PO_4 7.3	-60	1.6×10^{-2}	In	3.8	-2.2	- 36.6
D 3 - 4	PO_4 6.3	-63	3.3×10^{-2}	In	4.7	PO_4 7.3	-59	3.3×10^{-2}	In	3.6	-1.1	- 23.4
D 5 - 6	PO_4 6.3	-57	4.8×10^{-2}	In	4.4	PO_4 7.3	-55	4.8×10^{-2}	In	2.7	-1.7	- 38.6
D 7 - 8	PO_4 6.3	-58	6.2×10^{-2}	In	5.3	PO_4 7.3	-60	6.3×10^{-3}	In	1.6	-3.7	- 69.8
E 1 - 2	HCO_3-CO_2 7	-67	1.3×10^{-2}	Out	1.3	HCO_3-CO_2 6	-63	1.3×10^{-2}	Out	2.7	+1.4	+107.7
E 2 - 3	HCO_3-CO_2 6	-63	1.3×10^{-2}	Out	2.7	HCO_3-CO_2 5.6	-59	1.3×10^{-2}	Out	3.6	+0.9	+ 32
F 1 - 2	HCO_3-CO_2 7.1	-77	1.3×10^{-2}	In	1.4	HCO_3-CO_2 6	-73	1.3×10^{-2}	In	2.5	+1.1	+ 78.5
F 2 - 3	HCO_3-CO_2 6	-73	1.3×10^{-2}	In	2.5	HCO_3-CO_2 5.7	-71	1.3×10^{-2}	In	3.2	+0.7	+ 23

TABLE IV (Continued)

Muscle	Solutions and pH	E_m (mV)	I_a (μA)	Direction of I_a	R_e (x 10^5 ohm)	Solutions and pH	E_m (mV)	I_a (μA)	Direction of I_a	R_e' (x 10^5 ohm)	ΔR_e (x 10^5 ohm)	± %
G 1 - 2	HCO_3-CO_2 7.1	-63	9.5×10^{-3}	Out	0.3	HCO_3-CO_2 6	-63	9.5×10^{-3}	Out	0.8	+0.5	+166.6
G 2 - 3	HCO_3-CO_2 6	-62	9.5×10^{-3}	Out	0.8	HCO_3-CO_2 5.7	-62	9.5×10^{-3}	Out	1.0	+0.2	+ 25
G 4 - 5	HCO_3-CO_2 7.1	-61	2.6×10^{-2}	Out	0.6	HCO_3-CO_2 6	-64	2.6×10^{-2}	Out	0.8	+0.2	+ 33.3
G 5 - 6	HCO_3-CO_2 6	-64	2.6×10^{-2}	Out	0.8	HCO_3-CO_2 5.7	-63	2.6×10^{-2}	Out	1.2	+0.4	+ 50
H 1 - 2	HCO_3-CO_2 7.2	-60	6.4×10^{-3}	In	1.6	HCO_3-CO_2 6	-65	6.4×10^{-3}	In	2.3	+0.7	+ 43.7
H 2 - 3	HCO_3-CO_2 6	-65	6.4×10^{-3}	In	2.3	HCO_3-CO_2 5.7	-63	6.4×10^{-3}	In	3.0	+0.7	+ 30.4
H 4 - 5	HCO_3-CO_2 7.2	-61	1.2×10^{-2}	In	1.2	HCO_3-CO_2 6	-60	1.2×10^{-2}	In	2.1	+0.9	+ 75
H 5 - 6	HCO_3-CO_2 6	-60	1.2×10^{-2}	In	2.1	HCO_3-CO_2 5.7	-59	1.2×10^{-2}	In	2.2	+0.1	+ 4.8
H 7 - 8	HCO_3-CO_2 7.2	-57	1.4×10^{-2}	Out	1.6	HCO_3-CO_2 6	-60	1.4×10^{-2}	Out	2.6	+1.0	+ 62.5
H 8 - 9	HCO_3-CO_2 6	-60	1.4×10^{-2}	Out	2.6	HCO_3-CO_2 5.7	-59	1.4×10^{-2}	Out	3.2	+0.6	+ 23
H 10-11	HCO_3-CO_2 7.2	-58	3×10^{-3}	Out	2.0	HCO_3-CO_2 6	-59	3×10^{-3}	Out	2.9	+0.9	+ 45
H 11-12	HCO_3-CO_2 6	-59	3×10^{-3}	Out	2.9	HCO_3-CO_2 5.7	-58	3×10^{-3}	Out	3.9	+1.0	+ 34.5
H 13-14	HCO_3-CO_2 7.2	-56	6.8×10^{-3}	Out	1.1	HCO_3-CO_2 6	-59	6.8×10^{-3}	Out	2.2	+1.1	+100
H 14-15	HCO_3-CO_2 6	-59	6.8×10^{-3}	Out	2.2	HCO_3-CO_2 5.7	-63	6.8×10^{-3}	Out	3.0	+0.8	+ 36.3
H 16-17	HCO_3-CO_2 7.2	-64	1×10^{-2}	In	1.2	HCO_3-CO_2 6	-65	1×10^{-2}	In	1.9	+0.7	+ 58.3
H 17-18	HCO_3-CO_2 6	-65	1×10^{-2}	In	1.9	HCO_3-CO_2 5.7	-55	1×10^{-2}	In	2.9	+1.0	+ 52.6

1) The muscle fibres tested were different in size. The total input resistance would be greater in small than in big muscle fibre. This is due to the greater area of the cell membrane of larger fibres. However, since no attempt had been made to measure the sizes of the muscle fibres tested it cannot be estimated to what extent the sizes of muscle fibres would account for the discrepancy in the measured resistances.

2) The distance between two microelectrodes inserted in the same muscle fibre probably differed considerably in different experiments. This might also produce discrepancies between the results of the resistance measurements.

3) The membrane potential varied over a rather wide range. This might be due to the fact that cell membranes had been damaged to varying extents by the double penetration.

4) The rectification of cell membrane in frog sartorius muscle might also be responsible for the difference in the measured resistance between these two groups of experiments, since the property of the rectification varies with the membrane potential level and current density through the membrane.

In brief, although the difference in the measured resistance between the two groups of experiments seems to be signficant the comparison itself, however, is questionable. If experiments with single muscle fibres could be carried out most of the difficulties would be removed and the study on the effects of hydrogen ion concentration upon the membrane resistance would be clarified further.

6. Control experiments

Fig. 26 shows the results of one of the three control experiments. There was no measurable change in potential when microelectrode was inserted in the agar thread and the solutions at different pH levels were introduced.

The lack of response to changes in pH serves to exclude the possibility that the changes in membrane potential observed in the experiments with muscles might be due to the difference in the pH levels between the microelectrode inside the muscle fibre and the reference electrode in the extracellular medium. As has been discussed in Chapter I, Introduction, there is a pH gradient between the inside and outside of a muscle fibre when the external pH is decreased from 7 to 6 or 5.8. Probably, a similar pH gradient existed between the agar thread and external fluid in the control experiments. Therefore, if the observed changes in membrane potential in the experiments with muscles were due to the differences between pH levels in small regions surrounding the two electrodes used there should be detectable changes in potential in the control experiments.

The negative results also suggest that the observations in experiments with muscles are not artifacts arising from either the penetration of the microelectrode or switching from one solution to another. However, the control experiments did not tell anything about the possible effects of the diffusion of ions from KCl-filled microelectrodes and result of alteration of ion contents of the muscle fibres.

Fig. 26

Effects of the difference in pH level between the microelectrode and reference electrode. Plotted here are the results of one of the control experiments with agar fibres. Phosphate buffered solutions (solutions B and C) of pH 6 and 7 were used. KCl microelectrode.

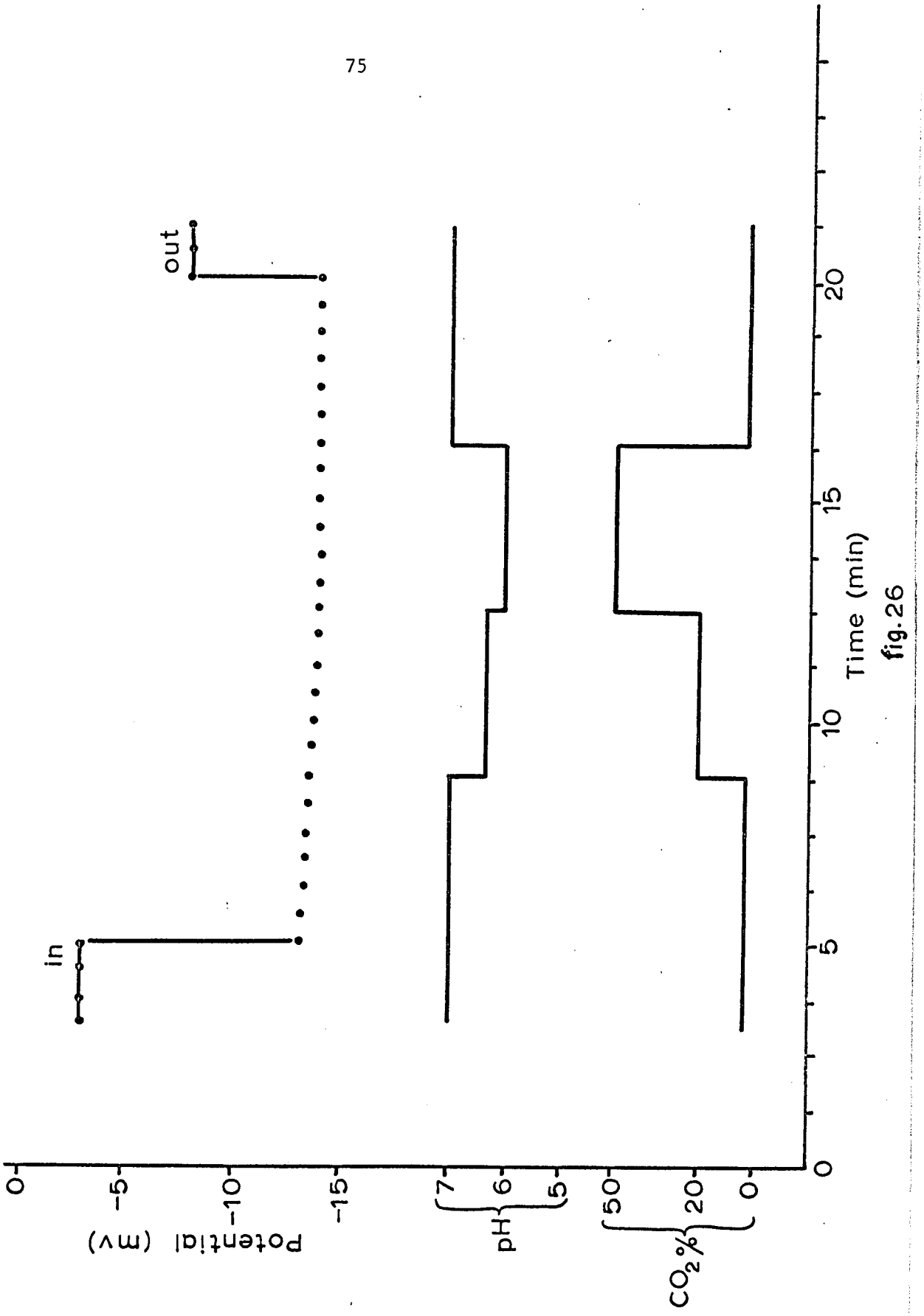

in
out
Potential (mv)
0
-5
-10
-15
pH
7
6
5
CO_2%
50
20
0
0
5
10
15
20
Time (min)

fig.26

The diffusion of Cl^- ions might, to some extent be responsible for the response with changes in membrane potential to pH alteration as will be discussed later.

CHAPTER IV

Discussion

1. Hyperpolarizing and depolarizing responses to H^+ ions

The results presented show that in frog sartorius muscle within a certain range of the resting membrane potential, i.e. -60 to -90 mV, lowering pH over the range of 7.0 to 5.8 results in a reversible increase in membrane potential. In some fibres the reversible change in the potential is superimposed on a slow decrease so that the membrane potential falls with successive cycle. This response occurs in either phosphate or bicarbonate buffered solutions. These findings imply that the change in H^+ ion concentration is the major factor responsible for the observed alteration of the resting membrane potential. This is contrary to the observations reported by some workers (Ling and Gerard, 1949; Kostyuk and Sorokina, 1960; Harris, 1963 and 1965; Mainwood, 1966). This apparent contradiction may result from differences in the time intervals over which the observations were made and differences in experimental conditions. The results reported here are the immediate effect of a pH change measured by continuous recordings of membrane potential from muscles in normal Ringer's solutions. Several of the observations which show the opposite effect are measured only after prolonged exposures to pH changes (Ling and Gerard, 1949; Harris, 1963). Furthermore, in such cases the effects were not demonstrated to be reversible. In other cases the pH fall was achieved by large increases in PCO_2 up to one atmosphere (Ling and Gerard, 1949; Kostyuk and Sorokina, 1960). In one case (Mainwood, 1966) the observations were not made in normal Ringer's solution but in a sucrose medium. Some workers have suggested that the depolarization observed at low pH due to raised CO_2 levels is the result of the loss of K^+ ions from

the fibres due to H^+ - K^+ exchange and accumulation of K^+ ions in the extracellular fluid (Fenn and Cobb, 1934; Shanes, 1948). In the present study, however, the focus was on the immediate response to the changes in pH.

High PCO_2 (50%) has resulted in reversible decreases in membrane potential in some of the experiments reported here (Fig. 7). It seems that in these cases the effect of high PCO_2 (Mainwood, 1966) is dominant over that due to the increase in H^+ ion concentration.

The conditions under which the present experiments were carried out are very similar to those with the same tissue described by Meves and Volkner (1958). The results reported here are in agreement with those of Meves and Volkner who found an immediate and reversible hyperpolarization in response to a pH fall.

2. Possible alternatives for the actions of H^+ ions

The main bulk of the evidence demonstrates that H^+ ions act through a change in conductance of the membrane, particularly in the conductance to Cl^- ions. However, before discussing this evidence, several possible alternatives must be considered.

(a) The possibility of artifacts due to pH effects on the junction potential of the electrodes -

The results of control experiments (Fig. 26) show that the difference in the pH levels between the microelectrode and reference electrode do not cause any detectable change in junction potential which may give rise to an apparent change in the membrane potential.

This may be taken as the evidence that the observed changes in membrane potential are not artifacts arising from the differences in H^+ ion concentrations around the two electrodes.

(b) Adsorption of H^+ ions on the membrane -

It has been claimed that most mammalian cells have negatively charged surfaces (Heard and Seaman, 1960). If it is true in frog sartorius muscle then in the experiments with phosphate buffers as the pH decreased probably there were more H^+ ions adsorbed on the outer surface of the cell membrane. The adsorption of H^+ ions would tend to increase the potential gradient within the membrane and the potential would be added to the total potential measured across the cell membrane (Hecht and Hutter, 1963). As a result the latter would become higher in magnitude. The present experiments cannot provide any information as to what extent the adsorption of H^+ ions may account for the changes in E_m upon the pH changes. However, it seems very unlikely that the adsorption is the only mechanism responsible for the observations, because the manner of the response altered as the concentration of ions on both sides of the cell changed even though the pH changes were the same.

(c) Potential arising from the concentration gradient of H^+ ions across the cell membrane -

Since the cell membrane is permeable to H^+ ions, a change in pH on one side of the cell membrane would cause an alteration in the concentration potential of H^+ ion (E_H) which is given by the equation:

$$E_H = \frac{RT}{F} \ln \frac{H_o}{H_i} \qquad \ldots\ldots\ldots\ldots\ldots (1)$$

However, because the absolute H^+ ion concentrations on both sides of the cell are so low (i.e. about 10^{-4} of the concentrations of the major current carrying ions in the tissue) and the membrane resistance to the cation is so high that E_H should be too small to affect the E_m.

(d) Effect of H^+ ions on the Na^+-pump -

Intracellular H^+ ions may compete with Na^+ ions for the sites concerned with the active Na^+ transport. This may result in an accumulation of Na^+ ions in the cells. Considering the fact that the results of the experiments with bicarbonate and phosphate buffered Ringer's solutions are comparable it seems that the inhibition effect of H^+ ions on the Na^+ transport can only be negligible.

The effects of H^+ ions described in (c) and (d) should reduce the membrane potential and are, therefore, opposite to the observed effect.

3. Effects of H^+ ions on the cell membrane

The most likely remaining alternative is that H^+ ions have an effect upon the membrane properties, in particular, the permeability of the membrane to the major current carrying ions. The evidence for this will now be considered.

(a) Site of H^+ ion action -

The results reported by many workers (Hill, 1955; Caldwell, 1958; Kostyuk and Sorokina, 1960) show that CO_2 causes rather obvious

changes in the intracellular pH of various tissues, including skeletal muscles, but other acidifying agents do not seem to change the pH readily in the absence of CO_2. Therefore, the similarity between the responses due to alteration of pH by phosphate and bicarbonate buffered Ringer's solutions provides strong evidence that H^+ ions must exert their effect by acting on the outer surface or on the sites near the outer surface of the cell membrane. The interpretation is in agreement with the one made by other workers (Vaughan Williams and Whyte, 1967; Hutter and Warner, 1967).

(b) The effect of H^+ ion concentration on membrane resistance or membrane permeability -

The observed changes in membrane resistance which result from a fall in pH thus appear to be due to the binding of H^+ ions by sites on the outer membrane surface. This is in agreement with the conclusions of other workers (Meves and Volkner, 1958; Hecht and Hutter, 1963; Hutter and Warner, 1967).

Electrical currents across the membrane are carried chiefly by Na^+, K^+ and Cl^- ions. Generally speaking any change in the membrane resistance can be looked upon as a change in membrane permeabilities to one of these ions.

According to Meves and Volkner (1958) the increase in membrane resistance due to protons is the result of a decrease in K^+ conductance. It seems to other workers (Brooks and Hutter, 1962, 1963; Hecht and Hutter, 1963; Hutter and Warner, 1967) that the increase in the membrane

resistance is due to the reduction of membrane permeability to Cl^- ions, the K^+ permeability of resting muscle fibres being little affected by H^+ ions. There are still other workers (Straub, 1956; Shanes, 1958) who propose that the changes in membrane potential are associated with changes in Na^+ conductance of the membrane.

The results of the present investigation allow us to judge which of the above mentioned working models fits best.

(c) Changes in the permeabilities of the membrane to Na^+, K^+ or Cl^- ions -

It is generally accepted that in frog skeletal muscles the major current carrying ions are Na^+, K^+ and Cl^-, and the membrane potential is given by the Goldman constant field equation (Goldman, 1943; Hodgkin and Katz, 1949).

$$E_m = \frac{RT}{F} \ln \frac{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i}{P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o} \quad \ldots\ldots (2)$$

From the known data concerning the ion concentrations (Table I) and membrane permeabilities to the ions, E_m in a normal muscle fibre would fall between the equilibrium potential of K^+ and Cl^- and much higher than the equilibrium potential of Na^+, that is, $E_K > E_m > E_{Cl}$ and $E_K \gg E_{Na}$. Also from the Goldman equation and the relations between E_m and the equilibrium potentials of these ions it can be seen that a decrease in Na^+ permeability, everything else being unchanged, would increase the E_m. Similarly, E_m should decrease or increase as K^+ or Cl^- permeability, respectively fall.

By changing the extracellular ion concentrations the relationship between the equilibrium potentials of the ions and E_m, as well as E_m itself can be altered. As a result changes in the conductance to any one ion species should have different relative effects on membrane potential depending on the equilibrium potential of that ion relative to the membrane potential. Therefore, if the change in concentration of an ion species is known the responses of the membrane potential to pH changes may provide some clues to the change in membrane permeability. This was the objective of the experiments shown in Fig. 16, 18 and 20.

(i) Effect of H^+ ions on the Na^+ permeability -

The experiments by Straub (1956) showed that in myelinated nerves the initial hyperpolarization by elevated H^+ ion concentration or PCO_2 was depressed in the absence of Na^+ ions. This finding suggests that increased H^+ ion concentration decreases Na^+ permeability. The idea is also implied by the findings in experiments with mammalian cortical neurons reported by Krnjevic, et al (1965). Hecht and Hutter (1963) proposed a similar effect of low pH in sheep Purkinjie fibres. Thus, in acid choline Ringer's solutions the E_m of fibres depolarized slightly. But in the presence of Na^+ ions hyperpolarization occurred as the bathing fluid was made acid.

The results of the present study, however, show that changes in pH have the same effect on the membrane potential in frog sartorius muscle in Ringer Solution or when choline is used to replace Na^+ ions. Furthermore, in the experiment shown in Fig. 16, with normal Ringer's solutions, the hyperpolarizing effect of H^+ions is reversed by increasing the K^+

concentration. In this experiment the E_{Na} remains unchanged and any decrease in Na^+ permeability should have a hyperpolarizing effect at high, as well as low K^+ concentration levels. The results, therefore, suggest that Na^+ ions are not involved. This conclusion is supported by the fact that the reversal of the H^+ ion effect is seen when the K^+ concentration is suddenly increased in choline Ringer's solutions where no Na^+ ion is present (Fig. 18).

(ii) Effect of H^+ ions on K^+ permeability -

The experimental results reported by some workers (Dettbarn and Stempfli, L957; Meves and Völkner, 1958; Hecht and Hutter, 1963) show that H^+ ions have some effect on the K^+ permeability. According to Meves and Völkner (1958) the K^+ permeability of frog skeletal muscle is decreased by low pH. The hypothesis can be discussed by considering the experiments with K^+-rich solutions at different pH levels.

In the following discussion, E_{m_1}, E_{K_1}, E_{Cl_1} and E_{Na_1}, and the ratios $\frac{Cl_{o_1}}{Cl_{i_1}}$, $\frac{K_{i_1}}{K_{o_1}}$, and $\frac{Na_{i_1}}{Na_{o_1}}$ represent the membrane potential, equilibrium potentials of K^+, Cl^- and Na^+ ions and the ratios $\frac{Cl_o}{Cl_i}$, $\frac{K_i}{K_o}$ and $\frac{Na_i}{Na_o}$, respectively, when muscle is soaked in Ringer's solutions containing 2.5 mM/l K^+. Muscle that was perfused with solutions containing high K^+ or low Cl^- concentration is indicated by the subscript 2 of each of the designations and ratios, for example, E_{m_2} or $\frac{Cl_{o_2}}{Cl_{i_2}}$. The figure 3 is used as the subscript in the cases when the solutions

containing normal K^+ or Cl^- ions are readmitted.

Upon switching to K^+-rich solutions (10 mM K^+) the membrane potential decreased. At the very instant when the new membrane potential is reached, assuming no redistribution of ions occurs, the ratios, $\frac{Cl_{o2}}{Cl_{i2}}$ and $\frac{Na_{i2}}{Na_{o2}}$ should be the same as those in normal Ringer's solution, while the ratio $\frac{K_{i2}}{K_{o2}}$ is reduced to 1/4 of $\frac{K_{i1}}{K_{o1}}$ theoretically. If the membrane does behave as a perfect K^+ electrode at this high K^+ concentration level, according to calculation the E_K should decrease by about 35 mV and the E_m should be very close to E_K. However, in the present study, the mean maximum decrease in E_m in 4 fibres at pH 6 within 5 minutes is 24 ± 2.7 (mean ± S.E.), while the decrease in 3 fibres at pH 7 is 16.1 ± 2. The discrepancy between the theoretical and observed values may be explained as follows. Upon the admissions of the K^+-rich solutions a non-equilibrium condition is set up under which K^+ and Cl^- ions are flowing into the cell. The E_m, E_K and E_{Cl} are related to each other by the following equations:

$$E_m = E_K - I_K R_K = E_{Cl} + I_{Cl} R_{Cl} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where I_K and I_{Cl} represent the currents carried by K^+ and Cl^- ions, respectively; R_K and R_{Cl} represent the membrane resistance to K^+ and Cl^- ions respectively. The difference between the calculated and measured decreases arises from the different membrane resistances to ions at the two pH levels. This will be discussed in more detail later.

In addition to the reason just discussed, an imcomplete displacement of solutions in the extracellular spaces might also be responsible for the discrepancy.

Consequently the relationship between the E_m and equilibrium potentials of all ions must change. Thus, $E_{Cl_2} > E_{m_2} > E_{K_2}$. Under this circumstance a decrease in K^+ permeability should increase E_m. However, the results reported here are just the opposite of those expected assuming H^+ ions decrease K^+ permeability. Therefore, the finding that low pH decreases the E_m, whereas high pH increases it, during K^+ depolarization contradicts the suggestion that K^+ permeability is sensitive to changes in pH.

On readmission of normal K^+ Ringer's solutions the membrane potential increased as predicted from the Goldman equation. During K^+-rich solution perfusion redistribution of ions must have occurred. Consequently, E_{K_3} was much larger than E_{m_3}. Under this condition, if increased H^+ ion concentration did reduce K^+ permeability we would expect a big decrease in membrane potential when the muscle was perfused with acid solution and a big increase in membrane potential when it was perfused with alkaline solution. Again, the opposite is what has been observed.

If the assumption is made the H^+ ions increase the K^+ permeability, the resultant changes in membrane potential would become explicable. But in so doing the increase in membrane resistance at low pH and the change in the rate of ^{42}K efflux at different pH levels as

reported by some workers (Brooks and Hutter, 1960; Hutter and Warner, 1967b) would become difficult questions to account for.

An alternative explanation would be that H^+ ions may have effects on both K^+ and Cl^- permeabilities and that the effect on the former may be overshadowed by the stronger effect on the latter, since under the condition described here changes in K^+ and Cl^- permeabilities in the same direction would have opposite effects on the membrane potential. However, the experiments just described are not able to distinguish between the effect on K^+ permeability and that on Cl^- permeability. The problem could be clarified better by comparing the experiment in which both intra- and extracellular K^+ concentrations are within normal range, to that in which all extra- and intracellular K^+ ions have been removed.

(iii) Effect of H^+ ions on Cl^- permeability -

The results of the experiments with low Cl^- solutions at different pH levels support the hypothesis that H^+ ions act on Cl^- permeability, although they by no means argue against the possibility that K^+ permeability is sensitive to H^+ ions.

On switching from normal K^+ and Cl^- to normal K^+ low Cl^- solutions the ratio $\frac{Clo_2}{Cli_2}$ is reduced. Therefore, E_{Cl_2} becomes much lower than E_{m_2}. On the other hand, $\frac{Ki_2}{Ko_2}$ remained unchanged, and since E_{m_2} decreases E_{K_2} becomes even bigger than E_{m_2}. If H^+ ions do decrease Cl^- permeability, the membrane potential at low pH should increase because it becomes less sensitive to the equilibrium potential of Cl^-. Obviously, the results

favor the suggestion that Cl^- permeability is sensitive to H^+ ions.

Because of the relatively high Cl^- permeability and the electrochemical gradient of the ion, redistribution of Cl^- ions was going on during the perfusion with low Cl^- solutions. Consequently, at the end of this period of time, as membrane potential gradually increased toward the normal level, the ratio $\frac{Cl_{o_2}}{Cl_{i_2}}$ and E_{Cl_2} increased as well. The result is the difference between E_{m_2} and E_{Cl_2} became smaller than at the beginning of the perfusion. This explains why the increased responsiveness, which was evident upon admission of low Cl^- solution, did not last for long but decreased with time.

Furthermore, as redistribution of ions took place, at the end of perfusion with low Cl^- solutions, the intracellular Cl^- concentration must have been lower than that in the normal fibre. Consequently on switching back to normal Cl^- Ringer's solutions, the ratio $\frac{Cl_{o_3}}{Cl_{i_3}}$ and E_{Cl_3} should be larger than those in normal fibre. Also, E_{Cl_3} would be larger than E_{m_3}. In this case, a decrease in Cl^- permeability would result in a drop in membrane potential, while an increase in Cl^- permeability would result in a rise in membrane potential. This is actually observed. The result, therefore, fits well with the suggestion that H^+ ions decrease Cl^- permeability of the membrane.

The experiment with K^+-rich normal Cl^- solutions can be explained equally well by assuming that H^+ ions decrease Cl^- permeability. Since $E_{m_2} > E_{K_2}$, $E_{Cl_2} > E_{m_2} > E_{Na_2}$ a decrease in Cl^-permeability due to an

acid solution would reduce the membrane potential. Alkaline solution which presumably restores the Cl^- permeability, would have the opposite effect on the membrane potential.

Because Cl^- ions pass into the cell during K^+ depolarization, repolarization on readmission of normal K^+ Ringer's solutions would set up a new relationship between E_m and E_{Cl}, that is E_{m3} becomes much larger than E_{Cl3}. Consequently, E_{m3} increased at low pH and decreased at high pH levels and the fluctuation of membrane potential in changing pH levels would be larger than that at the onset of the experiment.

If the above conclusion is true, it will lead to the confirmation that the E_{Cl} of frog sartorius muscle in vitro is lower than the E_m (Hodgkin, 1951, Horowicz, 1960). However, as the values of Cl_i and E_{Cl} of the tissue in vivo are still unsolved problems, it is of interest to investigate how the tissue would respond with changes in E_m to the pH alteration.

(d) Theoretical approach to membrane permeability reduction due to H^+ ions -

The previous discussion of the effects on each of the component resistances of the membrane can be pushed a little further by a theoretical treatment as follows:

If the total input resistance increases 40% when extracellular pH is lowered from 7 to 6, the membrane resistance of the muscle fibre would increase 2 times (refer to Chapter III). If the K^+ resistance (R_K) and Cl^- resistance (R_{Cl}) at pH 7.0 are assumed to be $1 \times 10^4\ \Omega\ cm^2$

and $5 \times 10^3\ \Omega\ cm^2$, respectively (Hodgkin and Horowicz, 1959), the surface area of a muscle fibre of 100 μ in diameter and 3 cm in length to be $3.8 \times 10^{-2}\ cm^2$, the total R_K and R_{Cl} would be $2.63 \times 10^5\ \Omega$ and $1.32 \times 10^5\ \Omega$, respectively. Since when a current is applied across the cell membrane the two component resistors are parallel, the total membrane resistance (R_{m7}) can be calculated as follows:

$$\text{since } \frac{1}{R_m} = \frac{1}{R_K} + \frac{1}{R_{Cl}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(4a)}$$

$$\text{or } R_m = \frac{1}{\frac{1}{R_K} + \frac{1}{R_{Cl}}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(4b)}$$

$$\text{hence } R_{m7} = \frac{1}{\frac{1}{2.63 \times 10^5} + \frac{1}{1.32 \times 10^5}}$$

$$= 0.88 \times 10^5\ \Omega$$

Since the membrane resistance at pH 6 (R_{m6}) is twice as large as that at pH 7, therefore

$$R_{m6} = 2 \times 0.88 \times 10^5\ \Omega$$

$$1.76 \times 10^5\ \Omega$$

Assuming all the change in membrane resistance upon altering pH is in the R_{Cl} it will be

$$R_{Cl} = \frac{1.76 \times 10^5 \times 2.63 \times 10^5}{2.63 \times 10^5 - 1.76 \times 10^5}$$

$$= 5.32 \times 10^5\ \Omega$$

which is an increase of about 303%. On the other hand, R_K remains the same as at pH 7, i.e. $2.63 \times 10^5\ \Omega$

Suppose that the E_K and E_{Cl} of muscle fibre soaked in 2.5 mM/l K^+ Ringer's solution are 98 mV and 88 mV, respectively (Hodgkin, 1951), the E_m at pH 7 can be calculated as follows. Since the R_{Na} in the membrane of muscle fibre is so great that the current carried by Na^+ is very small and can be ignored, consequently, the R_K and R_{Cl} are in series and E_m would be:

$$E_m = E_K - I_K R_K = E_{Cl} + I_{Cl} R_{Cl} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

By substitution of the values in equation (3),

$$E_m = 98 - \frac{98 - 88}{(2.63 + 1.32) \times 10^5} \times 2.63 \times 10^5$$

$$= 88 + \frac{98 + 88}{(2.63 + 1.32) \times 10^5} \times 1.32 \times 10^5$$

$$= 91.4 \text{ mV}$$

Similarly, the E_m at pH 6 can be worked out to be 94.9 mV. The difference between the E_m at these two pH levels is 3.5 mV.

When the muscle is transferred to 10 mM/l K^+ Ringer's according to calculation E_K would decrease by 35 mV while E_{Cl} remains the same as in 2.5 mM/l K^+ Ringer's, that is, E_K = 63 mV, E_{Cl} = 88 mV. By applying the same calculation discribed above, the E_m at pH 7 and 6 can be worked out to be 79.6 mV and 71.2 mV, respectively. The difference between them is 8.4 mV.

The values obtained by calculation are in agreement with those observed in the experiment shown in Fig. 16. The small divergence between the calculated and observed values, especially in 2.5 mM/l K^+ Ringer's may be attributed to the fact that K^+ resistance varies with the direction of electrochemical potential of K^+.

On the other hand, the hypothesis that all changes in the total input resistance is due to changes in the K^+ resistance of the membrane, is impossible because this would require that K^+ resistance becomes negative in order to satisfy the observed increase in the total membrane resistance when extracellular pH is changed from 7 to 6.

If the relation between E_m, intracellular Cl^- concentration, and the flow rates of Cl^- and K^+ is known the above described theoretical consideration may become more realistic and the whole course of changes in E_m can be plotted against time.

Fig. 27 shows the correlation of the flow rate of Cl^- ions, intracellular Cl^- concentration and the E_m of frog sartorius muscle soaked in solution at pH 7 (Courtesy of Dr. G.W. Mainwood). The graph is obtained by employing the following equation (Hodgkin and Horowicz, 1959):

$$P_{Cl} = M_{Cl} \frac{RT}{VF} \frac{1 - \exp(-VF/RT)}{Cl_o - Cl_i \exp(-VF/RT)} \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

where, M_{Cl} is the net flow of Cl^- through the muscle fibre; P_{Cl}, permeability coefficient of Cl^- ions; V, measured membrane potential; R, F and T have the usual meanings. P_{Cl} is considered constant at any given pH since it is little affected by changes in E_m or concentration of Cl^- ions, (Hodgkin and Horowicz, 1959). If different values are taken for M_{Cl} a family of curves representing the change in the intracellular concentration of Cl^- ions versus the change in E_m at different flow rates of the anion can be plotted. When E_m and intracellular Cl^- concentration are known the flow rate of Cl^- can be read off from the graph. Under non-

Fig. 27

The change in membrane potential and intracellular Cl^- concentration upon altering extracellular K^+ concentration. Single arrows (⟶) indicate the course of change in membrane potential on switching from 2.5 to 10 mM K^+ Ringer's solution. Double arrows (⟶⟫) designate the course of change in membrane potential during the restoration of 2.5 mM K^+ Ringer's. The solutions were at pH 7. Time courses are given by the vertical lines on both sides.

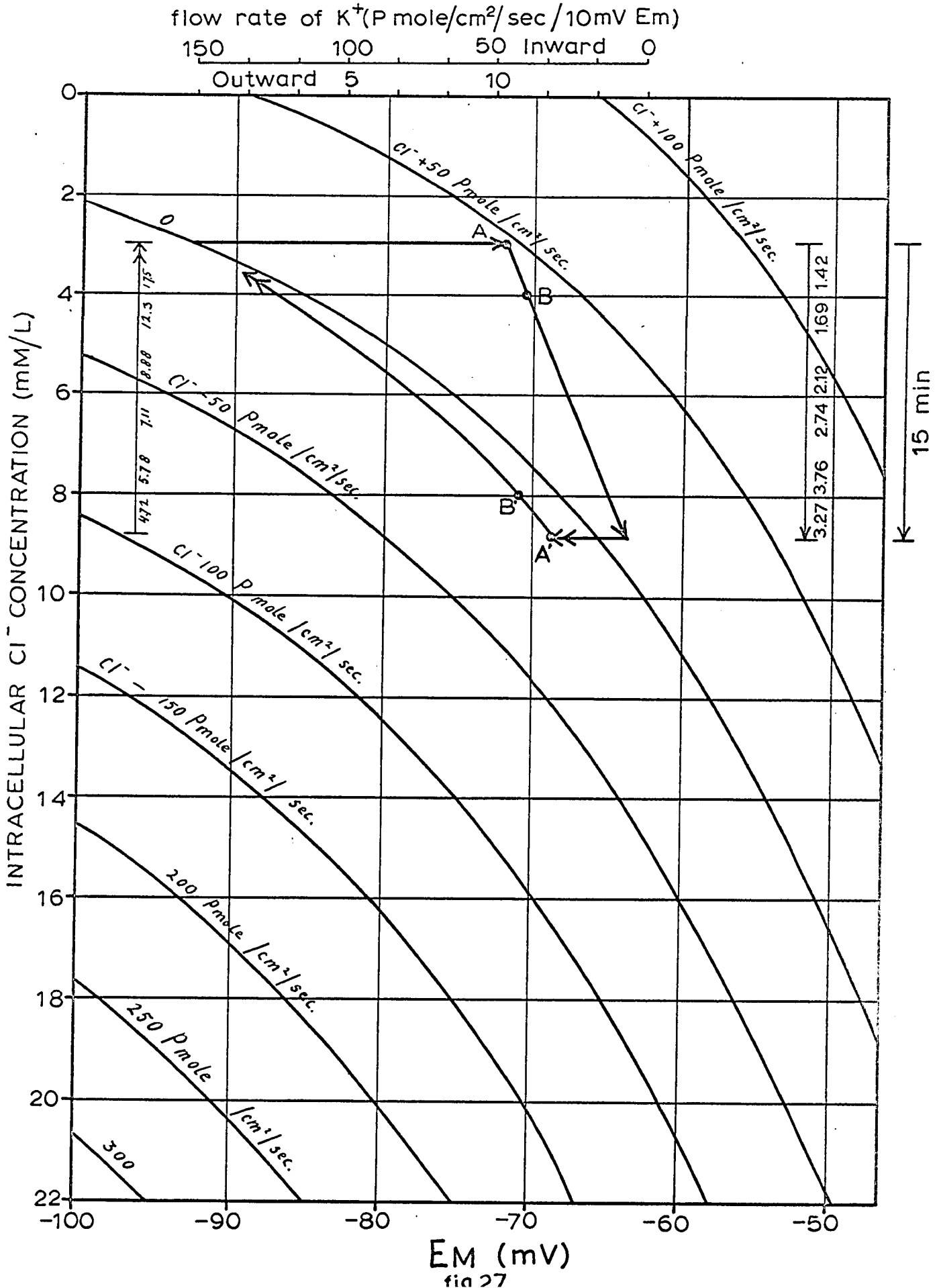

flow rate of K+(P mole/cm²/sec/10mV Em)
150
100
50 Inward
0
Outward 5
10
INTRACELLULAR Cl⁻ CONCENTRATION (mM/L)
0
2
4
6
8
10
12
14
16
18
20
22
-100
-90
-80
-70
-60
-50
EM (mV)
Cl⁻+50 Pmole/cm²/sec.
Cl⁻+100 Pmole/cm²/sec.
0
Cl⁻ 50 Pmole/cm²/sec.
Cl⁻ 100 Pmole/cm²/sec.
Cl⁻ 150 Pmole/cm²/sec.
200 Pmole/cm²/sec.
250 Pmole/cm²/sec.
300
A
B
A'
B'
15 min
1.75
12.3
8.88
7.11
5.78
4.71
1.42
1.69
2.12
2.74
3.76
3.27

fig 27

equilibrium conditions, e.g. when fibres equilibrate in normal Ringer's solution containing 2.5 mM K^+ are transferred to a solution containing 10 mM K^+, for example, the E_m at a given moment can be worked out by means of the graph by assuming $M_K = M_{Cl}$, if the intracellular Cl^- concentration is known.

By making use of the graph just described and using the values of R_K given by Hodgkin and Horowicz (1959) the inward and outward flow rates of K^+ can be shown to be 50 and 5 Pmole/cm2/sec/10 mV, respectively. The changes in E_m upon switching from 2.5 to 10 and then back to 2.5 mM/l K^+ Ringer's solutions (pH 7) have been calculated and are shown in Fig. 27. The calculation is described separately.

Fig. 28 shows the change in E_m when the muscle fibre is given the same treatment as that in Fig. 27, except that the extracellular pH is 6 instead of 7. Here again it is assumed that the total input resistance increases 40% in lowering the pH from 7 to 6 and that all changes are in the Cl^- resistance. Accordingly, the R_{Cl} at pH 6 will be 4 times as big as that at pH 7, as shown in Fig. 28.

Fig. 29 shows the plots of E_m obtained from Fig. 27 and 28, against time. As can be seen in the figure, 5 minutes after admission of 10 mM K^+ Ringer's solution the E_m at pH 7 is higher than that at pH 6 by about 2.6 mV. On the other hand, 5 minutes after switching back to 2.5 mM K^+ Ringer's, the E_m at pH 7 now is lower than that at pH 6 by about 11 mV. These figures are very close to the experimental values, (Fig. 16, 18).

Fig. 28

The change in membrane potential and intracellular Cl^- concentration upon alteration in extracellular K^+ concentration. The treatment is similar to that in Fig. 27 except that the pH level of solutions in the present case is assumed to be 6.

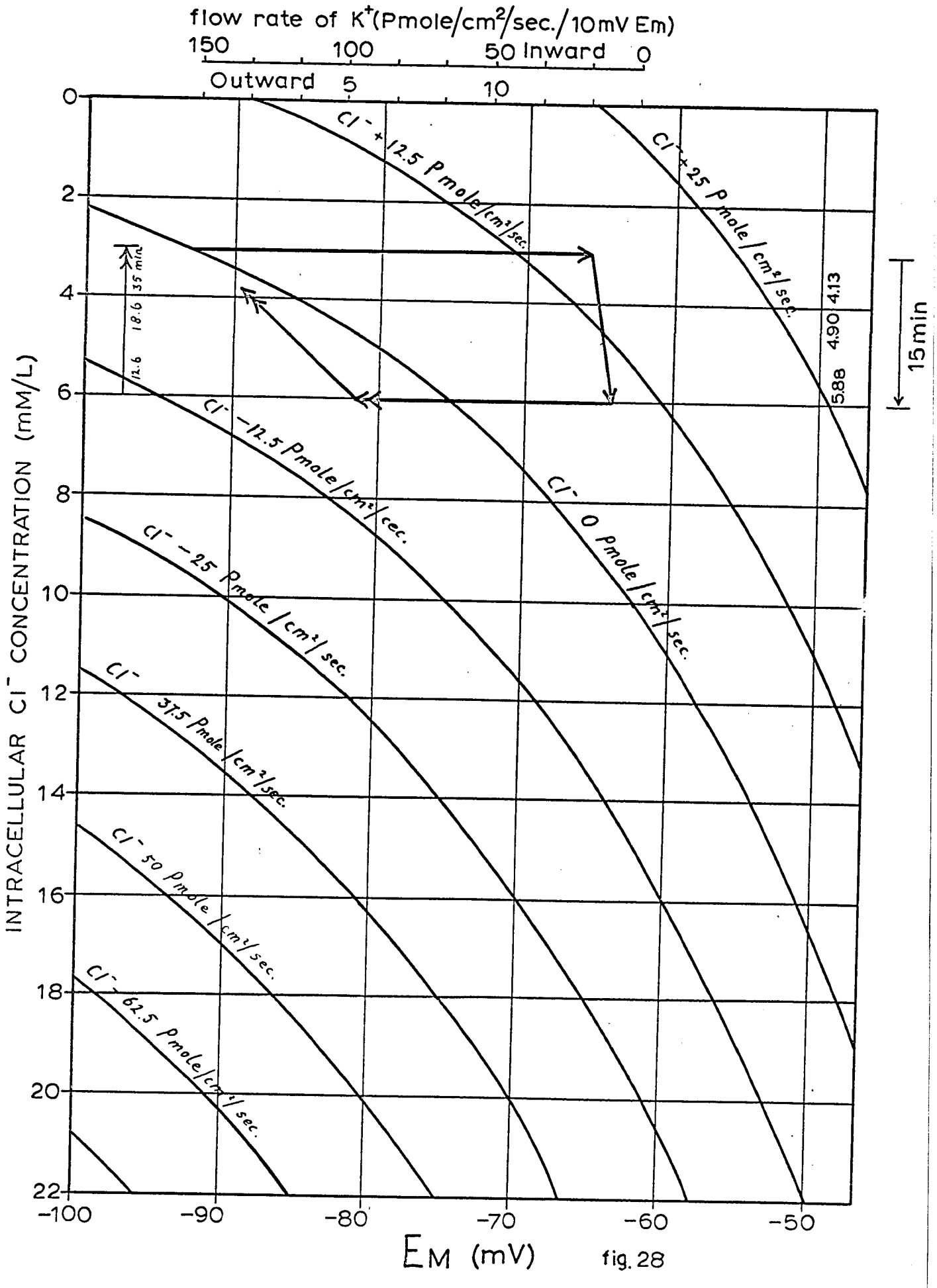

flow rate of K+(Pmole/cm2/sec./10mV Em)
150
100
50 Inward
0
Outward 5
10
Cl⁻ +12.5 Pmole/cm²/sec.
Cl⁻ +25 Pmole/cm²/sec.
35 min
18.6
12.6
4.13
4.90
5.88
15 min
Cl⁻ −12.5 Pmole/cm²/sec.
Cl⁻ 0 Pmole/cm²/sec.
Cl⁻ −25 Pmole/cm²/sec.
Cl⁻ 37.5 Pmole/cm²/sec.
Cl⁻ 50 Pmole/cm²/sec.
Cl⁻ 62.5 Pmole/cm²/sec.
INTRACELLULAR Cl⁻ CONCENTRATION (mM/L)
0
2
4
6
8
10
12
14
16
18
20
22
−100
−90
−80
−70
−60
−50
EM (mV)

fig. 28

Fig. 29

Plots of membrane potential against time. The course of change in membrane potential obtained from Fig. 27 is represented by continuous line, while that from Fig. 28, by broken line.

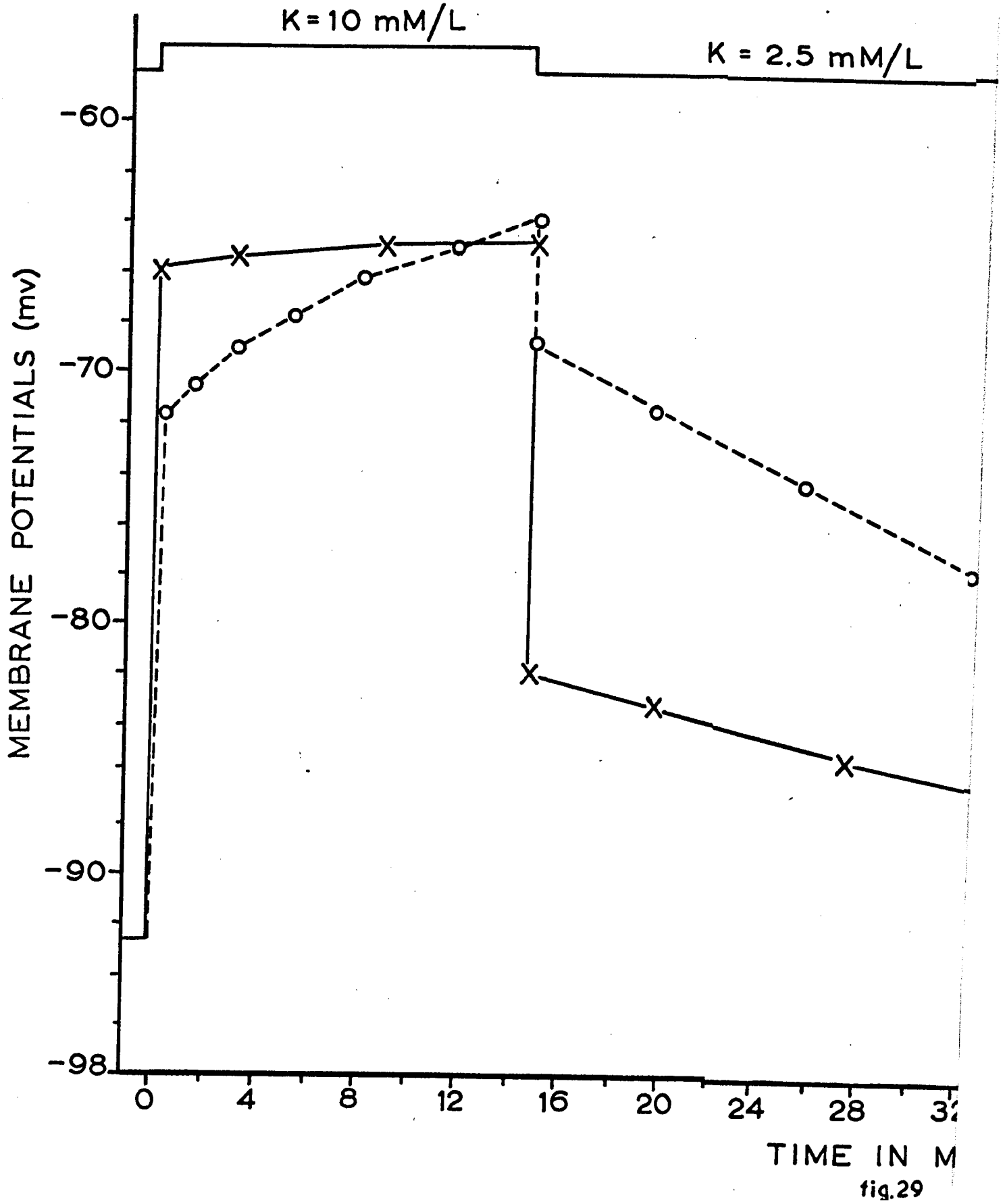

K = 10 mM/L
K = 2.5 mM/L
-60
-70
-80
-90
-98
MEMBRANE POTENTIALS (mv)
0
4
8
12
16
20
24
28
TIME IN M

fig.29

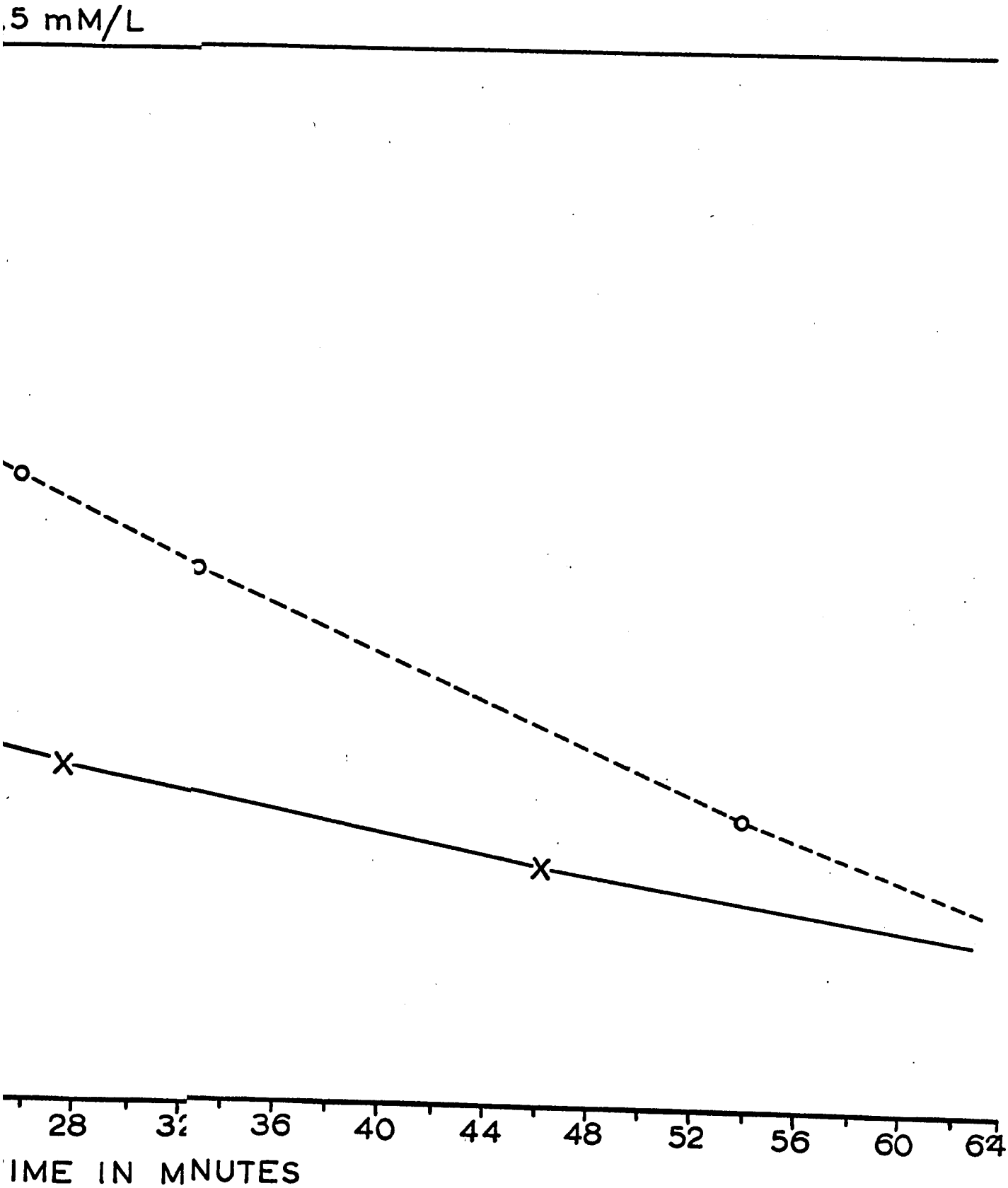


fig.29

Therefore, the results obtained both from observation and calculation point strongly to the possibility that H^+ ions act chiefly on Cl^- resistance of cell membrane of frog sartorius muscle, while a relatively small effect on K^+ resistance cannot be completely excluded.

(e) Some minor factors concerning the P_{Cl} reduction hypothesis -

Because many of the KCl microelectrodes used in the experiments presented here had resistance of about 5 M Ω, the flux of KCl from the electrode into fibre would be 0.06 pmole/sec (Hodgkin, 1951; Coombs, Eccles and Fatt, 1955). Let us consider a small region around the tip of the microelectrode inserted in the fibre. If the length of the part concerned is 1 mm and the diameter of the fibre is 80 μ, the volume of the region would be about 5×10^{-11} l. The rate of increase in Cl^- concentration in the region would be 0.72 mM/l/min. Assuming that the equilibrium of influx and efflux of Cl^- ions is reached in three minutes after the insertion of a 3 M KCl microelectrode, the net increase in the intracellular Cl^- concentration would be 2.2 mM/l, which is about 20% of the intracellular Cl^- concentration of the muscle which has been soaked in normal Ringer's for a couple of hours (Hodgkin, 1950). Consequently, the E_{Cl} in the muscle fibre would be lower than that of muscle fibre impaled by a 3 M K-citrate microelectrode. If H^+ ions do have effect on Cl^- permeability of cell membrane in the muscle the response with change in resting potential to the alteration of pH would be larger in a KCl microelectrode inserted than in a K-citrate microelectrode inserted muscle. However, in the experiments reported here, no significant difference can be demonstrated between the results obtained using KCl and

K - citrate electrodes. To avoid the possibility of Cl^- ion leakage through the electrode tip, K - citrate filled electrodes were used in most experiments after the preliminary ones.

Alternatively, Cl^- ions might also get into the fibre through the damaged cell membrane where the impalement is made. A regional increase in the intracellular Cl^- ion concentration would result. This would have the effect similar to that of KCl-filled microelectrode with low resistance. While the possibility of a slow inward leakage of Cl^- ions through the part of impalement cannot be excluded and may contribute to the increase in pH response with time, in many cells in which there was no apparent fall in membrane potential indicating a good seal between the electrode and membrane, pH effects were still observed.

4. Mechanisms of actions of H^+ ions on the membrane

The mechanism by which H^+ ions act on the membrane to change its permeability to ions is still an open question. So far several hypotheses have been proposed. According to one working model, which can now be considered a classic one, H^+ ions are adsorbed to the negatively charged groups on the membrane. In doing so they reduce the number of sites to which the major current carrying cations are adsorbed in their passage through the membrane. This is the basis of the hypothesis by Meves and Volkner (1958) which states that H^+ ions decrease K^+ permeability. But the model fails to explain the observations reported here as well as those made by others. For instance, when K^+ ions were the only current carrying ions on the both sides of muscle fibre, no change in membrane conductance

at different pH values was observed (Hutter and Warner, 1967a). Furthermore, it has been found that the sensitivity of Cl^- permeability to pH is not affected by Ca^{++}. On the other hand, as H^+ ions, certain foreign divalent cations, e.g. Cu^{++}, Zn^{++}, UO_2^{++} etc. can decrease the Cl^- permeability. All these findings have led Hutter and Warner (1967b) to the suggestion that binding of H^+ ions with macromolecules, probably those with imidazole groups in the membrane, results in a stronger binding of Cl^- by the protein molecules. In other words, the Cl^- permeability is dependent on a pH sensitive dissociation rate of the Cl^- macromolecule complex formed in the cell membrane. It seems that there are more sites remaining unoccupied by Cl^- ions at high than at low pH levels, and that the Cl^- permeability of the membrane is proportional to the number of the unoccupied sites. According to Harris (1965) the increase in H^+ ion concentration renders the transport of Cl^- ions through the membrane more difficult by increasing the number of cationic sites in proteins or other amphoteric molecules in the membrane.

5. Physiological significance of the pH response

So far no information is available concerning the physiological role of the change in E_m or Cl^- permeability upon pH alteration. However, a few speculations can be made along the lines as follows:

(i) Since E_m is sensitive to the changes in pH in the range of 6 - 7, which is not far on either side from the normal pH value, a decrease in pH of short time duration of the extracellular fluid, e.g. blood, under certain physiological or pathological conditions may cause

hyperpolarization in the muscle fibres. As a result stimulation which can produce bigger fluctuation of membrane current is required to bring the E_m toward the threshold level, otherwise, some fibres could not be fired at all.

(ii) During muscle activity the Cl^- content of the fibres should increase to a certain extent. Accordingly in active muscle fibres larger response to pH changes would be expected.

(iii) Reduction in Cl^- permeability as a result of H^+ ion accumulation in the extracellular fluid may prevent drastic osmotic changes which would otherwise result in swelling in muscle fibres.

Summary

1. Changes in the extracellular hydrogen in concentration have a definite effect on the resting membrane potential of frog sartorius muscle fibres. In normal Ringer's solutions a sudden change of pH from 7 to 6 results in a rapid but reversible increase in the resting membrane potential of the order of 1-3 mV.

2. When muscle fibres undergo depolarization in 10 mM K^+ solutions membrane potential can be further reduced by lowering pH from 7.0 to 6.0, thus reversing the normal pH response. Upon restoration of normal K^+ concentration the fibres again responded to changes in pH in the same directions as those in 1, but with a greater magnitude.

3. The changes in the membrane potential mentioned in 1. and 2. remained practically the same whether Na^+ is present or absent on the both sides of the cell membrane.

4. During the transient depolarization induced by soaking the muscle in 60 mM Cl^- Ringer's solutions the resting membrane potential increased at low but decreased at high pH. On switching back to normal Cl^- Ringer's solutions the manners of changes in membrane potential in response to altering pH levels reversed.

5. Membrane resistance altered reversibly upon changing pH. Lowering pH from 7 to 6 increased the membrane resistance by 54% in phosphate buffer to 77% in bicarbonate buffer.

6. An estimate of the effect of H^+ ions on each of the resistance components of the cell membrane has been made. It seems very likely that the ion acts chiefly on the Cl^- resistance, although the

possibility of a small influence of H^+ on K^+ resistance of the membrane cannot be completely ruled out.

7. It is concluded that the hyperpolarizing effect of hydrogen ions is a consequence of the decrease in chloride conductance of the cell membrane. This conclusion helps confirm that E_{Cl} is normally less than E_m under the experimental conditions reported here.

Appendix

Calculation of non-equilibrium potentials and chloride movements in high potassium solutions.

If the E_m of a muscle fibre soaked in normal Ringer's solution containing 2.5 mM K^+ is -93 mV, admission of 10 mM K^+ solution causes an immediate shift of E_m to a new level, -73 mV (point A in Fig. 27), at which the currents carried by Cl^- and K^+ ions are equal, that is, $I_{Cl} = I_K = 46$ pmole/cm^2/sec (from Fig. 27), while the intracellular Cl^- concentration, say 3 mM/l, is assumed unchanged at that instant. However, equal amounts of Cl^- and K^+ ions are flowing into the cell until E_m is equal to, or very close to E_K. If the volume of muscle fibre (V) is 1.5×10^{-4} cm^3 and the surface area (A) 3.8×10^{-2} cm^2, the time (T) required for the increase in the intracellular Cl^- concentration by I mM/l, say from 3 to 4 mM/l, is calculated as follows:

$$T = \frac{V}{I_{Cl} \times 60 \times 10^{-9} \times A \times 10^3}$$

$$= \frac{1.5 \times 10^{-4}}{46 \times 60 \times 10^{-9} \times 3.8 \times 10^{-2} \times 10^3}$$

$$= 1.42 \text{ min.}$$

Once the Cl^- concentration increases up to 4 mM/l, E_m will decrease to a new level (Point B) at which the I_{Cl} and I_K are again equal. By following the same procedure we can calculate the whole course of change in E_m and the time required as Cl^- and K^+ ions move into the cell. Finally a potential level will be reached at which I_{Cl} and I_K are equal to zero. This is the equilibrium potential of K^+ at 10 mM K^+ concentration.

Readmission of 2.5 mM K^+ Ringer's increases E_m and causes effluxes of Cl^- and K^+ ions. If the perfusing solution is switched back to 2.5 mM/l K^+ Ringer's solution at the 15th minute, E_m will immediately rise to 68.3 mV (point A'), while intracellular Cl^- concentration remains unchanged. At A', $I_{Cl} = I_K = II$ pmole/cm^2/sec. The time taken for the shift of E_m from A' to B', another point of equilibrium of ion fluxes.is calculated in the same way as that described previously. Here, since the decrease in Cl^- concentration is 0.8 mM, that is, from 8.8 to 8 mM/l,the time taken is equal to

$$T = \frac{0.8}{\frac{II \times 10^{-9} \times 3.8 \times 10^{-2} \times 60 \times 10^{3}}{1.5 \times 10^{-4}}}$$

$$= 4.7 \text{ min.}$$

Similarly, we can calculate all the changes in E_m and the time required until the equilibrium potential of K^+ at 2.5 mM K^+ Ringer's solution is attained.

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