SPONTANEOUS RECOVERY OF MAMMALIAN MUSCLE FROM DEPOLARIZING DRUGS

by

LEE DAVID MITCHELL

being a thesis submitted for the degree of

Doctor of Philosophy

in the

University of London

April 1978

Department of Physiology,
St. Mary's Hospital Medical School,
London W2 1PG.

ABSTRACT

It was found that the effects of depolarizing drugs in rat and guinea-pig diaphragm showed spontaneous recovery in the presence of the drug. To investigate possible mechanisms for this phenomenon, intracellular measurements were made from diaphragms of innervated rats and denervated guinea-pigs, by single fibre recording and by repeated sampling.

The end-plate regions were initially identified in rat diaphragm by the presence of miniature end-plate potentials. Later, it was found that structures which appeared to be end-plates could be visualised by the use of polarizing filters. This was supported by the presence of inverted miniature end-plate potentials, and by cholinesterase staining of some of these structures.

With decamethonium the median effective concentration in rat diaphragm was approximately 3μM by recordings of depolarization. When 100μM concentrations of carbachol, suberyldicholine or decamethonium were applied, the potential was reduced to approximately -55mV for these three drugs. There was an initial rapid partial recovery in some fibres, especially with decamethonium, and the membrane potential then showed complete or substantial recovery in a period of time which varied with the different drugs.

If ouabain in the 100μM range, or potassium free Krebs saline was used with the above compounds, the membrane at the end-plate became rapidly depolarized and then failed to recover. The effects of ouabain were reversible. It is inferred that an electrogenic ion pump may be one of the mechanisms responsible for the return of the membrane potential.

With high concentrations of decamethonium (3μM), the membrane potential showed recovery even in the presence of ouabain or potassium-free solution, and it was inferred that a different mechanism was involved.

Application of ouabain alone lead to a depolarization of the membrane by 9mV (median of 10 estimations by repeated sampling in 7 muscles). The effects of potassium-free solution were variable: many fibres became hyperpolarized, some became depolarized, and a few had a mixed response. It is suggested that the resting membrane potential in some cells may in part be maintained by the electrogenic pump.

With denervated guinea-pig diaphragm the initial depolarization with decamethonium (100μM) was followed by recovery which led to a transitory hyperpolarization. The recovery after the first 5 min was prevented by ouabain and potassium-free solutions.

It is concluded that in rat and guinea-pig diaphragm, the recovery of membrane potential at the end-plate may be attributed to one of two mechanisms. At drug concentration in the 100μM range, the sodium-potassium pump may repolarize the membrane. At higher decamethonium concentrations, there is a process for recovery which is insensitive to ouabain and to potassium-free solution.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor R. Creese for his constant encouragement, constructive supervision and personal friendship through the course of my study. My thanks are also due to Drs. Stanley Head and Paul Canfield for stimulating discussion and advice.

I would like to thank the entire technical staff, but particularly Mr. Alan Tivey who collaborated in staining and photography of end-plates, Mr. George Stolarczyk for diligent and competent technical service, and Mr. Nigel Randall of the Bioengineering Department for the development of the electrode amplifier. My thanks also to Mrs. Christine Turner for typing this thesis. The statistical tests have been taken from a paper by R. Creese and L. D. Mitchell, in preparation.

I am grateful to St. Mary's Hospital Medical School for awarding me the scholarship which made this period of study possible.

Finally, I would like to acknowledge my indebtedness to Miss Suzette Newman whose constant support aided the completion of this long effort.

CONTENTS

Title 1

Abstract 2

Acknowledgements 4

Contents 5

CHAPTER I INTRODUCTION 9

Spontaneous recovery in the presence of depolarizing drugs 10

a. Steady application of depolarizing drugs

b. Repeated application by iontophoresis

c. Changing effects produced by depolarizing drugs

d. Nomenclature of desensitization and related effects

e. Factors which affect desensitization.

Theories of desensitization 21

a. Entry theories

b. Rate theory

c. Time-dependent change in the drug-receptor complex

d. Desensitized state

e. Inactivation of the conductance mechanism

f. Metabolic effects

The electrogenic sodium pump: effects of ouabain and removal of potassium 25

a. Formulation of the electrogenic sodium pump

b. The electrogenic pump in frog muscle

c. The electrogenic pump in mammalian muscle

d. The effect of ouabain on sodium permeability

Dose-response Curves 29

a. Electrophysiological methods

b. Drug receptor interactions

c. Median effective concentration and maximum response

Fibre types 34

Identification of the end-plate region 37

Effects of denervation 38

a. Structural changes

b. Changes in permeability

c. Changes in electrical properties

d. Fibrillation potentials

e. Spread of acetylcholine receptors

f. Receptors in normal and denervated muscle

Plan of investigation 46

CHAPTER II METHODS 48

Muscle preparation

a. Rat diaphragm

b. Denervated guinea-pig diaphragm

Solutions 49

a. Standard Krebs' solution

b. Solutions with variable potassium

The experimental bath 49

a. The vertical bath

b. The horizontal bath

c. Another horizontal ~~bath~~ system

The electrical recording system 62

a. Interconnections between equipment

b. First and second stages

c. Differential amplifier (Grass)

d. Earthing system

The micro-electrodes 79

a. Silver/silver chloride electrodes

b. Agar salt bridge

c. Glass micro-electrodes

Standard method of recording 83

a. Cleaning

b. Micro-electrodes

c. Adjustment of amplification and capacitance

d. Continuous records

e. Processing of electrode recordings

List of drugs 87

Statistical procedures 87

CHAPTER III LOCALIZATION OF END-PLATES IN RAT DIAPHRAGM 88

Measurement of fibre diameter 88

Use of polarizing filters to visualise end-plates: miniature end-plate potentials 90

Staining of end-plates 96

Summary of Chapter III 100

CHAPTER IV EFFECTS OF POTASSIUM AND OUABAIN ON RAT DIAPHRAGM 102

Depolarization by potassium 102

Resting potentials in absence of potassium 102

Computer fit for the sum of two Gaussian curves 108

Miniature end-plate potentials in potassium-free solution 109

Effect of ouabain 109

Summary of Chapter IV 115

CHAPTER V CARBACHOL AND SUBERYLDICHOLINE ON RAT DIAPHRAGM 116

Effects of Carbachol (100μM)

100μM Carbachol in the absence of potassium 121

100μM Carbachol with ouabain 124

Depolarizing effects of ouabain after prolonged exposure to carbachol

Effects of suberyldicholine (100μM) 127

Suberyldicholine in the absence of potassium 130

Suberyldicholine with ouabain

Summary of Chapter V 135

CHAPTER VI DECAMETHONIUM ON RAT DIAPHRAGM 137

Concentration - effect curve 137

Effects of decamethonium (100μM) 145

100μM Decamethonium in the absence of potassium 147

100μM Decamethonium with ouabain 147

Effects of 3mM decamethonium 149

3mM Decamethonium with ouabain 149

3mM Decamethonium in the absence of potassium 151

Two rates of recovery with decamethonium 151

Summary of Chapter VI 154

CHAPTER VII DECAMETHONIUM ON DENERVATED GUINEA-PIG DIAPHRAGM 156

Resting potentials in normal and denervated muscle 156

Miniature end-plate potentials in innervated guinea-pig diaphragm 156

Effects of 100μM decamethonium in denervated guinea-pig diaphragm 158

Use of solution without potassium 158

Spontaneous depolarizing potentials 161

Effects of decamethonium in potassium-free Krebs' saline 161

100μM decamethonium with ouabain 166

Summary of Chapter VII 168

CHAPTER VIII DISCUSSION 169

Identification of end-plate regions 169

Miniature end-plate potentials 171

Spontaneous depolarizing potentials 172

Prolonged recording 173

The effects of potassium-free solution, and ouabain 173

Limited depolarization in rat muscle 176

Recovery of potential - two processes 178

REFERENCES 183

CHAPTER I INTRODUCTION

Acetylcholine acts on muscle end-plates by combining with receptors and inducing channels to open for the passage of cations: the resulting ionic fluxes lead to depolarization which triggers propagated action potentials in the fibre (see reviews by Katz, 1966, and by Ginsborg and Jenkinson, 1976). The transmitter is hydrolysed by cholinesterase at the end-plate, and may be studied by the use of stable compounds such as suberyldicholine, carbachol and decamethonium.

It was found by Burns & Paton (1951) that decamethonium produced depolarization in gracilis muscle of the cat, recorded by external electrodes, and Zaimis (1954) concluded that this compound blocks neuromuscular transmission by persistent depolarization. In some but not all cases it has been found that a degree of recovery of potential occurs in the presence of depolarizing drugs, and in this monograph the question has been investigated in rat diaphragm and also in denervated guinea-pig muscle.

This introduction is concerned mainly with the electrical characteristics of drug action studied with microelectrodes on mammalian muscle. The effects of neuromuscular block, which have been extensively reviewed by Zaimis (1976) will only be considered when relevant to the main theme. Some other topics which were encountered in the course of the investigation have also been included in the introduction.

Spontaneous recovery in the presence of depolarizing drugs

a. Steady application of depolarizing drug

Several studies using frog sartorius muscle indicate that during the application of transmitter analogues to the bath solutions, the response of the tissue reverts towards the original state. This was witnessed by Fatt (1950) as a recovery of potential following initial depolarization in the presence of the drug. Recordings using wick electrodes indicated that acetylcholine concentrations of 16.3μM induced a loss of sensitivity to this drug following the return of the membrane to a resting potential. At concentrations of 2.15μM or less the recovery of potential or the loss of sensitivity did not occur.

These findings were confirmed by Thesleff, first by intracellular voltage recordings and demarkation potentials (1955a) and later by measuring changes in resistance across the end-plate regions (1956). In the initial study the author found that in the presence of transmitter analogues in the 10 to 80μM range, there is a spontaneous recovery of potential within 15 min of the initiation of drug application and a complete neuromuscular block occurred only when the potential had reverted to its resting level. In the latter study Thesleff (1956) showed that restoration of the membrane potential occurs with a recovery of the resistance across the end-plate region.

Studies of changes in transmembrane resistance have also been conducted by Manthey (1966), and by Nastuk (1971). Both these authors were able to continue recording during depolarization as well as recovery. Both report that there is first a fall and then a recovery of resistance in the presence of carbachol, and the rate of recovery increases with drug concentration. Nastuk, however, claims that the recovery is never complete and that a small, but reversible,

lowering of resistance and potential remains during the drug's presence.

Jenkinson and Terrar (1973) used voltage clamp techniques to measure directly the action of applied 10μM carbachol on membrane conductance. Like membrane potential and transmembrane resistance, conductance spontaneously declines in the presence of the drug. The concept developed from these and other experiments is that in frog muscle the drug induces channels to open and the conductance increases, and the conductance later reverts towards the original value.

There is a paucity of similar studies in isolated mammalian preparations. Thesleff (1955b)used repeated sampling of intracellular membrane potentials from rat diaphragm to determine the effect of several transmitter analogues in the 100μM range. Prior to the addition of these drugs Thesleff found the resting potential to be about -97mV in 2mM potassium saline. One to three min after the addition of the drug, the membrane had depolarized to a value of -63 to - 68mV. After 30 min, the potential was -71 to - 75mV. Maximum depolarization occurred after 5 to 10 min. The extrajunctional region also depolarized to a lesser extent, from -99mV to a value of -73 to -76mV. Additions of any other transmitter analogues had no further effect, although miniature end-plate potentials could be seen throughout. However desensitization was reversible if the tissue was washed in fresh Krebs saline.

Perhaps the most thorough study using both applied drug solution to mammalian striate muscle has been conducted by Zaimis and Head (1976). They used isolated cat tenuissimus and rat extensor digitorum longus muscles to investigate the action of several transmitter analogues. Continuous intracellular recordings from the cat muscle showed that these drugs caused a depolarization from

-85mV to -42mV. In the rat muscle the depolarization was from about -80mV to -62mV. In neither case was there substantial recovery of potential or transmembrane resistance in the presence of these analogues. The drug effects were reversible with removal of the drug solutions. They demonstrated, however, that recovery of resting potential in fresh Krebs saline could be blocked by ouabain.

b. Repeated application by iontophoresis

Iontophoresis permits drugs to be repeatedly applied for short periods to the same site. These advantages have been exploited by Katz and his colleagues (Katz and Thesleff 1957, del Castillo and Katz 1957), and by Magazanik and Vyskocil (1970) in the investigation of desensitization in frog satorius muscle. Generally, two iontophoretic electrodes are used. One passes a small amount of drug for a relatively long period, causing little or no depolarization. The other electrode produces a series of short pulses which involves larger amounts of drug, and both electrodes pass drugs simultaneously.

The decline in depolarization produced by each succeeding short pulse is used as a measure of desensitization. Desensitization appears to occur within a second of the first impulse and all responsiveness is completely eliminated within a few seconds of continuous drug application. Axelson and Thesleff (1958) used the twin iontophoretic applications of acetylcholine to study desensitization in several different mammalian preparations including the cat tenuissimus, rat and guinea-pig diaphragm muscles. It was found that guinea-pig and rabbit muscles required a steady drug application for several seconds before any desensitization was noted. The rat diaphragm muscle and cat tenuissimus muscle appear to be particularly susceptible to desensitization, occurring within 10-20 m sec of continuous low level application.

There is a discrepancy in the effects produced by bath application and by iontophoresis, especially in cat tenuissimus muscle, and these differences may be due to the presumed high drug concentrations delivered by the iontophoretic electrode (Zaimis and Head 1976).

c. Changing effects produced by depolarizing drugs

The first report that a single drug could mimmick the action of both tubocurarine and acetylcholine on one muscle was made by E. Zaimis in 1953. She christened this phenomonen the 'dual block'. Further studies reported both in the above paper, and in subsequent publications confirmed that a given blocking drug may act as tubocurarine, or like a continuous application of acetylcholine, or by changing characteristics during its application. The species, and the particular muscle was shown to be the determining factor.

In subsequent studies, Jewell and Zaimis (1954) catagorized some of the differences distinguishing dual block from the depolarizing block. Two muscles in the hind limb of the cat, the tibialis, and the soleus were used in this investigation. This preparation has the advantage of permitting simultaneous recording and application of drug to both muscles. Decamethonium causes a depolarizing block in tibialis, and dual block in soleus.

Following on the work of Zaimis, Jenden (1955) investigated the nature of the neuromuscular block in the guinea-pig isolated phrenic nerve diaphragm muscle preparation. Two sets of stimulating electrodes were used. One set stimulated the muscle directly, while the other set was used to excite the phrenic nerve. By alternating the two stimulations, it was possible to distinguish between effects on neuromuscular transmission and direct effects on

the muscle. A series of transmitter analogues was applied. The observations were consistent with the dual block described by Zaimis and her co-workers. There was an initial phase during the first 10 min in which the drugs block the effect of indirect stimulation, and reduce the contraction due to direct stimulation. The response to nerve stimulation then recovered. A secondary block (Phase II) began after some 30 min and continued until the response to nerve stimulation was largely abolished while the response to muscle stimulation continued. Neostigmine was found to antagonize phase II block, while tubocurarine was found to mimmick it.

d. Nomenclature of desensitization and related effects

Several terms have been used to describe the observation that effects of end-plate depolarizing agents will wane with continuous application. The most common descriptive words are fade (Paton, 1961), tachyphylaxis (Goodman and Gillman, 1976 Burgen and Mitchell, 1968), and desensitization (Ginsborg and Jenkinson, 1976, Elmqvist and Thesleff, 1962, Cookson and Paton 1969).

There are probably as many definitions of the terms as there are authors. General, specific, and even theoretical definitions appear in the literature. Burgen and Mitchell define tachyphloxis as the loss of a drug's effect, with continuous application. Because the authors use electrophysiological methods, Ginsborg and Jenkinson talk of desensitization as the recovery of potential. Elmqvist and Thesleff produce the most explicit definition, incorporating a theory, the phenomenon, and an implication as to the method of measurement. They define desensitization as "a condition in which application of a depolarizing drug has made the chemoreceptors at the end-plate refractory to chemical stimulation. This phenomenon is associated with complete or partial repolarization of the membrane of the

end-plate region despite the continued presence of the drug".

Desensitization may incorporate not one, but several phenomena, each with a distinct origin. This possibility can be seen by noting the variation in the delay reported from application of the drug to the onset of desensitization. Katz and Thesleff (1957) report onset within 1 to 2 seconds after applying acetylcholine to the frog sartorius muscle. Magazanik & Vyskocil (1970, 1977, 1975) report onset as short as 1 second following intracellular applications of various desensitization-enhancing drugs. The recovery of end-plate membrane potential during the continuous application to certain mammalian muscles would fall within the definition of desensitization proposed by Ginsborg and Jenkinson and others. The full development of "Phase II block" (Jendon, 1955) may in some preparations take hours.

e. Factors which affect densitization

Except for the effects of temperature (Magazanik and Vyskocil 1975), the rate of recovery from desensitization in striate muscle is invariant (Rang and Ritter, 1970, Elmqvist and Thesleff 1962, Katz and Thesleff 1957) within a given preparation. In contrast, the rate of desensitization is easily altered. Ginsborg and Jenkinson in a review of neuromuscular transmission list several factors which influence it.

(1) the depolarizing agent

Even when the concentration of various drugs are varied so that their initial maximum effects are equal, the rate of decline of the response will differ (Rang and Ritter 1970). Some partial agonists (e.g. phenyltrinethylammonium and bistrimethylammonium) cause more desensitization than some full agonists (e.g. carbachol and suxamethonium): Parsons (1969) found that decamethonium, which acts like a partial agonist in frog muscle, produced more desensitization than carbachol.

(2) the preparation

Magazanik and Shekhivev (1970 quoted by Ginsborg and Jenkinson 1976) report that fast and slow frog muscles have different rates of desensitization. Jewell and Zaimis (1954) note that decamethonium on cat saleus muscle causes a "dual" block, but produces a constant depolarization block on the tibialis muscle. Thus in tibialis there is no desensitization as defined by Ginsborg and Jenkinson.

(3) temperature

Bigland & Zaimis (1958) found that to reduce the temperature from 36°C to 31°C reduced the rate of the change from a depolarizing to a competitive block in cat tibialis muscle in vivo, and this was confirmed by Alderson and Maclagan (1964). They found that on the cat hind limb, lowered temperature prolonged the depolarizing effect of decamethonium, but reduced the competitive blocking effect of tubocurarine. Magazanik and Vyskocil (1975) found that reducing the temperature reduced both the rate of desensitization, and the rate of recovery from iontophoretically applied acetylcholine to frog sartorius muscle. They found that the Q_{10} values for onset and recovery of desensitization follow the same time course, although the Q_{10} value for recovery is always smaller than the Q_{10} for onset.

(4) ion concentration and species

Frog sartorius muscle appears to be the only preparation used for the investigation of the influence of various ions on vertebrate striate muscle desensitization.

Nastuk and Lin (1966), and Manthey (1966) both investigated the role of calcium on desensitization to applied carbachol. Nastuk and Lin also observed its effect on neuromuscular transmission.

These latter authors noted that increasing concentrations of calcium to 1.8mM produced a steady increase in depolarization induced by either indirect stimulation, or applied carbachol. This effect was then reversed with further increases in the calcium ion concentration. At 30mM Ca^{2+}, with a five minute soak, neuromuscular transmission was completely blocked, and longer exposure caused slow depolarization. When both Ca^{2+} and carbachol were iontophorised together, Ca^{2+} led to a decline in the rate of depolarization and eventual block. A Lineweaver-Burk plot of carbachol with 1.8mM and 10mM Ca^{2+} gave the same intercept on the y axis, indicating that Ca^{2+} and carbachol are competing for the same site.

Manthey (1966), was able to demonstrate desensitization more clearly by measuring the change in effective resistance in the end-plate region with the application of 270μM carbachol from a diffusion pipette located 0.1mm above the preparation. With a bath concentration of 1.8mM Ca^{2+}, the half time of recovery following 5 to 10 mins of carbachol was 35 sec. When the Ca^{2+} concentration was raised to 10mM, the half time for recovery was reduced to 12 sec, and if Ca^{2+} was removed altogether, the half time was increased to 100 sec.

Later, Manthey (1970), demonstrated that if the carbachol concentration was substantially reduced, variation in Ca^{2+} would have no effect on the rate of desensitization. The preparations were bathed in a solution containing 165mM K^{+}. This increase in K^{+} concentration was meant to prevent contraction during excitation. It had the further advantage of preventing any voltage-dependent changes in extra-junctional membrane resistance.

Manthey (1972) has subsequently found that both Na^{+} and K^{+}

oppose the Ca^{2+} effect. The antagonism between K^{+} and Ca^{2+} was particularly carefully studied and this led Manthey to propose an ion exchange model for desensitization. Ginsborg and Jenkinson also point out that there is some evidence that divalent cations may reduce the affinity of quaternary ammonium compounds (i.e. carbachol) or have complex actions on the drug-receptor inter-action.

In an earlier study, Takeuchi (1963) also found that there was an interactive effect between Ca^{2+} and Na^{+} in voltage-clamped end-plates of frog muscle. He found that increasing the Ca^{2+} concentration lead to a reduction in the Na^{+} end-plate current, but had no effect on K^{+} end-plate current induced by the action of the neuro-transmitter.

Whether Na^{+} current is selectively diminished during desensitization is presently being debated. Kuba and Koketsu (1976) using frog sartorius muscle, iontophoresis, and voltage clamp, demonstrate a shift in the acetylcholine reversal potential during desensitization (a 25% reduction in end-plate current in 20 seconds). They ascribe this change to the Ca^{2+} effect on Na^{+} conductance. Katz and Miledi (1977) using bath applications and iontophoresis of acetylcholine on frog sartorius muscle either pretreated with glycerol or placed in hypertonic solutions find no shift in the end-plate equilibrium potential during desensitization. Apart from the obvious differences in technique no explanation can be offered for the differences in results.

To determine the effect of other multi-valent cations on the rate of desensitization, Magazanik and Vyskocil (1970) carried out experiments using Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Al^{3+} and La^{3+}. The authors either added or substituted these ions for Ca^{2+}. Desensitization

was induced by pulsing iontophoresised acetylcholine onto the end-plate region of the ubiquitous frog sartorei. One iontophoretic electrode was used to produce a long lasting plateau of depolarization, while another superimposed short depolarizations on top of this plateau. The rate of diminition of the short pulse heights was used as a measure of desensitization. The authors were then able to place the desensitization abilities of the ions in the following order: $Mg^{2+} \ll Ca^{2+} \ll Ba^{2+} \ll Sr^{2+} \ll Al^{3+} < La^{3+}$.

(5) application of special compounds

Some compounds can enhance the rate of desensitization produced by agonists. Adams (1974) used the barbiturate thiopentone to induce a desensitizing effect on the action of carbachol. Following application of thiopentone the second application of carbachol was markedly depressed in these experiments, and the effect decayed exponentially with a time constant of 165msec. Vyskocil and Magazanik (1972) infused a series of compounds which included SKF 525A (diethylaminoethyl diphenyl propylacetate) inside the end-plate region of frog muscles and found that desensitization was enhanced. The work has been reviewed by Magazanik and Vyskocil (1973); the fact that these compounds can affect desensitization from inside the fibre, though the receptors are known to be accessible only from the outside (del Castillo and Katz 1955) makes it unlikely that they are acting by combining with the receptor sites.

(6) membrane potential

Influenced by previous reports that increased potassium concentrations lead to a slowing in the rate of desensitization, Magazanik and Vyskocil (1970) investigated the effects of high potassium and membrane depolarization.

They used the same externally applied duel iontophoresis of acetylcholine to induce desensitization that has been described previously. When the extracellular potassium concentration was increased to 15mM, the membrane potential declined to -52mV, and the rate of desensitization was slowed by 80% of its control value. However, when the membrane potential was returned to -90mV by means of an intracellular current-passing electrode, the rate of desensitization returned to its control value although the external potassium concentration was still 15mM. The authors therefore concluded that the half-time is dependent upon the state of the membrane potential as well as other factors.

(7) drug concentration

The two most consistent findings in desensitization studies are that the rate of onset is concentration-dependent and the rate of recovery is invariant (Rang and Ritter 1970). It also appears that the rate of onset in frog muscles is measured in seconds or on occasion in minutes, while in mammalian preparations the changes which are observed may be measured in hours (Jenden, 1955; Taylor and Nedergaard 1965). This difference between isolated preparations may be due either to the drug concentrations used, or to differences in the mechanisms of recovery.

In frog sartorius muscle, many of the studies have used iontophoresis. While it is impossible using this method to determine the drug concentration at the affected receptors, two papers using this technique have pointed out that the rate of desensitization is concentration-dependent. Katz and Thesleff (1957) found that when they used moderate conditioning doses, the half time was 5 sec, but that with large conditioning doses, the half time was cut to 1 to 2 sec.

Katz and Thesleff point out that desensitization does not depend upon the height of depolarization, as it occurred with depolarization as low as 0.5mV. These findings may be consistent with the report by Magazanik and Vyskocil (1975). Using similar techniques and the same preparation, they found that the onset of desensitization was independent of the frequency of duration of iontophoretic pulses, but depended upon the total amount of drug released.

(8) chloride concentration

Jenkinson and Terrar (1973) have shown in frog muscle that chloride ions affect the repolarization after carbachol. Replacement of the chloride content of the Ringer solution by the isethionate abolished the slow phase of the carbachol response. It is likely that chloride redistribution can alter the rate of the response, and may complicate the interpretation of the dose-response relationship.

Theories of desensitization

Many hypotheses have been devised to explain the findings that drugs which are in contact with tissues may become less effective with time, and the main concepts which are applicable to muscle end-plates are summarized below.

a. Entry theories

The entry theories are probably the oldest. In 1907, Straub, (Waud 1968) proposed that the action of an excitatory drug is due to its flux across the membrane, and hence as the concentration of the drug equibrated its effect would be reduced. Later, Taylor and Nedergaard (1965) proposed in the case of decamethonium that depolarization depended upon its entry into the fibres, and when the entry ceased, the drug action ceased.

Many arguments can be raised against this theory. The intracellular accumulation of decamethonium proceeds at a steady rate in guinea-pig diaphragm for many hours (Nedergaard & Taylor, 1966). Intracellular loading of quaternary salts did not increase desensitization in the experiments of Nastuk, Manthey & Gissen (1966). And desensitization to one drug causes desensitization to others acting on the same receptors (Ward 1968).

b. Rate theory

Paton (1961) proposed that the effect of a stimulant drug is proportional to the rate of association between drug and receptor; as the drug-receptor complex is formed, the effect declines and "fade" is observed.

Stimulant drugs which act on receptors are charged organic cations and it is likely that their rates of association are extremely rapid (Burgen, 1966; Anderson & Stevens, 1973), and the reactions have not been directly measured. At the neuromuscular junction the rate of action of drugs seems to be determined by the rate of access to the receptor and not by the rate of a drug-receptor reaction (Waud, 1968, 1969).

c. Time-dependent change in the drug-receptor complex

Zaimis (1953) had found that decamethonium in cat soleus muscle produces neuromuscular block having at first the characteristics of depolarization, and which later causes to resemble the kind of block produced by tubocurarine. Zaimis has proposed that there is a time-dependent change in the drug-receptor complex to account for these pharmacological effects. The concept is descriptive and a mechanism at the receptor level has proved difficult to formulate.

d. Desensitized state

The concept which has probably gained most adherents is that

the drug-receptor complex can form a "desensitized state", as proposed originally by Katz & Thesleff (1957) and considered exhaustively by Rang & Ritter (1970). The hypothesis can be formulated in several different ways, but in most cases it is postulated that the receptor can exist in three states - a resting state with ion channels closed, an excited state when combined with drug such that ion channels open, and a "desensitized" state in which the drug is combined with receptor but the channels are again closed. A series of formal models have been considered, with sequential, parallel and cyclic kenetics. The mathematical models mostly predict that the onset of desensitization should vary with different drugs but that the offset should be similar for all drugs. This prediction has indeed been verified in several cases (Rang & Ritter, 1970) and this experimental finding has to be explained by any hypothesis. At present the methods available for analysing the drug-receptor complex are not sufficiently developed to enable a decision to be made regarding the existence of the "desensitized state".

e. Inactivation of the conductance mechanism

In these models the desensitization is attributed not to changes in the receptor but to the inactivation of ionic channels. Vyskocil and Magazanik (1972) injected iontophoretically into frog muscle the compound SKF525A (diethylaminoethyl diphenyl propylacetate). This produced desensitization as tested by externally applied acetylcholine. The authors propose that desensitization is a post-receptor phenomenon, and that it is caused by Ca^{2+} binding to channel phospholipids which block conductance.

Manthey (1972) noting that the rate of desensitization to carbachol was enhanced by Ca^{2+} and antagonized by K^{+}, conducted a

series of experiments relating the effect of one ionic concentration to the other. Based on this interaction, an ion exchange mode of desensitization was developed. The agonist is thought to expose an ionic site and Ca^{2+} and K^{+} compete for this site. When Ca^{2+} occupies it, the mechanism is occluded and desensitization sets in. Since the interaction between carbachol and the receptors takes milli-seconds, and desensitization requires seconds, Manthey proposes that this anionic site is located in the channel itself. Nastuk & Parsons (1970) have also attributed desensitization to inactivation of the conductance mechanism associated with accumulation of calcium at anionic sites in the channels. Sarne (1976) has shown that desensitization also occurs in crustacean muscle in response to gamma-amino-butyric acid, which increases permeability to chloride ions. The desensitization is enhanced by calcium, so that the mechanism proposed by Nastuk & Parsons (1970) would seem inappropriate in this case.

Adams (1974) has reported evidence suggesting that some barbiturates may block channels associated with nicotine receptors. In a later publication (1975) he studied the onset and offset of desensitization in voltage-clamped frog end-plates, and proposed that desensitization is due to channel blockade by the agonist molecule itself. Some support for this type of model may be gained from the measurements of the entry of labelled decamethonium at the junctional region of depolarized diaphragm in the rat (Creese & England, 1970). With high concentrations in the mM range the uptake of decamethonium is inhibited, and channel block is a possible mechanism.

f. Metabolic effects

Jenkinson & Terrar (1973) found that ouabain, which is known to inhibit the "sodium pump", had no appreciable effect on desensitization in frog muscle. Zaimis & Head (1976) found in cat muscle that the

repolarization following removal of decamethonium was slowed or halted by ouabain, and this type of experiment is considered in later sections.

The electrogenic sodium pump: effects of ouabain and of removal of potassium

The sodium pump may be defined as a glycoside-sensitive exchange of sodium for potassium across the cell membrane, which involves the enzyme adenosine triphosphatase (Glynn, 1975). The pump is stimulated by an increase in intracellular sodium, and requires external potassium. The glycosides act by either inhibiting dephosphorylation of the phosphoenzyme or phosphorylation of the dephosphoenzyme. These glycosides act on an external site, and there is a parallel inhibition for transport and ATPase activity. Na^+, Ca^{2+}, Li^+ increase the glycoside binding affinity, K^+ and Rb^+ decrease it. There may be one or two binding sites per molecule, and this is at present unclear. But the reaction can be described by first order kinetics, which suggests that the glycosides act directly on the pump.

a. Formulation of the electrogenic sodium pump

The ratio of potassium and sodium exchange during equilibrium ultimately establishes the membrane potential in the absence of net ionic movement. As long as there is no net passive movement of any other ion (i.e. Cl^-) the steady state potential can be described in the following way (Thomas 1972).

$$E = \frac{RT}{F} \ln \frac{rK_o + bNa_o}{rK_i + bNa_i}$$

where r = pump ratio of Na^+ to K^+. With the exception of a pump ratio differing from unity, this equation is identical with the constant field equation, with the symbols having their conventional meaning.

If the electrogenic component is to be distinguished from the diffusion potential, the following derivation may be used:

$$E_p = \frac{RT}{F} \ln \frac{rK_o + bNa_o}{rK_i} - \frac{RT}{F} \ln \frac{K_o + bNa_o}{K_i}$$

where bNa_i has been removed due to its low value.

Hence $$E_p = \frac{RT}{F} \ln \left[\frac{1}{r} \cdot \frac{rK_o + bNa_o}{K_o + bNa_o} \right]$$

The largest negative value is when

$$E_p = \frac{RT}{F} \ln \frac{1}{r}$$

Then assuming a pump ratio of 3Na to 2K,

$r = 1.5$, and $E_p = 10mV$.

Thomas (1972) points out that this applies only to a steady state: the contribution of the sodium pump could be much larger if the pump is stimulated.

b. The electrogenic sodium pump in frog muscle

The electrogenic pump has been well established in frog muscle, and Thomas (1972) has reviewed the major findings which are summarized here.

Kernan in 1962 depleted the potassium content from isolated sartorius muscles by storing them in cold potassium-free solutions overnight. When they were subsequently placed in a high potassium solution at room temperature, the muscles hyperpolarized by 10mV within 20 minutes. Frumento in 1965 showed that the potential change upon warming the Na-loaded and K-depleted muscle was directly related to the measured extrusion of sodium. Kernan concluded from his experiments that the pump was purely electrogenic and that it operated by extruding sodium, with a passive potassium entry. However, Keynes and Rybovo in 1963 showed that there was a substantial reabsorption

of potassium even when the membrane potential did not exceed E_K during recovery. Further studies by Adrian and Slayman (1966) on the pumping of sodium into recovery solutions containing Rb and K were interpreted as showing that only 10% of sodium extrusion was electrogenic, and 90% of the sodium was exchanged with external cation. Hence there is clearly coupling between Na and K.

Frog muscle hyperpolarizes when the external medium is changed to solution containing no potassium (see Thomas, 1972). Ouabain has little effect on the resting potential (Jenkinson & Terrar, 1973). It would appear that the sodium pump makes very little contribution in the case of resting frog muscle. However, Henderson (1970) has shown that removal of external potassium in glycerol-treated frog muscle produces depolarization, which indicates that there is an electrogenic component of the membrane potential in muscles treated in this manner.

c. The electrogenic pump in mammalian muscle.

Akaike (1975) has shown that rat soleus muscles which were sodium-loaded and potassium-depleted became hyperpolarized when external potassium was restored, and the membrane potential then exceeded the potential equilibrium potential. However studies designed to determine the electrogenic component of the potential in resting muscle have given conflicting results.

Hall, Hilton and West (1972) showed that a change-over to potassium-free solution, maintained at 24°C caused hyperpolarization in rat diaphragm muscle. Den Hartog and Mooij (1976) using chloride-free bath solutions found that at room temperature a change-over to potassium-free saline caused depolarization in the same preparation.

Akaike (1975) using rat soleus muscle found that at 37°C the changeover to potassium-free saline caused hyperpolarization. However if the muscle was sodium loaded by being placed overnight in cold potassium-free solution, the above treatment caused depolarization. There is little consistency in these results.

Analysis of the muscles' electrical response to ouabain has lead to equally ambiguous findings. McArdle and Albuquerque (1975) found that in mouse extensor digitorum longus and soleus muscle, 197μM ouabain caused a depolarization of 13 - 14mV. Bray, Hawken, Hubbard, Pockett and Wilson (1976) found that 1-5mM ouabain produced a fall in rat diaphragm resting potential of approximately 13mV. Robbins (1977) estimated that the depolarization caused by ouabain in rat extensor digitorum longus muscle could be explained by an increase in sodium permeability, and he concluded that the sodium pump does not directly contribute to the resting potential in this preparation.

d. The effect of ouabain on sodium permeability

Rogus and Zierler (1973) used the exchange of labelled sodium and its extrusion to examine the partition of sodium and water within subcellular structures of rat extensor digitorum longus muscle. Their findings indicated that 10μM ouabain increased the sodium content of the sarcoplasm, but did not, at this low drug concentration affect the rate of extrusion of sodium. They concluded that ouabain affects sodium permeability at low concentrations, and only blocks the sodium pump when applied in mM doses.

Robbins (1977) measured the Li^+, Na^+ and K^+ content of the same preparation to estimate the relative permeability. He then determined the membrane potential in 1mM potassium and concluded that

it could be accounted for by a diffusion potential. Ouabain in the 100μM range was found to increase the sodium content without affecting the intracellular potassium concentration. The depolarizing effects of 100μM ouabain, could according to this author, be accounted for by increased sodium permeability.

Dose-response curves

Dose-response curves are generally used to obtain information about the response produced by a drug, the concentration for half-maximal effect, and to give some insight into drug-receptor interactions.

a. Electrophysiological methods

Until recently, most curves were produced using the ultimate response of the tissue, such as force or degree of contraction (Rang and Ritter, 1970; Waud, 1968). However the use of micro-electrophysiology permits a more sensitive examination of the first steps in the tissue's response. The drugs can be presented by bath application and by electro-phoresis, when the drug can be pulsed onto the tissue. The responses which can be measured include depolarization, and also conductance which involves the use of voltage-clamp methods.

Although voltage is the biological stimulus causing the response, the first step in the process is an increase in conductance (Ginsborg and Jenkinson 1976). If the depolarization is recorded then the change in conductance may be estimated in relative terms from the observed depolarization v by applying the factor $(1 - v/V)^{-1}$, V being the resting potential minus the equilibrium potential of the drug (Martin, 1955, Jenkinson & Terrar, 1973).

Weighted as it is with resting conductance and driving force, voltage is obviously a less discriminating measure of a drug's effect

than is conductance. The use of the voltage clamp method to measure conductance has several limitations however. A glass micro-electrode can only deliver nano-amps of current. This may not be sufficient to hold a large receptor region at a specific voltage. This will be particularly relevant when high drug concentrations are used to produce maximal conductance (Peper, Dreyer & Muller, 1975).

Thus to examine a wide range of drug concentrations voltage measurement may be the only method feasible, while for discrimination of responses to small drug concentrations, the voltage clamp technique is superior.

A similar distinction exists between bath and iontophoretic drug applications. Bath applications ensure that the drug concentration is known. However, the delivery to the tissue may be relatively slow in comparison with receptor response. This can mean that the onset of drug desensitization may interfere with the recording of the maximum response (Adams, 1975).

Iontophoretic application can be delivered rapidly, and discretely. However, the ultimate concentration delivered to the receptor is generally unknown, as the exact location of the electrode tip is of the utmost importance here. Lately, methods have been developed to overcome these limitations, but they are of use only when applied to end-plates in which the geometry of the receptor distribution is known (Peper, Dreyer & Muller, 1975). Where initial responses to drug applications are to be considered, iontophoresis is the method of choice. When however, a dose response curve is to be prepared, bath application is usually the only method for ensuring that the final drug concentration is known.

b. Drug-receptor interactions

Simple adsorption kinetics would predict that conductance would increase linearly with receptor occupation. This would imply that a dose-response curve, on a linear scale, should be described as a rectangular hyperbola (Rang 1971).

The well known Langmuir equation relates the proportion of receptor occupation to molecular concentration

$$P = \frac{X}{X + K}$$

P = proportion of the receptors occupied

X = drug concentration

K = dissociation constant .

The most common strategy to determine if a dose-response curve deviates from this relationship has been to use the slope of the Hill plot:

$$(\text{Log} \frac{E}{E_{max} - E}) / \text{Log } X$$

E = effect measured

E_{max} = maximum effect (most often estimated)

X = drug concentration

If E/E_{max} is proportional to occupation and the Langmuir equation is obeyed, then the Hill plot should be linear and have a slope of unity.

It has been almost universally reported that conductance does not increase linearly with concentration (Colquhoun 1975). In frog muscle a Hill slope greater than unity has been found by Rang (1971), Johnson & Parsons (1972), and Adams (1975) who used bath applications, and by Peper et al (1975) who used iontophoresis. The results in

mouse muscle by Dreyer, Muller, Peper and Sterz (1976) lead to similar conclusions.

These deviations from a unity Hill slope can be explained by several different theories. Two or more molecules may bind to a receptor to produce activation; the receptors may respond allosterically (i.e. occupation of one receptor increases the probability that the second molecule will become bound); or there might be two populations of receptors (Johnson & Parsons, 1972).

There is little experimental evidence to discriminate between the two theories. The Hill slopes reported so far rarely have slopes of whole integers as would be expected of receptors with multiple binding sites. Secondly, even with multiple binding sites it would still be expected that the dose-response curve would be a rectangular hyperbola with the following modification to the Langmuir equation (Rang 1971)

$$P = \frac{X^n}{X^n + K}$$

P = proportion of receptors occupied

X = drug concentration

K = dissociation constant

n = number of drug molecule binding to one receptor.

Colquhoun (1971) in a review of the literature noted that whole integer slopes are rarely reported. One study where the application of acetylcholine was most carefully controlled (Hartzell et al 1975) showed that in the skin muscle of the garden snake, the foot of the dose-response curve was sigmoidal and not hyperbolic.

While it would seem that the results so far obtained favour the allosteric model for drug-receptor interactions, these

findings are too sparse to warrant any firm conclusions. The electrophysiological parameters of drug-receptor interactions in mammalian muscle are still unknown.

c. Median effective concentration and maximum responses

Very few complete electrophysiological dose-response curves have ever been produced. This is due to most authors' preference for the limited, but very sensitive voltage clamp methods. This has meant that most reports have dealt with only the foot of the curve.

Parsons (1969) and Johnson and Parsons (1972) produced dose response curves on frog sartorius muscle. Both studies used pipettes to perfuse the end-plate with a known concentration of drug. In the study by Parsons 0.01 to 10.0 mMolar decamethonium and carbachol were compared. Carbachol depolarized the end-plate region to -15mV at 500μMolar. He found decamethonium much less effective; 1mM depolarized the end-plate region to -30mV.

In the later study Johnson and Parsons again used carbachol. Maximum depolarization was 70mV with 270μM and the median effective dose appeared to be about 19μM. The Hill coefficient was calculated by the authors to be 1.57.

In the rat diaphragm muscle, Thesleff (1955b) found that 100μM decamethonium caused a depolarization to 63mV. This was assumed to be the maximum response and the effects of no other concentrations were reported.

Since the uptake of decamethonium is apparently related to conductance, it may offer an alternative method of determining the first steps in the tissues' response to the drug. Creese and

England (1970), referred to previously, found a bell shaped relationship between the concentration of decamethonium and the rate of uptake in the junctional region of depolarized diaphragm of the rat. The median effective concentration was 4μM; the maximum uptake was at 100μM. In high concentrations in the mM range the rate of entry declined with half-saturation of 0.4mM.

The studies by Parsons, and Johnson & Parsons suggested that decamethonium in frog muscle is a partial agonist, while carbachol is more fully effective. Whether this is true for the electrophysiology of mammalian end-plates remains to be seen. The response to 100μM decamethonium has been recorded by Thesleff and work by Creese and England seems to suggest that this drug concentration will give the maximum effect. However a broader examination of the electrophysiological response to varying concentrations of any drug must be made before any conclusions can be made at present.

Fibre types

Extraocular muscles of the cat have been shown to contain two types of fibres, twitch and slow: the twitch fibres show all - or - none impulse activity, propaged spikes, end-plate potentials and spontaneous miniature end-plate potentials, while the slow fibres have multiple endings, and display spontaneous miniature slow junctional potentials anywhere along the muscle length, and respond to depolarizing drugs by contracture. Two types of muscle fibre which differ in their fibrillar structure were shown by Krüger (1949). One type of fibre showed a pattern in which the fibrils are regular, distinct and punctate (Fibrillinstruktur), while the other had large irregular poorly defined fibrils (Felderstruktur). In several muscles of the frog and chicken and in mammalian extraocular muscle it has been shown that the twitch fibres have the Fibrillinstruktur while

the slow fibres have Felderstruktur (see Hess & Pillar, 1963). This type of arrangement seems however to be uncommon in mammalian muscles, and although Felderstruktur fibres have been described in soleus, diaphragm and elsewhere (Günther, 1952; Krüger & Günther, 1955, Muscholl, 1957) there is no evidence in these muscles for the existence of slow fibres as in extraocular muscles and the significance of Felderstruktur is unclear (Hess & Pillar, 1963).

Muscles may also be characterized according to the isometric contraction, and are classified as slow-twitch or fast-twitch (e.g. Buller, 1972). These two types of fibre respond differently to depolarizing drugs. Jewell and Zaimis (1954) noted that "red" and "white" muscles of the cat hind limb differ in their response to indirect stimulation and drug applications. White muscles (tibialis like) are rapid contracting, fuse only at relatively high frequences of stimulation, 30µg/kg decamethonium causes a depolarizing block, and the muscle was relatively insensitive to tubocurarine. Red muscles (soleus like) are slow contracting, fuse at lower frequencies, 70µg/kg decamethonium causes a dual block, and the muscle is relatively insensitive to tubocurarine.

Three kinds of motor units have been found in the soleus and extensor digitorum longus of rats (Close, 1967). The majority of motor units in soleus muscle are slow-twitch with an average contraction time of 38msec with the remainder having an intermediate value of 18msec. The extensor digitorum longus is a much more uniform muscle with all its motor units having a contraction time of 11msec. The rate of contraction was inversely related to the fusion frequency.

The diaphragm of the rat is a muscle which shows great heterogeneity of fibre structure. Muscholl (1957) found large fibres

with Fibrillinstruktur and smaller fibres with Felderstruktur, in a ratio of 53:47. Gauthier and Padykula (1966) classified fibres on the basis of their mitochondrial content, into small fibres rich in mitochondria ("red" fibres) and large fibres with less mitochondrial content ("white" fibres).

Rat diaphragm is a mixed twitch muscle, 60% of the fibres are red, 20% are white, and the remaining 20% are of the intermediate type. The fibre diameters are inversely related to their occurrence. In osmium tetroxide or glutaraldehyde fixed preparations the red fibres are 27μM in diameter, the white are 44μM and the intermediate are 34μM (Padykula and Gauthier, 1970). The heterogeneity seen typically in rat diaphragm seems to be absent in diaphragm of very small mammals such as the shrew, where the fibres are "red", and large mammals such as the cow in which the fibres are "white" (see Gauthier and Padykula, 1966).

Muscles can also be classified by histochemical criteria. Dubowitz and Pearce (1960) examined a variety of striate muscles from rat, human and pidgeon, and they classified them into Type 1 and Type 2. The first type are red and have relatively small diameter fibres. They are rich in oxidative enzymes associated with the Krebs cycle, have the expected high amounts of myoglobin, contain many mitochondria, and have a high lipid content. The type 2 fibres are relatively large diameter fibres and are white in colour. They contain large amounts of glycogen and phosphorylase with a relatively low lipid content. Naturally they are expected to utilize the Embden-Meyerhof anaerobic metabolic pathway. Type 1 are expected to be slow-twitch fibres and type 2 fast- or intermediate-twitch fibres.

The studies or rat diaphragm muscle fibres have been extended

by Sreter and Woo (1963), and by Zolovick, Norman, and Fedde (1970). Sreter and Woo examined the sodium, potassium and water content of intracellular and extracellular space in red and white muscles. They found that the sum of intracellular sodium and potassium concentrations were the same in all muscles, and there was no distinct demarcation between white and red fibres in mixed muscle based upon these considerations.

Zolovick, et al., found three types of fibres in rat diaphragm muscle based upon histochemical analysis of myosin adenosine triphosphatase activity. They found that there was no relationship between the radius of fibres and transverse membrane resistance. The results suggest to these authors that the membrane's electrical characteristics are independent of fibre types in rat diaphragm muscle.

Identification of the end-plate region

When recording the effects of depolarizing drugs, the exact location of the electrode is crucial (Ginsborg and Jenkinson, 1976). Chemosensitivity falls off steeply with distance. In rat diaphragm, where there is some extrajunctional sensitivity, depolarization may decline 1000 fold for every 100μM distance from the most sensitive site (Miledi, 1960).

The procedure generally used to locate the area of maximum chemosensitivity is to identify the end-plate region. Beside staining, the only methods in current use for identifying end-plate regions is by searching with a microelectrode, or by Nomarski differential interference microscopy. Peper and McMahan (1972), and Hartzell, Kuffler & Yoshikami, (1975) have demonstrated that by this means end-plates can be directly visualized. However this method

requires a preparation of only a few fibres in thickness, and is not suitable for diaphragm muscle (Dreyer, Muller, Peper & Sterz, 1976).

To identify the end-plate region with microelectrodes, in diaphragm muscle, the area around the small nerve branches is explored to find miniature end-plate potentials (mepps). Then the electrode is moved back and forth until the point with mepps of the highest voltage is found (Auerbach and Betz, 1971). In the rat diaphragm, the most sensitive area is defined by the location of "focal receptors" where mepps rise to peak voltage in 1 - 2msec (Miledi, 1960).

Within the area of the focal receptor, the point of peak conductance is referred to as the "active spot" (del Castillo and Katz, 1956). These spots are identified by the appearance of inverted mepps recorded from the external surface of the muscle membrane. Since in the rat diaphragm there are few active spots within an end-plate region, 3% of the total number of mepps (Liley, 1956) in this preparation, the appearance of well defined mepps has generally been considered sufficient for the identification of the areas of maximum chemosensitivity.

The effects of denervation

Chronic denervation of skeletal muscle leads to profound changes in its structure and responsiveness. There is a change in the size, and type of fibres comprising the muscle. There is a rise in membrane resistance and a fall in membrane potential, probably associated with changes in the permeabilities to sodium and potassium. Action potentials in denervated muscles are resistant to tetrodotoxin. A spontaneous type of spike potential occurs which can be blocked by tetrodotoxin and ouabain. And finally there is an increase in the numbers and spread of acetylcholine receptors: these new receptors

may have a different affinity for d-tubocurarine, and different "noise" characteristics, but may have the same equilibrium potential as the junctional receptors.

a. Structural changes

It has long been known that diaphragm muscle shows initial hypertrophy and an increase in protein synthesis following denervation (Stewart, 1955). Yellin (1974) made a careful examination of changes in the rat hemidiaphragm following denervation. Using two different means of classification, five types of fibres were observed. The first system was based on the number and arrangement of mitochondria, and the second system depended upon the alkalinity or acidity of myosin ATPase staining: the two systems are not mutually exclusive. One week after denervation all fibre types increased in diameter. After the first week, there was a general decline in the diameters of all the fibre types. After 28 days type "A" was down to 70% of its innervated value. Type "B" had returned to its original size. And type "C" was still 5% greater than its innervated value. There was an increase in the number of normally infrequent cells which have a stable reaction to both alkali and acid ATPase stain. On several occasions within one fibre the type of reaction varied. In one animal, islands of robust fibres were found 30 days after denervation, indicating that some fibres may receive their innervation from a source other than ipsiliateral phrenic nerve.

The physiological and pharmacological inferences of such changes cannot yet be made. However, they do demonstrate that not only do denervated fibres undergo substantial changes, but that innervated and denervated muscles contain different proportions of the various fibre types.

b. Changes in permeability

Denervation in rat diaphragm is accompanied by a decrease in the rate of exchange of potassium (Klaus, Lullmann & Muscholl, 1960) and an increase in the rate of exchange of sodium (Creese, El Shafie & Vrbova, 1968).

c. Changes in electrical properties

Denervation in rat diaphragm produces a decrease in membrane potential (Lullmann, 1958) and an increase in membrane resistance. Albuquerque and Thesleff (1968) compared a fast muscle, the extensor digitorum longus (E.D.L.) and a slow muscle, the soleus of the rat in vitro. The E.D.L. membrane potential fell from -72 to -57 mV and the soleus muscle fell from -69 to -55 mV. The membrane resistance of the E.D.L. muscle doubled while that of soleus remained much the same. The electrical time constants almost tripled in each case from 1.7m sec. to 4.4m sec., and 1.7m sec to 4.8m sec for E.D.L. and soleus respectively.

These findings were later confirmed and extended by Albuquerque and his colleagues (Albuquerque and McIsaac 1970, McArdel and Albuquerque 1975), who also found that ouabain caused no further depolarization on denervated muscle in mouse.

A study by Bray et al. (1976) gives support to the concept that the depolarization found in denervated rat diaphragm is due to "turning off of an electrogenic pump". In denervated diaphragm ouabain produced little depolarization in 15 min; the resting potential of denervated diaphragm could be largely restored by addition of dibutyryl cyclic adenosine monophosphate, and ouabain then produced a marked depolarization, as in innervated diaphragm.

The most consistent findings about the electrical characteristics of denervated mammalain muscles are the reduction in membrane potential, the increase in membrane resistance, and the reduction in the difference between fast twitch and slow twitch fibres.

d. Fibrillation potentials

Fibrillation potentials will be defined as spontaneous action potentials appearing in chronically denervated mammalian skeletal muscle (Smith and Thesleff, 1976), and can be considered as a manifestation of instability of the membrane (Li, 1960). Two distinct types of fibrillation potentials have been observed. The first type consisted of rhythmic oscillations originating from small depolarizations, similar to anode break excitation, probably associated with a progressive removal of sodium inactivation (Thesleff & Ward, 1975). The other type were irregular depolarizations, discrete, and appeared to be stimulated by randomly occurring excitations. They were first seen after 3 days denervation in mouse diaphragm and reached a maximum frequency between 9 and 12 days (Smith and Thesleff, 1976). It was found by these authors that anything that would stimulate the sodium pump will increase the frequency of these potentials, while reducing the pump's activity will prevent their occurrence. Reducing the temperature to 14^{o} C increased the amplitude and time course, but reduced their frequency by 30%. Reducing the calcium concentration (presumably increasing sodium permeability) increased the frequency of fibrillation potentials, as did adding various catcholamines. Removing the potassium from the bath saline caused an initial increase in frequency for the first $1\frac{1}{2}$ hours followed by a fall. Ouabain 10^{-4}M and TTX 10^{-7}M also blocked activity. Breaking up the tubule system by glycerol treatment or high osmolarity saline also prevented their occurrence. Smith and Thesleff concluded that these potentials were due to regenerative sodium activity in the

system. Catcholamines and ouabain could affect this system either directly through an action on membrane excitability or indirectly via the sodium pump.

Belmar and Eyzaguirre (1966) used denervated rat gracilis anterior muscle to determine the site of origin of the fibrillation potentials. They found that in vivo or in vitro applications of acetylcholine or noradrenaline could increase the frequency of the potentials only when applied to the old end-plate region. Further, direct stimulation changed the frequency of the fibrillation potentials also only when applied to the end-plate region. They concluded that the junctional region is the pacemaker site in denervated muscle for fibrillation potentials.

Fibrillation potentials in rat muscle disappear in vitro (Belmar & Eyzaguirre, 1966). Smith & Thesleff, 1976, found that fibrillation potentials could be recorded in mouse muscle in vitro if the diaphragm was perfused on both sides.

Fibrillation potentials can be distinguished from action potentials. The former appear to originate in the junctional tubule system of denervated muscle. They occur spontaneously and their frequencies increase or decrease by variables which would be expected to affect the sodium pump. Finally, fibrillation potentials are sensitive to TTX while action potentials in denervated muscles are relatively insensitive to it (Grampp, Harris & Thesleff, 1972).

e. Spread of acetylcholine receptors

Following denervation there is a spread of acetylcholine sensitivity along the entire length of the fibre. This is not due to a wider distribution of the same receptors, but rather an increase

in their number. The junctional region remains the most densely packed. In the denervated guinea pig diaphragm muscle there may be a more uniform distribution of receptors.

Albuquerque and McIsaac (1970) used iontophoresis and intracellular recording to plot the spread of acetylcholine sensitivity, following denervation. The rat E. D. L. and the soleus were used to compare the effects on the fast twitch and slow twitch fibres respectively. In innervated muscles, Ach sensitivity was restricted to the end-plate region of E. D. L. whereas in soleus sensitivity could be detected along the entire fibre. Following denervation, sensitivity increased simultaneously along the fibres of soleus, whereas sensitivity developed more slowly in E. D. L. There was no decrease in the sensitivity of the end-plate region.

Prior to denervation, Miledi (1960) using the same technique found that in the rat diaphragm acetylcholine sensitivity was found in an area 520μM long at the junctional region.

Radioactive α-bungarotoxin has been used either with autoradiography or scintillation counting to determine the number of acetylcholine receptors in rat diaphragm muscle. Assuming that one molecule of toxin binds to one receptor, Miledi and Potter (1971) counted 4.7×10^7 sites per end-plate. Fambrough and Hartzell (1972) found 1.14 to 8.70×10^7 receptors per end-plate, or $1.3 \times 10^4/\mu m^2$. Later Fambrough, Drachman & Satyamurti (1973) counted $2.81 \times 10^7 \pm 0.07$ with the exact number of receptor sites increasing with the size of the rat. Chang, Chan & Chuang (1973) counted 1.9 to 2.2×10^7 receptors per fibre, but found that the number increased 30 fold 18 days after denervation. Hartzell and Fambrough (1972) found that in

innervated fibres, there are fewer than 5 receptors/μm^2 in the extra junctional region. The maximum density in this region reached is in 14 days with 1695 receptors/μm^2. At 45 days the numbers had diminished to 529 receptors/μm^2.

Hartzell and Fambrough also report that from appearance it seems that slow twitch fibres in the rat diaphragm have more receptors over them than the large presumably fast twitch fibres, and further, that after denervation the number of receptors in the end-plate does not decrease. Using the uptake of labelled decamethonium, Creese, Taylor and Case (1971) demonstrated that the receptor areas had spread following denervation. Even after 12 days, however, there was a marked peak of uptake at the old end-plate region. However, this peak did not occur in 12 day denervated guinea pig diaphragm. This suggests that in the latter preparation there may be a more uniform distribution of receptors.

6. Receptors in junctional and extrajunctional regions and denervated muscle

Due to the paucity of studies on the differences between acetylcholine receptors in innervated and denervated studies, references are made to a variey of preparations from both mammals and amphibians. There is some evidence that the biochemical structures of the two classes of receptors, innervated and denervated, are somewhat different. An early study indicates that the equilibrium potentials are the same for acetylcholine. However, the affinity to d-tubocurarine may be altered as are the receptor "noise" parameters to acetylcholine and nicotine.

Using toxin-receptor complexes, Brockes and Hall (1975) were able to isolate receptors from the junctional region of innervated and

the extra junctional region of denervated rat diaphragm. The two complexes were indistinguishable by gel filtration, or zone sedimentation in sucrose gradient. They had identical precipitation curves with rabbit anti-serum to the eel acetylcholine receptors. Further tests indicate that they are both glycoproteins. Affinity binding by d-tubocurarine showed a dissociation constant of 4.5×10^{-8}M in the junctional receptors, and 5.5×10^{-7}M in the extrajunctional receptors. Isoelectric focussing indicates that extrajunctional receptors focus approximately 0.15pH units higher than junctional. The authors concluded that while the two types of receptors were similar they were distinguishable.

Dreyer,Walther & Peper (1976) used spectral analysis of membrane "noise" to distinguish between innervated and denervated receptors in frog cutaneous pectoris muscle. They found that two Lorenzian curves are required to fit the power spectrum of junctional receptors, which to these authors suggests that even in the innervated state, there may be two types of receptors, and that denervation causes an increase in one of these types.

They also found that the time constant was 2.4msec with acetylcholine, and 0.45msec with nicotine. In extrajunctional denervated receptors, the time constant increased respectively to 5.0msec, and 2.1msec. Channel conductance was reduced to less than half in denervated extrajunctional receptors.

As noted previously, Brockes and Hall found a reduced affinity to d-tubocurarine in the extrajunctional receptors in denervated rat diaphragm muscle. A similar phenomonen has previously been noted in the same preparation by Beranek and Vyskocil (1967), who found the

equi-effective concentration ratio for d-tubocurarine to atropine was 0.0005 at the end-plate region, but 0.003 for denervated receptors.

An early paper by Jenkinson (1960) on the frog toe muscle showed that while the affinity to d-tubocuarine increased upon denervation, the affinity to acetylcholine and carbachol was further enhanced. He concluded that this represents an increase in the number of receptors, and that in reality the affinity of d-tubocurarine to the new receptors had been reduced.

Denervated and extrajunctional receptors appear to be very similar, but not identical to end-plate receptors. The equilibrium potentials may be the same. However, analysis of membrane noise suggests that denervated receptors have a longer time constant and a reduced conductance. There is also general agreement that in various preparations, denervated receptors have a reduced affinity to d-tubocurarine.

Plan of investigation

In pilot experiments it was found that decamethonium produced depolarization followed by some degree of recovery in the presence of the drug. A decision was made to investigate this phenomonen. Rat diaphragm muscle was chosen as the experimental preparation because of its use in parallel investigations of end-plate permeability by the use of labelled markers as carried out in this department (Creese, Franklin, & Mitchell, 1976, 1977).

Potassium free Krebs saline and ouabain were used to determine the effect on the recovery process of the sodium-potassium pump. Therefore it was necessary to determine their effects prior to the application of depolarizing drugs. Several different transmitter

analogues were used so that the results could be more generally applicable.

Both continuous recordings from single fibres and repeated sampling from the muscle were used to measure the effects of the drug. The latter method was of advantage for long lasting experiments. Because of the requirement for repeated sampling, a method for detecting end-plate regions after the application of depolarizing drugs was sought. These regions are generally identified by the occurence of miniature end-plate potentials. However these transitory events are eliminated by depolarizing drugs. Several different techniques were attempted before a method for visual identification was developed.

In the early experiments, the muscle was suspended vertically in a tissue bath. This had the advantage of ensuring adequate oxygenation to both sides of the tissue, and permitting a direct comparison with studies of the uptake of labelled sodium in which a similarly mounted muscle was used (Creese, Franklin & Mitchell, 1977). Most of the recordings from single fibres were obtained from muscles which were pinned horizontally in a bath with flowing saline.

Parallel experiments were conducted using denervated guinea-pig muscle, in which spontaneous recovery from decamethonium was demonstrated. This preparation obviated the necessity for identifying end-plate structures, and again permitted the results to be more generally applicable.

CHAPTER II METHODS

In this chapter the methods used for electrical recordings from isolated diaphragm are described. Microscopy and histochemical procedures are given in Chapter III.

Muscle preparation

a. Rat diaphragm

All the rats used in these studies were albinos weighing 150-180g. The rats were stunned, the thorax opened, and a left anterior strip of the diaphragm dissected by two parallel cuts through the rib and tendon. The diaphragm strip was then removed and transferred either to a 100ml dish containing cold physiological saline, or mounted directly on the bath. In the dish, aeration (95% oxygen, 5% carbon dioxide) was affected by means of sintered glass distributors. In both cases time from stunning to immersion was about 90 sec so that the preparation could be set up within a short time of the cessation of circulation.

b. Denervated guinea pig diaphragm

Denervation of the left hemidiaphragm of the guinea pig was carried out under ether anaesthesia with weanling animals weighing 100-140g. Two lengths of cotton thread were passed through the ribs, approximately 15mm apart. An incision was then made between the fourth and fifth rib so that a hook could be inserted into the thorax. The phrenic nerve was then pulled towards the incision in the chest wall, the nerve cut, and the threads tied to seal the thorax. The denervated muscles were used after fourteen days in the manner described above.

Solutions

a. Standard Krebs' solution

The physiological saline solution used is the same as that described by Creese and Northover (1961) (Table 2.1).

A stock solution containing all the salts except for calcium chloride was made up in ten litre quantities. A separate stock solution of calcium chloride was made up fresh once a week. Daily, the calcium chloride and glucose was added to the stock solution. it was then bubled with 95% (v/v), 5% carbon dioxide. The pH was 7.45.

b. Solutions with variable potassium

Modified Krebs' solutions with varying amounts of potassium were prepared as shown in table 2.2, the potassium content being increased by the addition of potassium methylsulphate. The solutions were buffered with bicarbonate and 5% carbon dioxide. For solutions with 141mM potassium the bicarbonate was reduced and 100% oxygen was used (Table 2.2).

The solutions are designed to give uniform osmolarity. In experiments on the effects of external potassium, particular concentrations were selected to permit comparison of results with studies on the uptake of radioactive sodium (Creese, Franklin and Mitchell (1976).

The Experimental Baths

a. The Vertical Bath

In this arrangement the diaphragm is mounted vertically on a plastic frame as seen in Fig. 2.2. The vertical bath, and the position of the microscope within the Faraday cage are shown in Fig. 2.1. The dissecting bath and the reservoir for the heated water are seen outside the cage on the left hand side of the photograph.

TABLE 2:1 PHYSIOLOGICAL SALINE

(Modified Krebs' Solution)

Krebs & Henseleit 1932 Hoppe-Seyl. Z. 210: 33.

This is made by mixing certain columes of stock salts which all have the same molar strength (0.154 M).

Modified Krebs' Solution

NaCl	0.9g/dl	100 ml
H_2O		0.75 ml
$NaHCO_3$	1.3g/dl	21 ml
KCl	1.15g/dl	4.25 ml
$CaCl_2.2H_2O$	1.2g/dl	3 ml
$MgSO_4.7H_2O$	3.82g/dl	1 ml
Glucose		0.26 g
$NaH_2PO_4-2H_2O$		0.096 g
		130 ml

Na	145 mM	Cl	126 mM
K	5	HCO_3	25
Ca	1.9	SO_4	1.2
Mg	1.2	$HPO_4^{2} + HPO_4$	1.2

Bubble with 5% CO_2, 95% O_2 pH 7.45

Calcium chloride (deliquescent): make up fresh solution each week. See Krebs & Henseleit 1932.

Changes in solution used by Krebs & Henseleit 1932:

(1) K^+ — 5.0 mM
(2) All K^+ as KCL: used $NaH_2PO_4.2H_2O$
(3) Ca^{++} — 1.9 mM (7.5 mg %)

TABLE 2:2 SOLUTIONS WITH VARIABLE POTASSIUM

Potassium mM	0	2.5	5	10	25	60	141
NaCl 0.9 g/dl	100	100	100	95.75	83	53.25	
H_2O	5	2.875	0.75	0.75	0.75	0.75	
$NaHCO_3$ 2.1 g/dl	21	21	21	21	21	21	5
KCl 1.15 g/dl	-	2.125	4.25	4.25	4.25	4.25	
$KMeSO_4$ 2.31 g/dl	-	-	-	4.25	17	46.75	119
$CaCl_2.2H_2O$	3	3	3	3	3	3	6
$MgSO_4$ 3.82 g/dl	1	1	1	1	1	1	
			130 ml. + 5% CO2, 95% O_2				130 ml. + 100% O_2

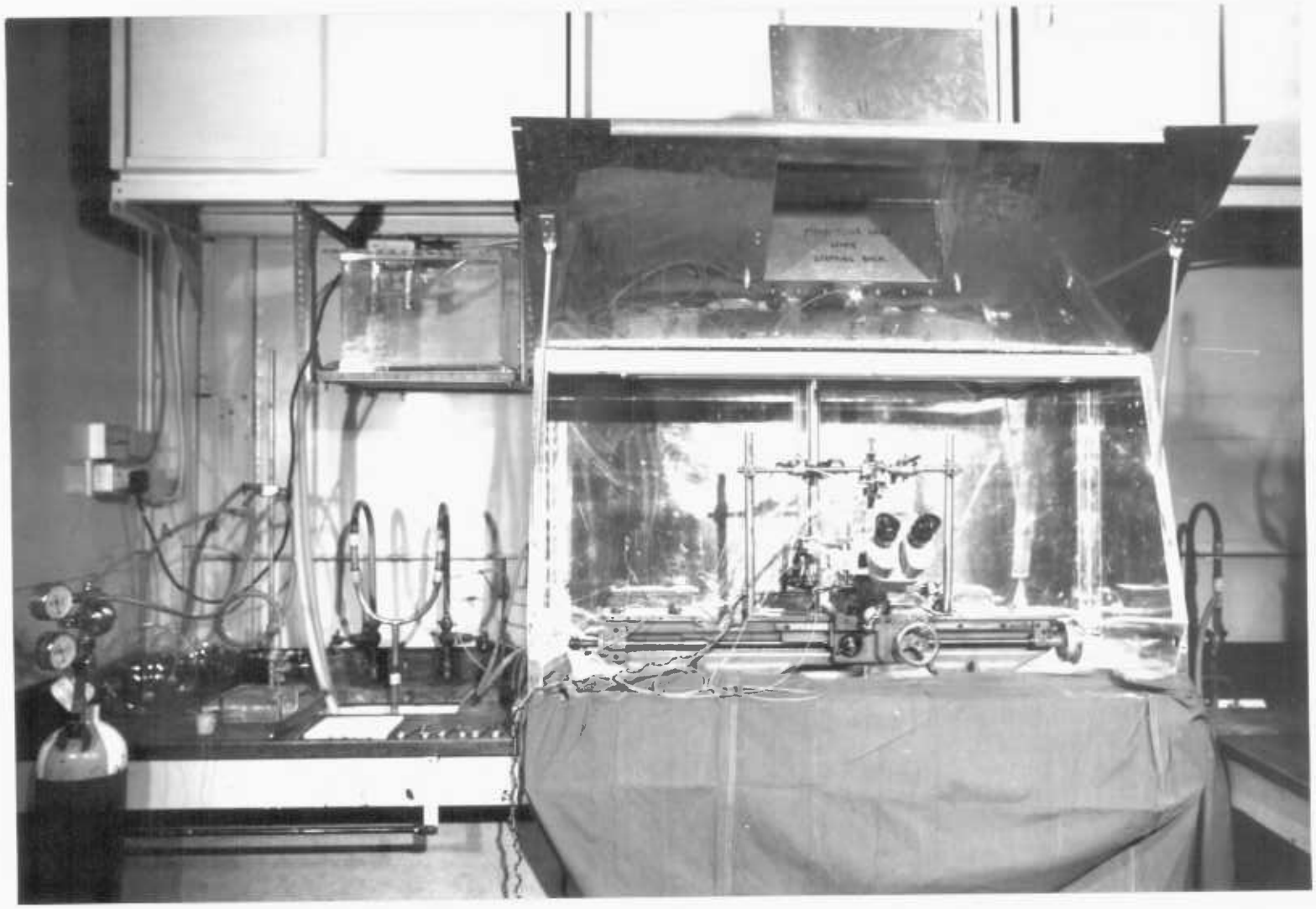
$KMeSO_4$ mol.wt. 150 (Hopkin & Williams)

Solutions contained glucose and NaH_2PO_4 as in Table 1.

Volumes are given in ml.

Fig. 2.1

Screened box used for the vertically mounted preparation. The tissue bath is fixed to the lathe bed, the microelectrode manipulator on the cross bar above it. The microscope is in the horizontal plane. The heating reservoir is left of the box, and the dissecting apparatus is beneath it.



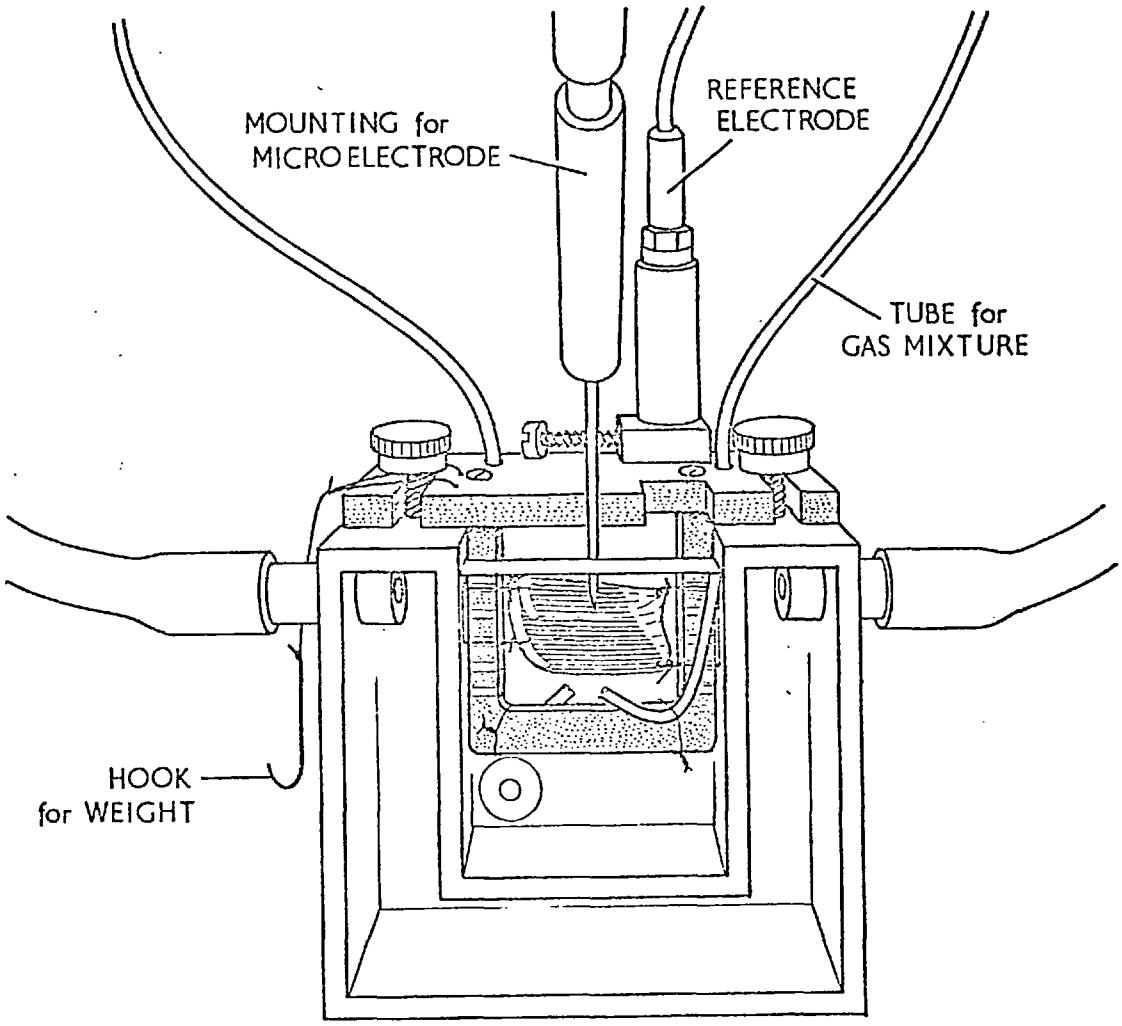


Fig. 2.2

Arrangement for preparation mounted vertically in jacketed bath. Inner chamber contains preparation and solution. The diaphragm is kept taut by addition of weight to hook on left.

The diaphragm and the vertical frame were placed in cold Krebs saline in a dissecting bath. The tendon was attached to the right post of the frame with sutures. Two sutures were attached to the rib and then threaded through holes to the left post of the frame and passed over the top of the frame to a hook.

The frame was then returned to the vertical bath and a weight of 30g was attached by the hook. The oxygenating gas was conveyed by plastic tubes whose ends contained plugs of glass fibres which had previously been soaked in ethylene diamine tetra acetic acid for 24 hours to remove heavy metals. The plastic tubes were attached to the frame with platinum wire. A steady mist of bubbles could then be produced which were directed over the medial portion of either side of the muscle.

Fig. 2.3 shows the arrangement used for changing solutions. A 'Y' joint attaches the back of the tissue bath with two 100 ml jacketed water reservoirs with taps. At the rear upper right hand corner of the tissue bath, an outlet connected the tissue bath by a tube with a Bunsen water pump. By operating the water pump and turning the appropriate tap, the fluid in the tissue bath could be exchanged for either fresh Krebs' or drug solution. Plastic tubing with fibre glass tips were used to oxygenate the reservoirs.

The tissue bath is jacketed on three sides. The bath and the two reservoirs are inter-connected and attached to a 15 litre water bath with a heating unit (Circon) fitted with an electric noise suppressor. The tissue bath and the two reservoirs were maintain to within a half a degree of 38°C by this means.

The muscle was transilluminated by a Bars and Stroud Type

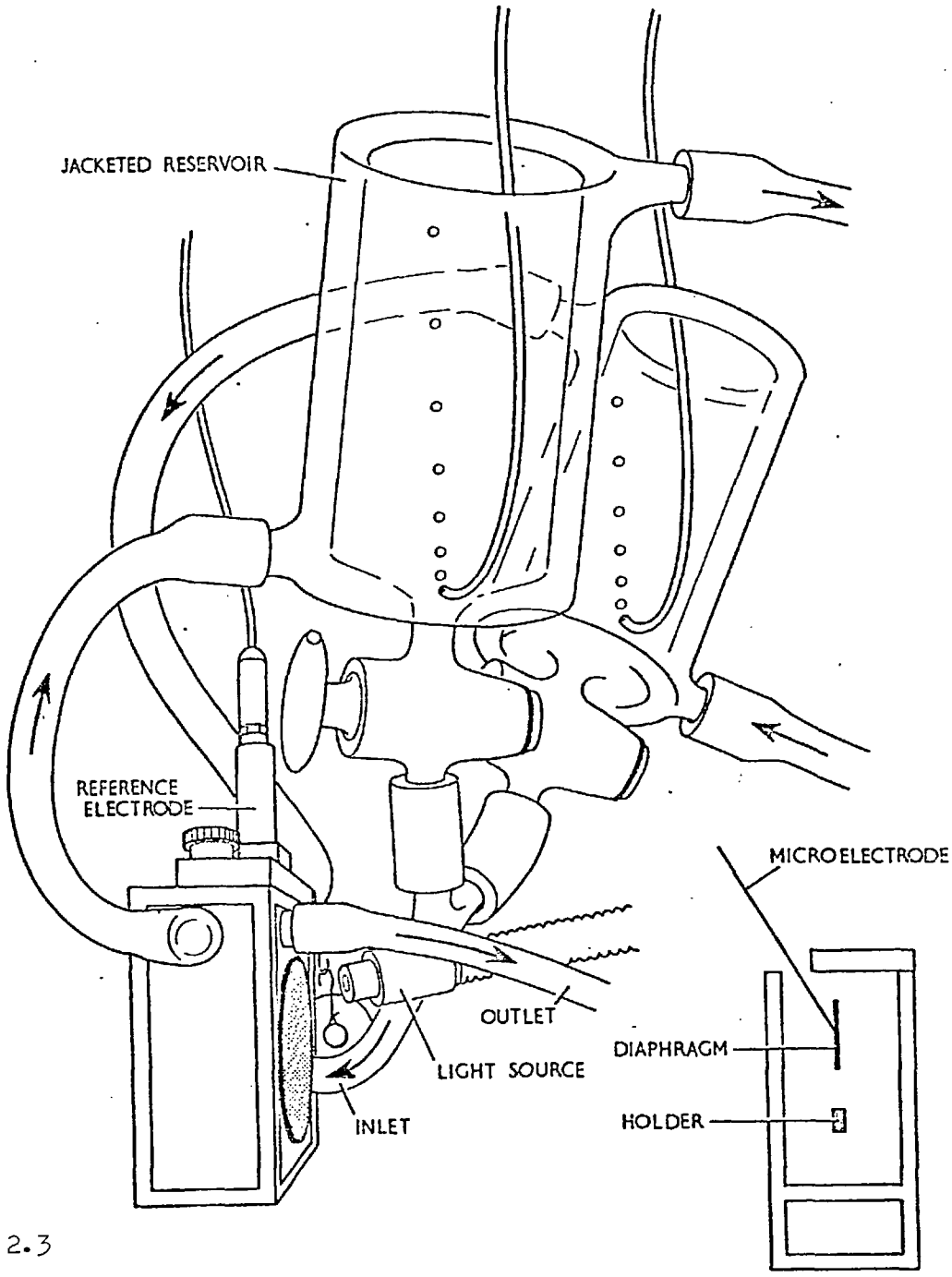


Fig. 2.3

Heated reservoirs for Krebs saline and drug solutions, connected to inner chamber at inlet. Arrows indicate direction of flow in jacketed vessels. Outlet removes solution from inner chamber. Fibre optic light source and polarizing filter are shown at the back of the tissue bath. Insert cutaway of side view showing location of micro-electrode, preparation, and horizontal bar of holder. Location of micro-electrode can be altered by vertical alignment of electrode, and by moving tissue bath forwards or sideways.

LS2 fibre optic light source. The fibre optic was held in place by an 'X' block, and was made to shine through a polarizing filter into the tissue bath. By this means an 80 watt light source could pass cold polarized light through the muscle.

A Nikon SMZ-6 stereoscope was mounted in the horizontal plane facing the front of the tissue bath. The second polarizing element and a 1.5X nose piece was attached in front of the objective. Either 10X or 20X eye pieces were used depending upon magnification desired.

To position the microelectrodes in the vertical bath, two separate systems were operated together. The bath was mounted on the saddle of a lathe to produce left - right, and forward - back movements.

A Narishige SM -19 micromanipulator was mounted so that the microelectrode was angled towards the muscle (Fig. 2.3 inset). The micromanipulator was mounted on a horizontal cross member supported by two vertical bars (Fig. 2.1). The vertical bars were in turn rigidly attached to the lathe bed.

The muscle was mounted such that the fibres ran horizontally (Fig. 2.2). The nerve then ran vertically. By moving the entire bath back or forward, or by vertical movement of the micromanipulator, it was possible to move from one muscle fibre to another. It was also possible by moving the bath from left to right to locate the microelectrode on various parts of the same fibre.

b. The Horizontal Bath

The horizontal bath and associated equipment can be seen in Fig. 2.4. The heating bath and reservoirs for the salines are to

the left of the Faraday cage. The peristaltic pump used to return the saline is to the right and rear of the cage. The three way tap used to direct the returning saline is to the right foreground. The micro-manipulators, the pedestal and bath, and the arrangement of the micro-scope can be seen inside the Faraday cage.

The horizontal tissue bath is mounted onto a Nikon microscope stage and stereoscopic microscope base. This allowed the fibre optic light source to be mounted directly under the tissue bath. There is a recess in the stage for one of the polarizing filters.

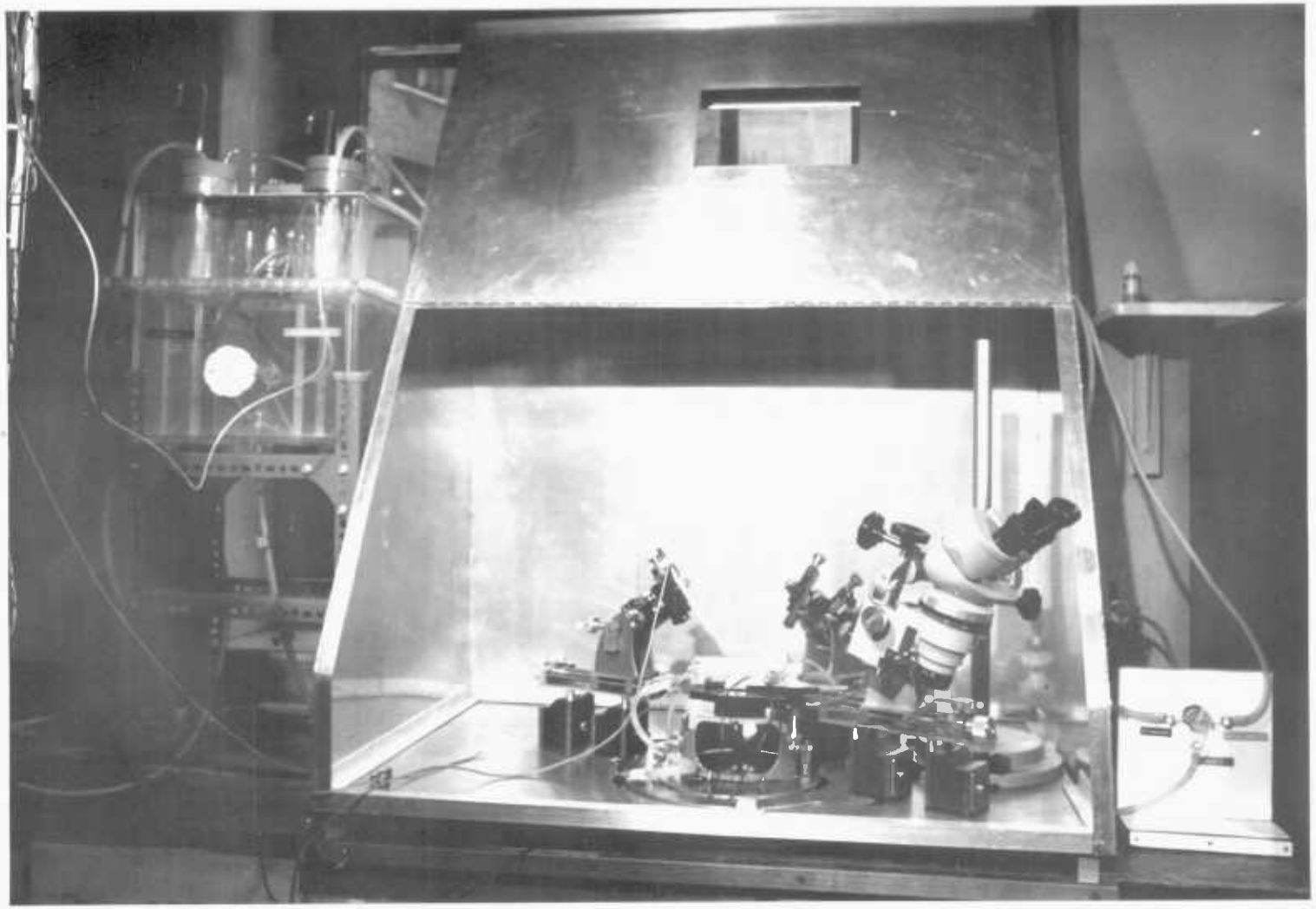
The trough of the horizontal bath contained a bridge (1 x 4.9 cm) made of translucent Sylgard resin (Fig. 2.5). A 1 cm wide strip of diaphragm muscle was pinned to the bridge with cut down hedgehog quills.

The system used to heat and exchange fluid consisted of five parts (Fig. 2.4). Two reservoirs for the saline and the drug solutions were housed within a 20 litre perspex tank, and interconnected with a three-way tap. The tissue bath was jacketed, and connected to the tank and reservoirs with co-axial tubing. The outer tubing carried heated water from the bath to the jacket of the tissue bath. The inner tube carried saline from the reservoirs to the inner trough of the tissue bath. A two channel Watson and Marlow peristaltic pump returned the heating water to the tank, and pumped the experimental fluid to a three-way tap. The tap was used to return the saline solutions to their reservoirs or send them to waste.

A Circon heater with electrical hum suppressor was used to heat the contents of the water bath. A Mullard VA3700 miniature bead thermister was encapsulated in the jacket of the tissue bath and was used to drive a meter. The heater was adjusted until the temperature

Fig. 2.4

Screened box for the horizontally mounted preparation. The tissue bath is fixed to a modified microscope stage. Micromanipulators are held by magnets to the floor of the box. Left is the heating reservoir for the saline solutions, and selector tap. The pump which returns saline and water to the reservoir is behind the screen.



in the trough of the tissue bath was 38°C as measured with a mercury thermometer and the thermister meter was then adjusted to read 38°C.

The exchanging of one solution with another is similar to the problems encountered in the analysis of indicator methods for measurement of blood flow. The change of indicator concentration as a function of time can be considered to be governed by two components, mass transport (flow) and radial diffusion. When the mass transport component dominates, the flow becomes laminar, with the greatest concentration of indicator in the centre of the flow and progressively less concentration towards the periphery of the stream. The length of time required to reach full and uniform concentration is then maximal.

When the radial diffusion component dominates, the difference in radial concentration is minimal and the difference in concentration along the line of flow is maximal. The consequence is that under these conditions the change from no indicator present to maximal concentration of indicator occurs in the shortest period of time (Bates, Rowlands and Sirs, 1973). Thus by judiciously increasing the number of constrictions in the flow-tube, the time required for changeover of solutions can be considerably shortened.

The time for changeover was measured by placing a photocell system over the Sylgard bridge in the tissue bath and measuring the rate of change from the standard Krebs saline to a haemoglobin solution. The response was recorded on the storage oscilloscope and photographed.

The clamps and the twists on the tubing were then changed until the rate of change was maximized. The rate of change was then found to reach 80 per cent in two seconds (Figs. 2.6; 2.7). The

rate of flow was found to be 65 ml per min.

Fig. 2.5 shows the tissue bath mounted on a microscope stage and the microelectrode held by a micromanipulator. The MM-3 micromanipulator could be moved in all three dimensions, with an extra micrometereadjustment in parallel with the microelectrode. The micromanipulator was mounted on a carriage, and the frame was supported on two magnetic bases.

c. Another horizontal system

In some cases a system which incorporated a Prior 20-30 model micromanipulator, a differential valve cathode follower with ME 1400 Electrometer valves (Keith Copland design, University College, London), and an eight way tap with bubble trap was used. The remainder of the system consisted of design features described as for the horizontal bath (above).

The Electrical Recording System

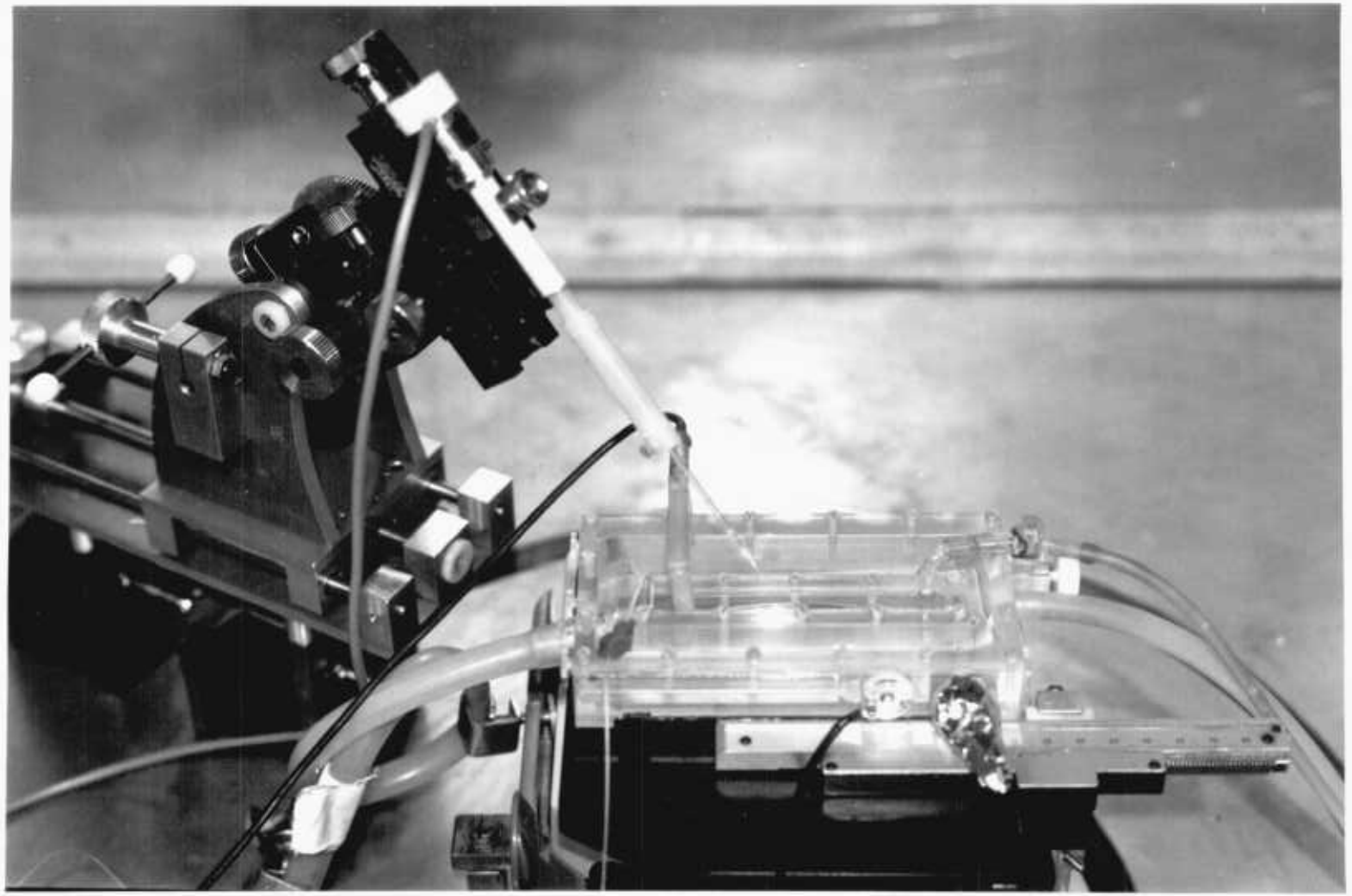
a. The inter-connections between equipment

The equipment was arranged so that membrane potential could be simultaneously observed and recorded by an ultra violet trace, while miniature end-plate potentials could also be observed and photographed. At selected intervals a digital record of the membrane voltage could also be made and the interval marked on the trace. This approach had the advantage of constantly calibrating the trace, and obviating the need for a zero potential line on the record.

The organisation of the equipment is shown in Fig. 2.8. The output of the second stage was connected to the Grass P18 pre-amplifier. The output of the Grass amplifier was connected in parallel to a 8000A Fluke Digital Multimeter, a 305 and a 502A

Fig. 2.5

Bath for the horizontally mounted preparation. The tissue is mounted in the trough, which is surrounded by a hot water jacket. Left is a coaxial tube which passes saline through the inner core, and heated water through the outer tube. Right are the tubes for removing the heated water and saline. Reference electrode is mounted on the back of the bath, in front are the thermistor and earth point for the heated water. Micro-electrode system is on the manipulator having facilities for movement in three planes and an arc.



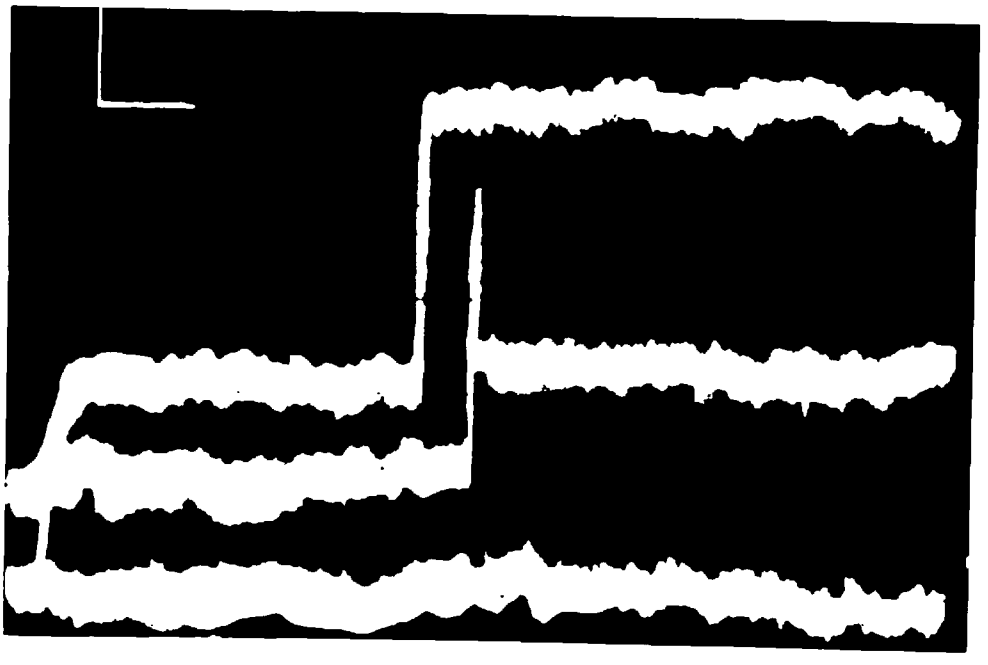


Fig. 2.6

Response time of photocell mounted over horizontal tissue bath.

Upper trace: interruption of light beam by paper sheet

Middle trace: interruption of light beam by Wratten filter

Lower trace: continuous recording

Vertical calibration 2 mV

Horizontal calibration 2 sec.

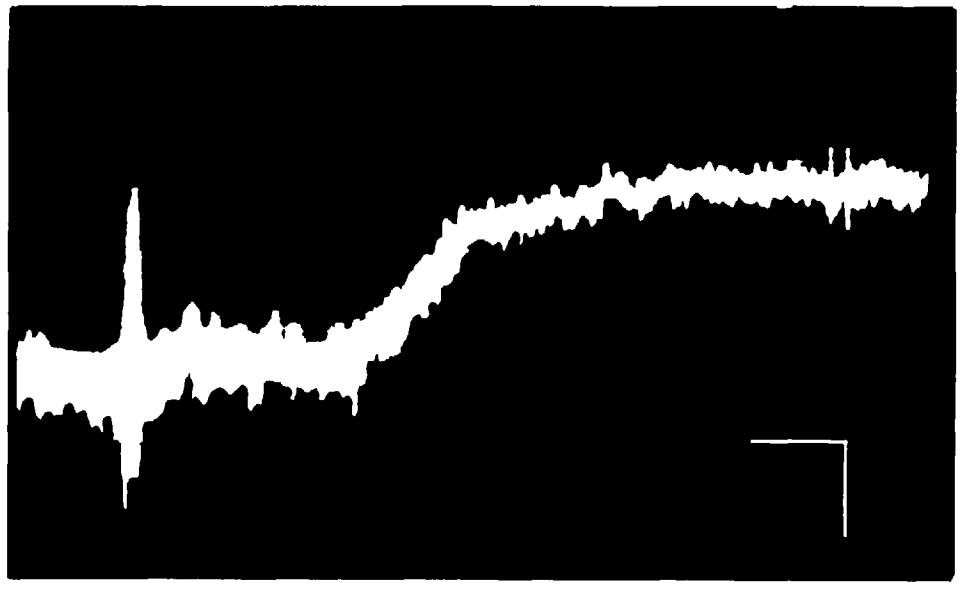


Fig. 2.7

Response of photocell to change from saline to haemoglobin solution.

80 percent response occurred within 2 sec.

Vertical calibration: 2 mV. Horizontal: 2 sec.

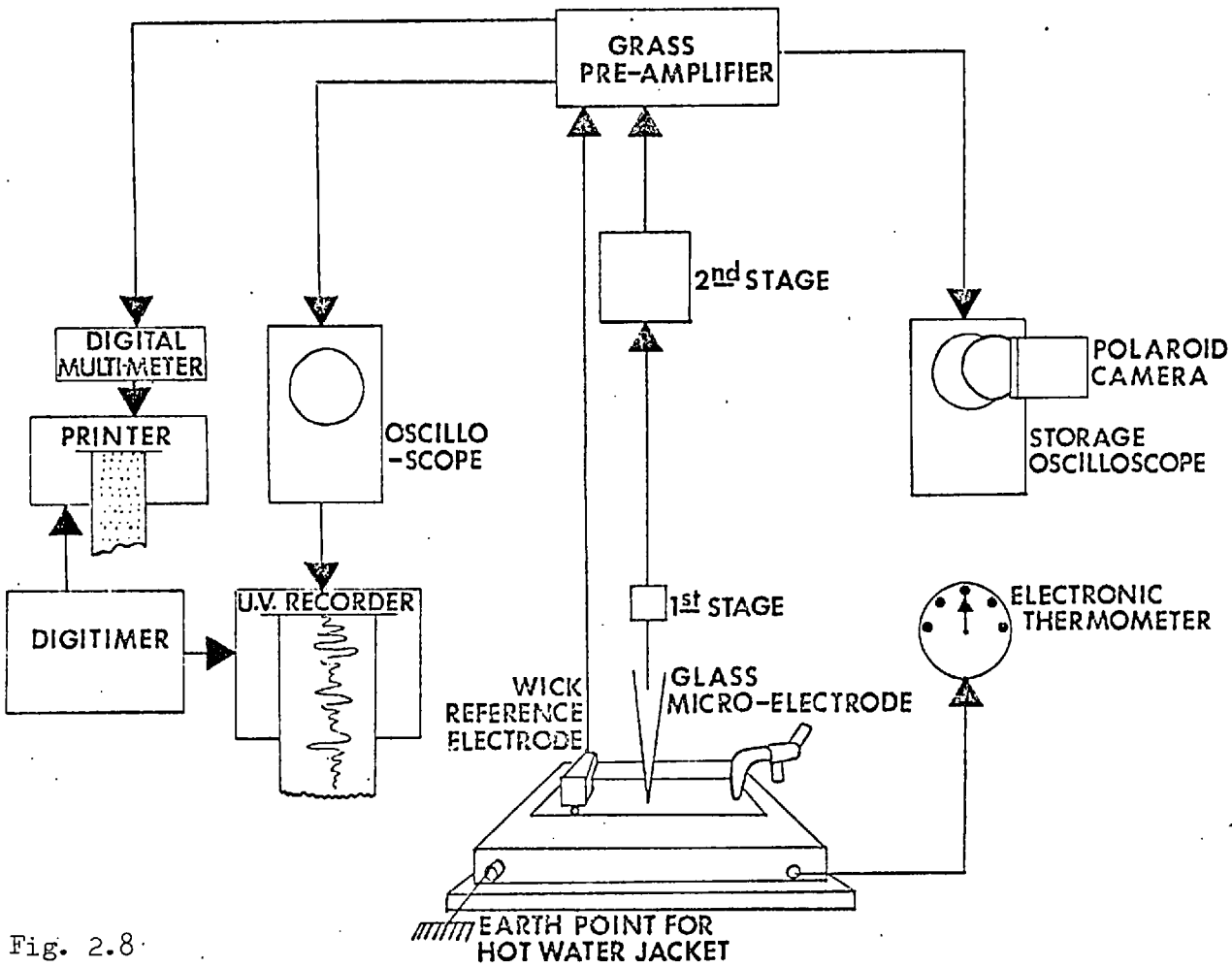


Fig. 2.8

Arrangement of electronic equipment. Arrows indicate the direction of "information flow".

A two-stage unity-gain amplifier connects the glass micro-electrode to the Grass pre-amplifier.

Tektronix oscilloscope. The 502A oscilloscope was modified to drive an S E Laboratories 2100 Ultra violet recorder. A C-27 Tektronix camera was used to photograph the face of the 305 oscilloscope. The output of the digital multimeter was recorded by an Anadex D P-500-18 printer. A D4030 Digitimer was used to drive a vertical line across the ultra violet trace and to trigger the printer.

b. The first and second stages

The circuit was composed of two parts (Figs. 2.8; 2.10). The first stage was incorporated into the electrode assembly (Fig. 2.12). while the second stage, which included the power supply, X 1 amplifier, voltage backoff system and an electrode capacity compensation was rack mounted and connected in turn to the Grass pre-amplifier (Fig. 2.8).

The first stage consisted of an LH0022 National Semi Conductor operational amplifier with an FET (field effect transistor) input, and a metal shield which covered about half the length of the Perspex electrode holder (Fig. 2.12). A brass screw, brazed onto the silver/ silver chloride electrode and projecting from the Perspex holder, screwed into a nut which was soldered onto the input of the amplifier.

This operational amplifier was connected in the non-inverting unity gain mode, making the effective input resistance 10^{12} ohms and the effective output impedence less than 75 ohms. The manufacturer states that the input capacity of the operational amplifier is 4 pf.

A cap when placed in position at the back of the first stage (Fig. 2.12), closed the electrode resistance measuring circuit by connecting the input of the LH0022 operational amplifier via a line to a 10^9 ohm resistor and 1 V power supply. By placing the connector as close as possible to the operational amplifier, the

Fig. 2.9

Recording system. Left rack, top shelf, ultraviolet recorder: middle shelf, storage oscilloscope with polaroid camera, oscilloscope to drive U.V. recorder: lower shelf, light source for fibre optic. Centre rack, top to bottom: electrical thermometer connected to tissue bath thermistor, digitimer, digital printer, digital voltmeter, second stage amplifier, iontophoresis system, membrane polarizer system, power supply.

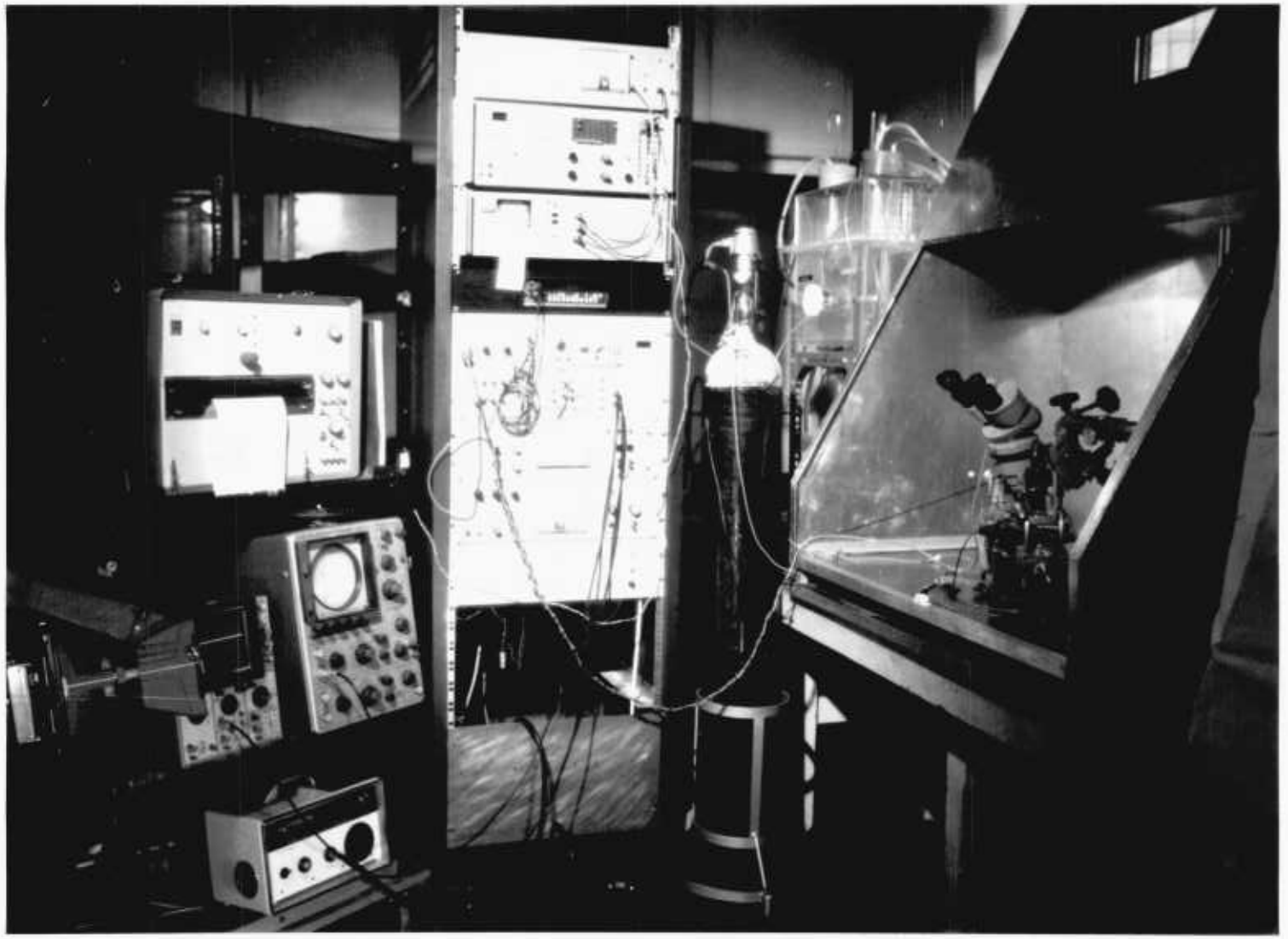
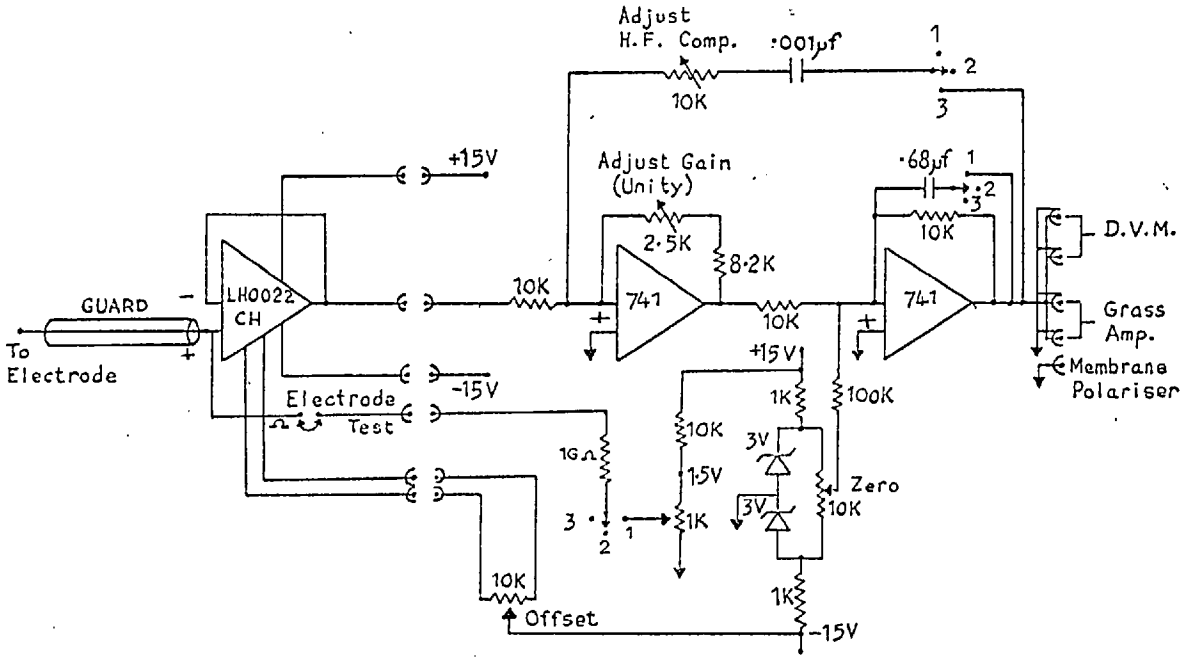


Fig. 2.10

The two stage amplifier with unity gain. The left hand operational amplifier, LH0022, is the first stage and is close to the micro-electrode. The second stage is rack-mounted and connects to a digital voltmeter, and Grass pre-amplifier. A device used to measure electrode resistance is in the first stage. In the second stage, the gain of the left 741 amplifier was set to unity. The right 741 amplifier is arranged to allow zero balancing and incorporates a high-frequency cut-off capacitor. There is overall positive feed back to compensate for signal loss due to electrode and input capacitance.

MICRO-ELECTRODE AMPLIFIER



SPECIFICATION

SUPPLY ±15V
RISE TIME 0.5 mS normal
20µS H.F. Comp. (10M Ω source imp.)
NOISE ⩽200 µV normal 0-1 MHz
⩽500 µV H.F. Comp. 0-1 MHz.
INPUT IMPEDANCE 10^{12} Ω
STABILITY TYPICAL 0.5mV/hour after 15 mins warm up.
ZEROING RANGE ±300mV.

SWITCH POSITION (Ganged)

1. Electrode Ω
2. Normal use
3 With H.F. Comp.

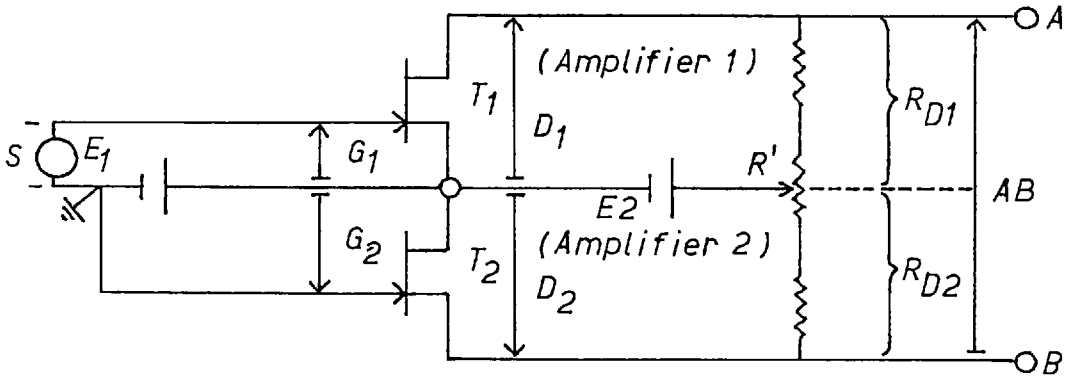


Fig. 2.11

Model diagram of the connections to the Grass amplifier, to illustrate that with one input earthed, there will be common mode rejection, and compensation for changes in the power supply (diagram from Electronics Department, Chelsea College). S is the signal from the second stage. The reference electrode from the bath is connected directly to the earth point of the Grass amplifier. AB is the output.

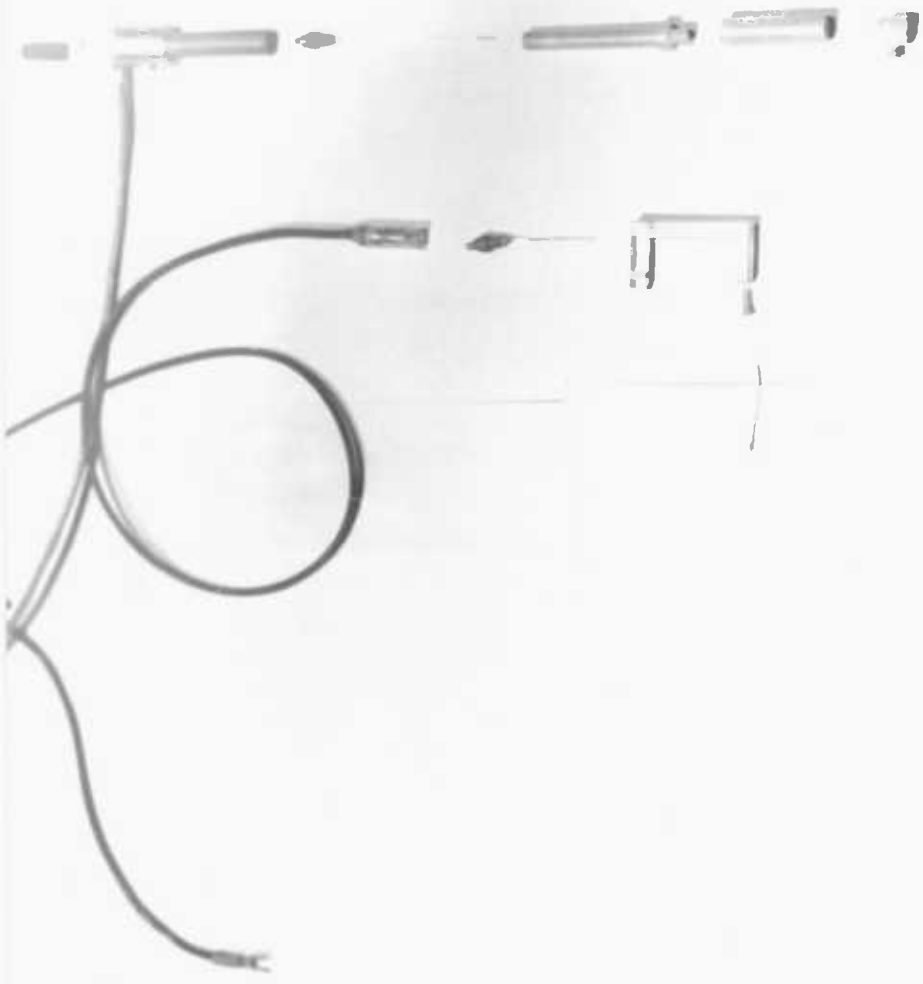


Fig. 2.12

Electrode system - top, left to right: first stage of follower system, chlorided silver wire, silver wire holder, agar bridge holders, microelectrode holder, glass micro electrode.

Bottom, reference electrode: cable with nut to hold silver wire, chlorided silver wire, agar bridge and wick holder.

capacitance of the line was prevented from interfering with the normal functioning of the system. When connected 1mV was produced for every M ohm of electrode resistance, with a test current of 1 nA. This voltage was then monitored on the digital voltmeter.

A separate 15 V source, located outside the Faraday cage provided the first stage with an offset voltage and a power supply. The offset voltage line was connected through a 10 K ohm variable resistor to the first stage. The variable resistor was located with the remainder of the controls outside the cage. This resistor was used to set the output of the amplifier to zero volts. The first stage was connected to the remainder of the circuit with a multicore screened cable.

The second stage (Fig. 2.10) was composed of two 741 operational amplifiers, and $\pm$ 3 volt power supply. This power supply circuit consisted of a separate 15V source, two 1 K ohms resistors, two 3 V Zener diodes and a 10 K ohm variable potentiometer. The two diodes were connected in series with an earth point in between (Fig. 2.10) to yield $\pm$ 3 volts. The variable potentiometer was connected in parallel with the two Zener diodes and thus could be used to select a voltage within that range.

The two 741 operational amplifiers were connected in series. In both cases, the input resistance was matched to a resistor of equal value in the negative feedback configuration. The feedback resistor on the first 741 amplifier was a variable potentiometer to permit it to be adjusted to the same value as the input resistance.

The input of the second 741 amplifier was also connected through a fixed value resistor to the 10 K ohm variable potentiometer

of the $\pm$ 3 V power supply system. This is the signal summing configuration and permits a fine adjustment to remove a standing D C potential. The use of the signal ~~averaging~~ SUMMING configuration on the last amplifier and the variable potentiometer in the negative feedback configuration on the first 741 operational amplifier allowed the complete system to be adjusted with no standing D C voltage and an amplification factor of one.

Two capacitors were also connected in feedback configuration. The output of the second 741 amplifier was fed back through a 0.001μF capacitor and variable potentiometer to the input of the first 741 amplifier. This permitted a variable positive high frequency feedback, which was used to compensate for signal loss due to the electrode and input capacitance. A second capacitor (0.68μF) was connected across the second 741 amplifier in parallel with the 10 K ohm resistor when measuring electrode resistance. This latter arrangement was used to eliminate any 50 cycle electrical interference from the cable in the resistance-measuring circuit.

The measured responses of the system were as follows: risetime 20 μS with a 10 M ohm source resistance; 200μV noise level at 0-1 M Hertz; input resistance of 10^{12} ohms, output impedence 75 ohms; and a 0.5mV per hour drift with a fifteen minute warm up time.

The entire system was designed to produce an amplifier which would have a high input resistance, a low output impedence, have no D C potential of its own, would neither amplify nor diminish a signal, and would produce an accurate representation of the event it was recording.

c. Differential amplifier (Grass)

A model which can be used to illustrate the connections to the Grass pre-amplifier is given in Fig. 2.11 (laboratory notes from Chelsea College Electronics course). The signal(s) in the diagram would be the output of the second stage. The bath reference electrode would be represented by the line connected to earth. Then the output of the second stage is connected across the input of amplifier 1 (T_1) and earth. The bath reference electrode connects the input of amplifier 2 (T_2) to earth.

Due to the low output impedence of the second stage, both amplifiers of the Grass are connected to low resistance sources. Any electrical interference should then effect the voltage on the lines to the two Grass inputs to the same degree.

The transistor circuit T_1 is amplifying a signal in the conventional manner (its output is V_{D1}). An identical amplifier (2) has NO signal applied to it, but uses the same supply of batteries E_2 and E_1. The output from the two amplifiers is taken in a differential manner, i.e. $(V_{D1} - V_{D2}) = V_{AB}$. With zero signal applied to amplifier 1, adjustment is made of the potential divider R until $V_{AB} = 0$. The system is said to be balanced. The total load resistance of T1 is now R_{D1} and of T2 is R_{D2}.

Suppose that a steady potential from a source is being measured. The value of V_{D1} changes, but V_{D2} remains unchanged. Hence V_{AB} alters by an amount depending on the signal applied to the system. If there is a change in the power supplies E_1 or E_2, V_{D1} and V_{D2} are both affected by the same amount so that at balance V_{AB} remains unchanged. As long as both transistors have the same characteristics, then changes in power supplies do not alter the

output V_{AB}. It is discriminating against changes in power supplies.

There are also electric and magnetic fields in the environment of the amplifier particularly at 50 Hz. Owing to the mechanical symmetry of the system such fields produce potentials of the same magnitude of both inputs simultaneously. An inphase signal causes V_{D1} and V_{D2} to change by the same amount and in the same direction and so V_{AB} remains unchanged. The amplifier discriminates against inphase signals. Thus, although one side of the Grass was earthed it still operated as a differential amplifier.

d. The earthing system

(1) common earth point

The common earthing point for all electrical equipment was a $\frac{1}{4}$ inch diameter, multiple stranded copper wire which was connected to a large copper plate buried in the earth. This line is used by few laboratories at the Medical School and is thus relatively free from electrical interference.

(2) the Faraday cage

The preparations, micromanipulators, microelectrode and the first stage system were contained within 5 sided aluminium cages. For the vertical preparation (Fig. 2.1) the tissue bath was mounted on a lathe which was bolted to a heavy wooden platform. A sheet of aluminium was placed under the platform. The sheet, the cage and the lathe were interconnected with unscreened electrical wire.

The apparatus used for recording from horizontally placed preparations was mounted on a half inch steel plate (Fig. 2.4). The steel plate and the aluminium cage were also connected together with the unscreened electrical wire. Both cages were connected to the common earthing point. The boxes thus formed acted as Faraday cages

and aided in preventing electrostatic changes from interfering with the recording.

The front of the boxes were divided into sections and the sections hinged together. This permitted most of the cage to be closed off while the microscope and the micromanipulators were being used.

(3) jacketing fluid

The plastic tubes carrying heated water to warm the tissue baths were interrupted on the outside of the boxes by a length of copper piping. These pipes were also connected to the common earthing point, and prevented electrical noise from the water heater entering the Faraday cages.

(4) remainder of equipment

All other electrical equipment except for the U V. Recorder, oscilloscopes and light source, were housed within a five sided sheet steel electronics rack. The rack and all the earth lines for the housed equipment were connected to the common earthing point.

The U.V. Recorder was found to be particularly sensitive to electrical disturbances. These were overcome by placing the recorder within a cardboard box lined with sheets of antimagnetic nickel alloy. In this case no connections to the common earthing point were made.

The microelectrodes

Fig. 2.12 shows the assembly for the microelectrode and the reference electrode. Both electrodes were contained within a Perspex tube drilled and shaped to accept the silver/silver chloride wire, and either a glass microelectrode, or a cotton wick in the case

of the reference electrode. Both housings were filled with agar and potassium chloride to act as a bridge between the microelectrode or cotton wick and the silver/silver chloride wire.

a. Silver/silver chloride electrodes

The Silver/silver chloride electrodes were constructed from 0.022 inch 999 standard silver wire brzed to 6BA brass screws which were held in electrical sockets. The silver wire was prepared for chloriding by scraping the surface with coarse and then fine abrasive paper, washing the surface with diluted nitric acid then wiping the electrode dry.

The silver wires for the reference and the recording electrodes were chlorided together with a thick silver wire which acted as the cathode. The silver wire was chlorided to conform to the silver chloride salt density proposed by Geddes (1972). In terms of current this amounted to 500mA sec^{-1} cm^{-2} of electrode surface. This was achieved by passing of 6 mA of current for 8 min in a 0.9% sodium chloride solution.

Due to the increasing resistance of the silver wire as it accumulated a silver chloride salt coat, a special constant current circuit was constructed (Fig.2.13).

The silver wire was cleared and rechlorided once a week. It was then tested with a digital voltmeter to determine the potential difference between the pair of electrodes. If the electrodes had developed a potential greater than 100μV the wires were cleansed again and rechlorided. Such electrodes are very stable and non-polarizable (Katz, 1966).

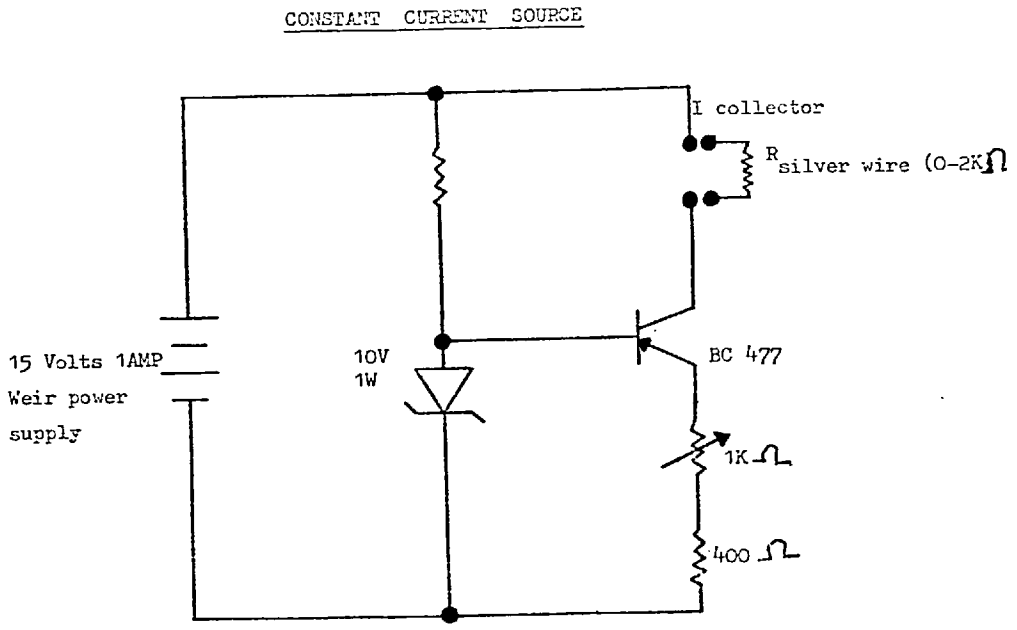


Fig. 2.13

Circuit of constant current source used to chloride the silver wire at a constant rate.

b. Agar salt bridges

The Agar bridges were made up of 1.5 per cent agar (w/v) with 2.5M potassium chloride solution. The agar was melted in a beaker suspended in boiling water. This procedure prevented the agar from burning. The hot liquid was then pipetted into the Perspex electrode housing. By using the same salt concentration for both agar bridges, and microelectrode electrolyte, diffusion potentials between the two were kept to a minimum.

c. Glass microelectrodes

The glass microelectrodes were pulled by a Narishige microelectrode puller. The electrode material was Clark Electro-medical filament glass (2mm external, 1mm internal bore). The glass as delivered by Clark does not require cleaning and was used without any additional treatment.

Because this glass contains a filament which assists filling by capillary actions, the microelectrodes could be filled in less than 10 min.

The electrodes were filled by 2.5 M KCl under reduced pressure at 97°C. The electrolyte was made up in bulk from "Analar" grade KCl salt and distilled water, and stored in brown Winchester bottles. The solution was filtered with number 4 filter paper prior to storage.

When electrodes were being made up, the specific gravity of the solution was measured, and deionized water was added until the hydrometer indicated 1.105 units. The electrolyte was then filtered by 0.22 μM Millipore filters to remove dust.

The containers used for heating the solution and the rod used to hold the electrodes were washed and rinsed in distilled water to prevent any dust contaminating the electrodes while they were being filled.

Standard method of recording

a. Cleaning

All the parts of the system that were in contact with the tissue, the Krebs solutions, or the microelectrodes were taken apart once a week and cleaned. The glass reservoirs and the tissue baths were washed in Tepol and then rinsed three times in distilled water before drying in an oven. The rubber tubing was boiled in a sterilizing tank, and the Perspex electrode holders were boiled in a Tepol solution to remove agar from the inside surfaces. The tubing in the Watson-Marlow pump was replaced each week to prevent unexpected breaks and leaks.

b. Microelectrodes

The electrode holders were filled with a fresh agar-salt solution, and the silver wire electrodes were rechlorided once a week also. Two sets of twenty glass microelectrodes were pulled and filled at the same time.

Every day prior to recording, the system was connected together, the reservoirs filled with fresh Krebs solution and the glass microelectrodes put into place. The recordings systems were switched on, and the gas tanks opened up. The system was then run for half an hour to allow the equipment to warm up. For a second half an hour the system was checked for electrical drift. If a drift greater than 2mV per hour was recorded, the microelectrode

assembly was replaced with an alternative one.

The glass electrodes used for recording had resistance of 15 to 20 M ohm. During experiments in which recordings were made by repeated insertions into different parts of the muscle the electrodes were tested after each set of insertions and if the resistance was outside the above range the electrodes were replaced.

c. Adjustment of amplification and capacitance

Having been switched on for an hour, the amplification and capacitance of the recording system, with microelectrode in place, was tested. Any voltage due to 'tip potential' (Geddes, 1972; Lavallee, 1969) was first backed off at the first stage (Fig. 2.10). A known voltage was then inserted from the millivolt calibrator of the Grass pre-amplifier. The voltage was displayed on the digital voltmeter.

The Fluk Digital Multimeter, and the millivolt calibrator were themselves tested with the aid of a standard resistor and standard one cell battery. Any change in the gain of the recording system could be adjusted at the first 741 integrated circuit of the second stage. However, this was rarely necessary since the output never varied by more than 100μV.

The capacitance of the system was tested by connecting a Grass SD9 square wave stimulator in series with the glass microelectrode and observing the output of the Grass pre-amplifier. The signal from the square wave stimulator and the pre-amplifier were both displayed on the 305 storage oscilloscope (Fig. 2.14). The capacity of the entire system was adjusted at the high frequency potentiometer of the microelectrode amplifier (Fig. 2.10). When the compensated signal

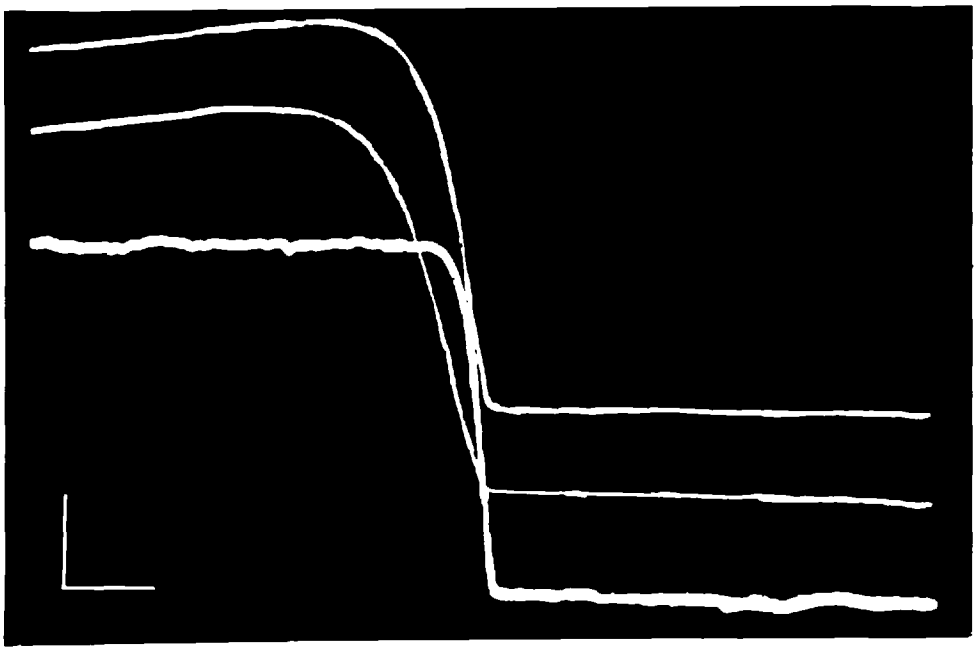


Fig. 2.14

Response time of the recording system to square wave pulses.

Lower trace: direct from stimulator into oscilloscope (end of square wave)

Middle trace: applied in series with micro-electrode.

Capacity compensation. 10Hz and 10KHz filters applied.

Upper trace: as middle trace but without filters.

Vertical calibration: 10mV

Horizontal calibration: 50μsec.

of the recording system was as close as possible to the directly observed signal from the Grass stimulator, the filters of the oscilloscope were then set at 10 Hz and 10K Hz. This caused very little distortion in the response of the system (Fig. 2.14) but was seen to improve the clarity of the observed miniature end-plate potentials (Fig. 3.5 below).

d. Continuous records

For continuous records an ultra-violet trace was made as well as a printout from the digital voltmeter. The Digitimer was used to coordinate a line on the ultra violet trace with the printer, so that the two recording systems could be inter-related.

A superficial fibre was selected for recording if miniature end-plate potentials were observed and if there had been a change less than 1mV min^{-1} in the first 5 min preceeding the introduction of the drug solution. When the tap was turned to introduce the drug solution, the ultra violet trace was marked and a switch was operated which changed the colour of the print face on the output of the voltmeter.

After a drug solution was introduced, the solution was sent to waste or in some cases recirculated. When repeated insertions were made, the potentials were recorded on a sheet of paper, which also contained other information about the experiment.

e. Processing of electrode recordings

The ultra violet traces were kept in a light-proof box and a photocopy was made the morning following the experiments. This was found to be unsuitable for reproduction by photography or by zerox: transparent paper was placed across the photocopy and the trace was outlined with Indian ink. The transparencies were photographed with reduced size and the glossy photos were zeroxed.

List of drugs

The drugs which were used are given, with their molecular weights in parentheses. Carbamylcholine chloride (carbachol: Koch-light, 182.6). Decamethonium bromide (Burroughs Wellcome Co., North Carolina, 418.4). Suberyldicholine iodide (gift to Professor Creese from Dr. A. Ungar, University of Edinburgh: 346). Strophanthin-G (ouabain, as octahydrate: BDH: 728.8). Tetrodotoxin (Sankyo, 319.3). The decamethonim was stored in a desiccator before use to prevent moisture accumulating.

Statistical procedures

The median and its 95% confidence limits have been used when the distributions were not known (Tables 4.1; 4.2; 5.1; 5.2; 6.3), the 95% confidence limits being found from tables given by Colquhoun (1971).

In Chapter IV a biomodal distribution was found for the resting potentials of fibres in solution with zero potassium, and the method for obtaining a computer fit of the results is given in this Chapter.

In Chapter VI the dose-response curve (Fig. 6.4) has been plotted for the effect of decamthonium on the membrane potential of rat diaphragm at the end-plate region, and the mean depolarization at different concentrations have been analysed by conventional probit methods (Goldstein, 1964).

CHAPTER III LOCALIZATION OF END-PLATES IN RAT DIAPHRAGM

While measuring the diamter of muscle fibres for calculation of sodium fluxes (Creese, Franklin & Mitchell, 1977) it was noticed that end-plates (or similar structures) could be seen with polarized lights. This was further explored as shown below.

Measurement of fibre diameter

Four pieces of equipment were used to measure fibre-diameter. These were the vertical bath, the zoom microscope, a set of polarizing filters, and two graticules. By using the vertical bath, the largest number of fibres could be viewed in one living preparation.

One of the filters was on the rear wall of the bath, between the fibre-optic light source and the preparation (Fig. 2.3). The second filter was set in a swivel ring attached to the nose-piece of the microscope (Fig. 2.1). By turning the second filter, the amount of polarization could be varied. This system enhanced the identification of individual fibres.

The measurements were made by using two graticules (each line divided into 50μm units), one mounted in the right eye-piece of the microscope, and a standard which was mounted on the front surface of the vertical bath. The diameters of individual fibres were expressed in eye-piece units. After a set of fibres had been measured, the microscope was focused onto the second graticule, without adjusting the magnification, and the two graticules were compared. In practice 100μm was equivalent to 6.5 eye-piece units (Table 3.1). The fibre diameters were measured in eye-piece units and converted into μm.

Table 3:1 Fibre diameters in rat diaphragm

Units	µm	No.	Cumulant	%	Probit	µm	log µm	No
0.25-0.75	3.8-11.5			0				6
0.75-1.25	11.5-19.2	6	6	2.2				
1.25-1.75	19.2-26.9	6	12	4.6				61
1.75-2.25	26.9-34.6	55	67	25.7	4.35	34.6	1.54	
2.25-2.75	34.6-42.3	38	105	40.2	4.75	42.3	1.62	89
2.75-3.24	42.3-50	51	156	59.8	5.25	50.0	1.70	
3.25-3.75	50 -57.7	27	183	70.1	5.53	57.7	1.76	55
3.75-4.25	57.7-65.4	28	211	80.8	5.53	65.4	1.82	
4.25-4.75	65.4-73.1	14	225	86.2	6.09	73.1	1.86	38
4.75-5.25	73.1-80.8	24	249	95.4				
5.25-5.75	80.8-88.5	4	253	96.9				5
5.75-6.25	88.5-96.2	1	254	97.3				
6.25-6.75	96.2-103.8	6	260	99.6				7
6.75-7.25	103.5-111.5	1	261	100				

5 rats were used (weight range 85-115g). The microscope was focussed such that 100µm in the reference graticule = 6.5 units in the eyepiece.

50-61 fibres were measured in each muscle.

The results are shown in Table 3.1 and in Fig. 3.1. In 261 fibres from 5 rats the median fibre diameter was 46μm. The range was from 15 to 108μm. The distribution is asymetrical (Fig. 3.1). When the cumulative percentage was plotted on a probit scale against log diamter a straight line was produced (Fig. 3.2). It is concluded that 96.9% of the fibre diameter in rat diaphragm muscle has a log-normal distribution. Extreme values in the range were not used in the calculations due to their small sample size.

Use of polarizing filters to visualise end-plates: miniature end-plate potentials

When polarizing filters were used to examine individual fibres small spots were seen either on or near nerve fibres. In Figs. 3.3 and 3.4 the electrode has been positioned so it points towards these structures. The objects were only observed when polarizing filters were used, and they were photographed with a Nikon camera which was attached to the dissecting microscope.

Intracellular recordings were then made from 67 of these spots in 5 rat diaphragm muscles, the microelectrode being positioned as accurately as possible. In each case miniature end-plate potentials were recorded, most with inverted forms when the electrode was withdrawn so that it was on the external membrane surface of the fibre (Fig. 3.5).

The characteristics of miniature end-plate potentials were measured by examination of polaroid photographs of the stored image on the 305 Tekronix oscilloscope. The rise time is taken as the time for miniature end-plate potential to reach maximum potential.

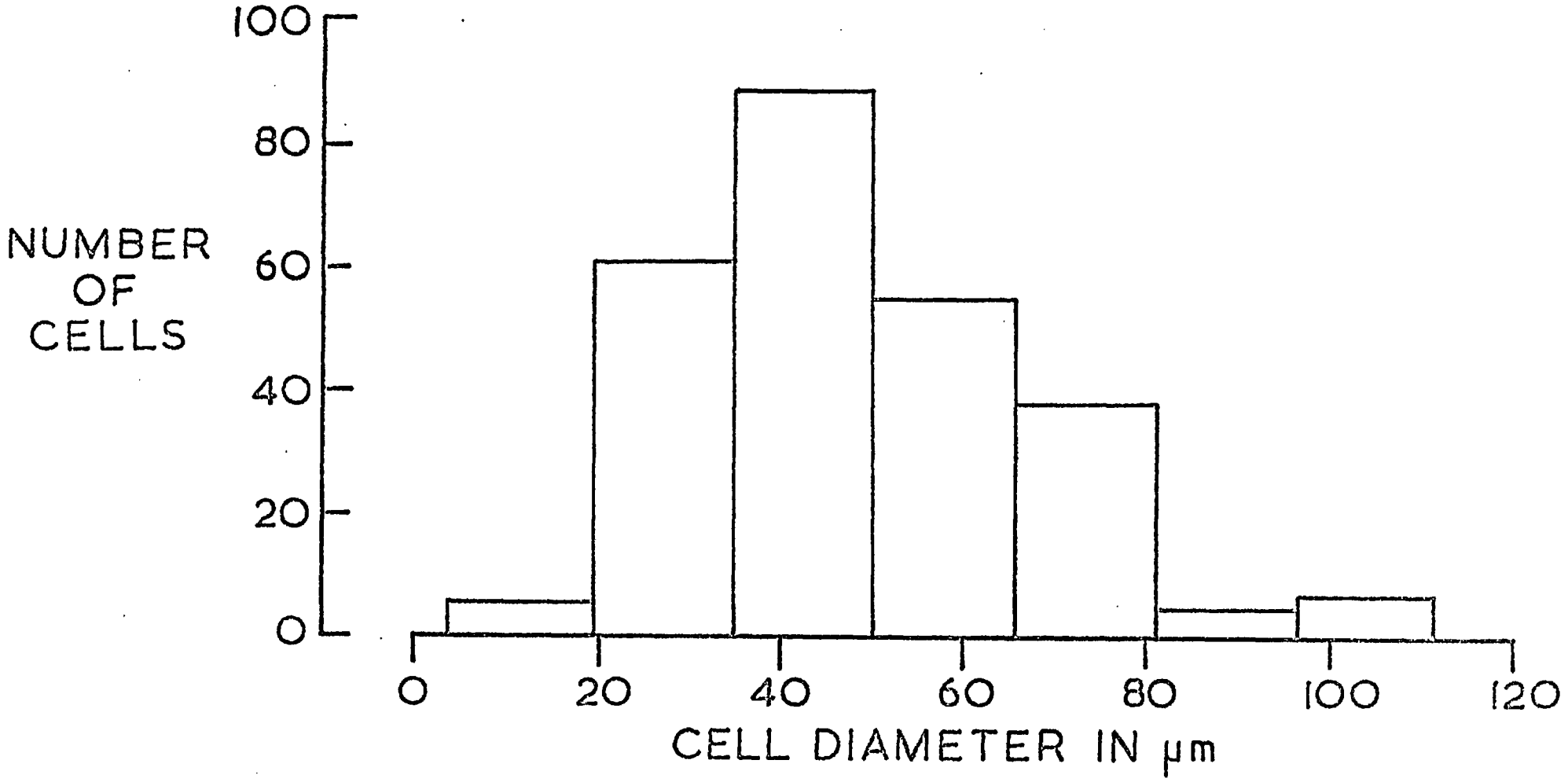


Fig. 3.1

Histogram to show distribution of fibre diameters

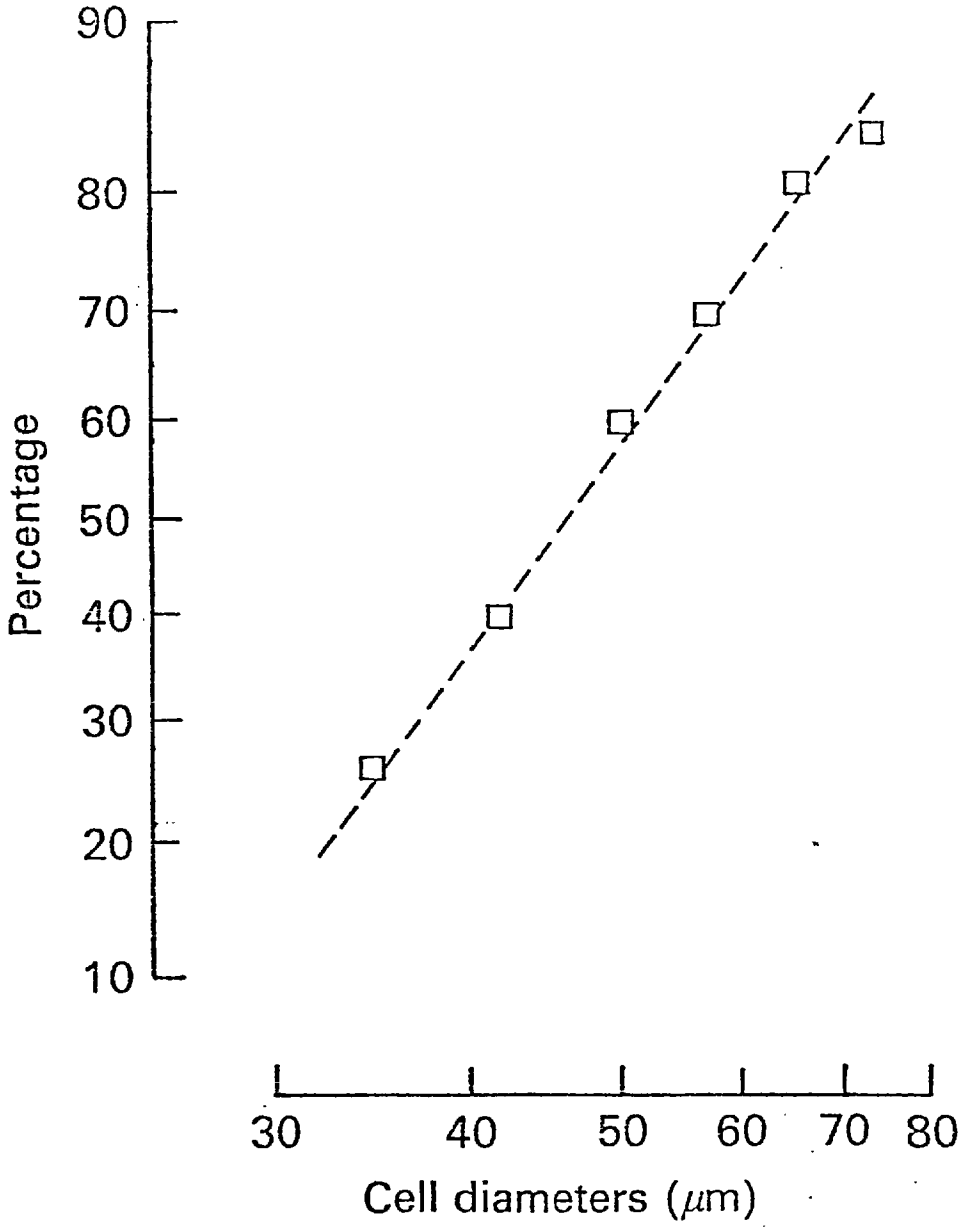


Fig. 3.2

Distribution of fibre diameters. Cumulative percentage is plotted on probit scale against log diameter, so that a straight line indicates a lognormal distribution.



Fig 3·3

Fig. 3.3. Appearance of rat diaphragm in polarized light. The microelectrode is above the end-plate region with the muscle in the horizontal bath. Top of photograph shows meniscus formed by bath solution. The vertical muscle fibres are crossed by nerve trunks, which are out of focus. The tip of the electrode points towards a dark spot which is in focus. Magnification as in Fig. 3.4 A.

Fig. 3.4 A. As Fig. 3.3. The electrode points towards three dark spots which are in association with a branch of the nerve.

Fig. 3.4 B. As A at higher magnification. The three dark areas are believed to indicate the sites of end-plates.

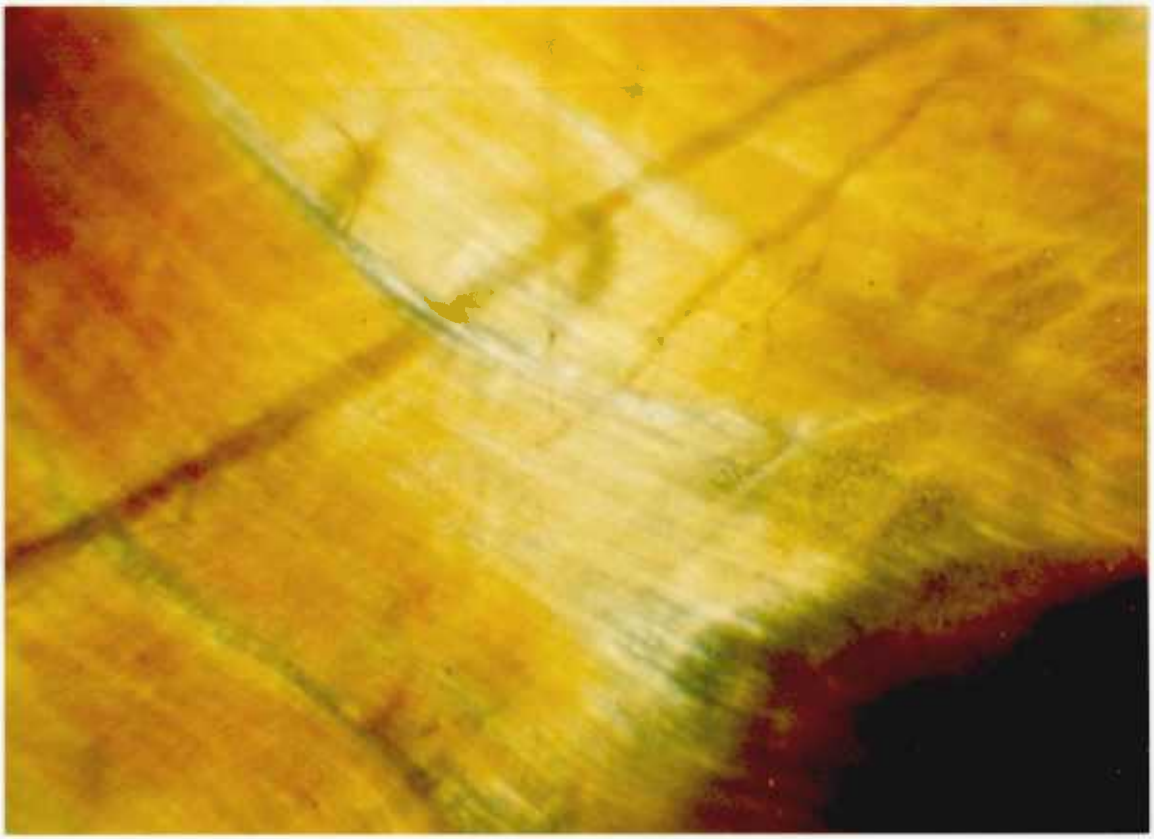


Fig 3·4 A

100 µm



Fig 3·4B

100 µm

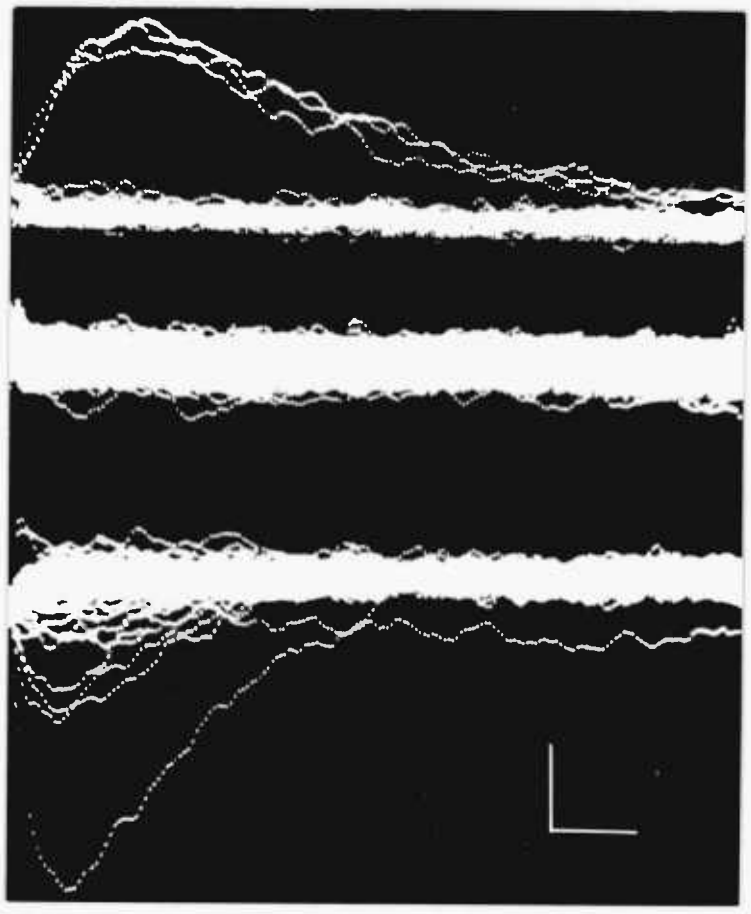


Fig. 3.5

Fig. 3.5

Miniature end-plate potentials and inverted miniature end-plate potentials recorded from same site.

A: miniature end-plate potentials - internal recording

B: electrical noise level recorded externally

C: inverted miniature end-plate potentials recorded externally at same site.

a.c. recording with 10H and 10KH filters.

Vertical calibration: 0.2 mV

Horizontal calibration: 0.2 msec.

The fall time was taken as half the time required for the recovery of resting potential. The rise time of miniature end-plate potentials was 0.45msec (median of 12, range 0.3msec - 1.5msec). The site used for Fig. 3.5 also gave inverted potentials when the electrode was moved to the outer surface of the fibre (Fig. 3.5c), and this is an example of focal recording (del Castillo & Katz, 1956). A short rise-time was found when inverted potentials could be demonstrated, and it is possible that the longer rise-times which were occasionally found were recorded from sites which were distant from the point of focal recording. The 50% fall time was 0.6msec (median of 12). The rise time of the inverted miniature end-plate potentials was 0.2msec (in four cases where adequate photographs were available) and the fall time of the inverted miniatures was 0.25msec (range 0.2msec to 0.4msec).

Staining of end-plates

Staining of cholinesterase-containing structures was undertaken to further determine the relationship between observed spots and end-plates. Strips of diaphragm muscle were dissected in the usual manner, and the rib was then removed. The strips were maximally stretched and pinned tightly to individual rectangles of "Sylgard" encapsulating resin glued to microscope slides. The muscles were kept in refrigerated Krebs saline until photographed 3 - 24 hours later. The pinned strips of diaphragm were then placed under the microscope. The polarizing filter and the muscle were manoeuvred until small spots associated with nerve fibres came into view. A Gillett and Silbert Conference microscope with polarizing lenses and attached 35mm camera (objective 10X, body magnification 8X) was used to photograph the end-plate regions. This system was much more stable than the stereo-zoom microscope used to implant microelectrodes, permitting the longer exposure times required to photograph stained diaphragm muscles. The setting of the stage micrometers was noted

Fig. 3.6 A. Diaphragm mounted on Sylgard resin, attached to a microscope slide and viewed in Krebs saline with polarized light. Nerve trunks can be seen. The arrows point to two dark areas.

Fig. 3.6 B. As A. Same preparation stained for cholinesterase. The arrows show staining of structures which had previously been identified with polarized light.

Fig. 3.7 A,B. Another muscle treated as in Fig. 3.6, showing appearance before and after staining. The arrows point to a structure which was identified with polarized light, and subsequently stained. Magnification as Fig. 3.6



Fig 3·6A

100 µm

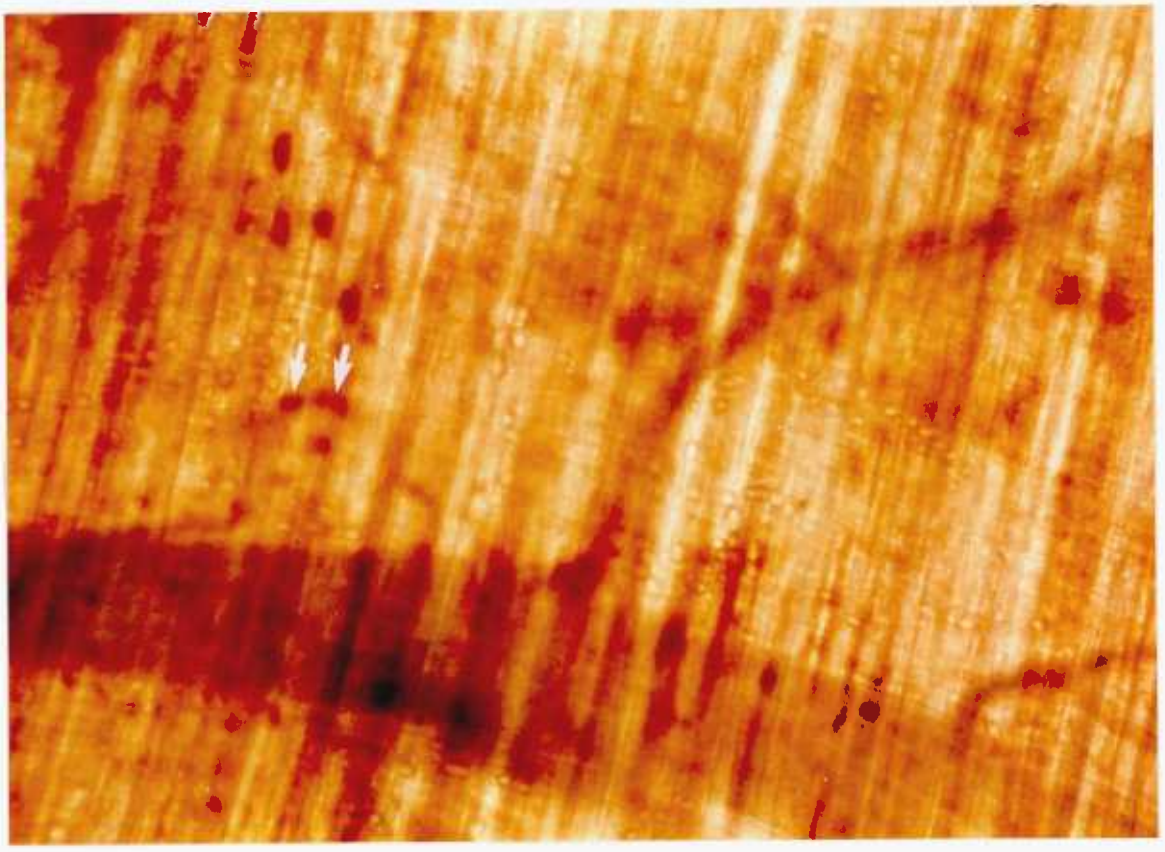


Fig 3·6B



Fig 3·7A

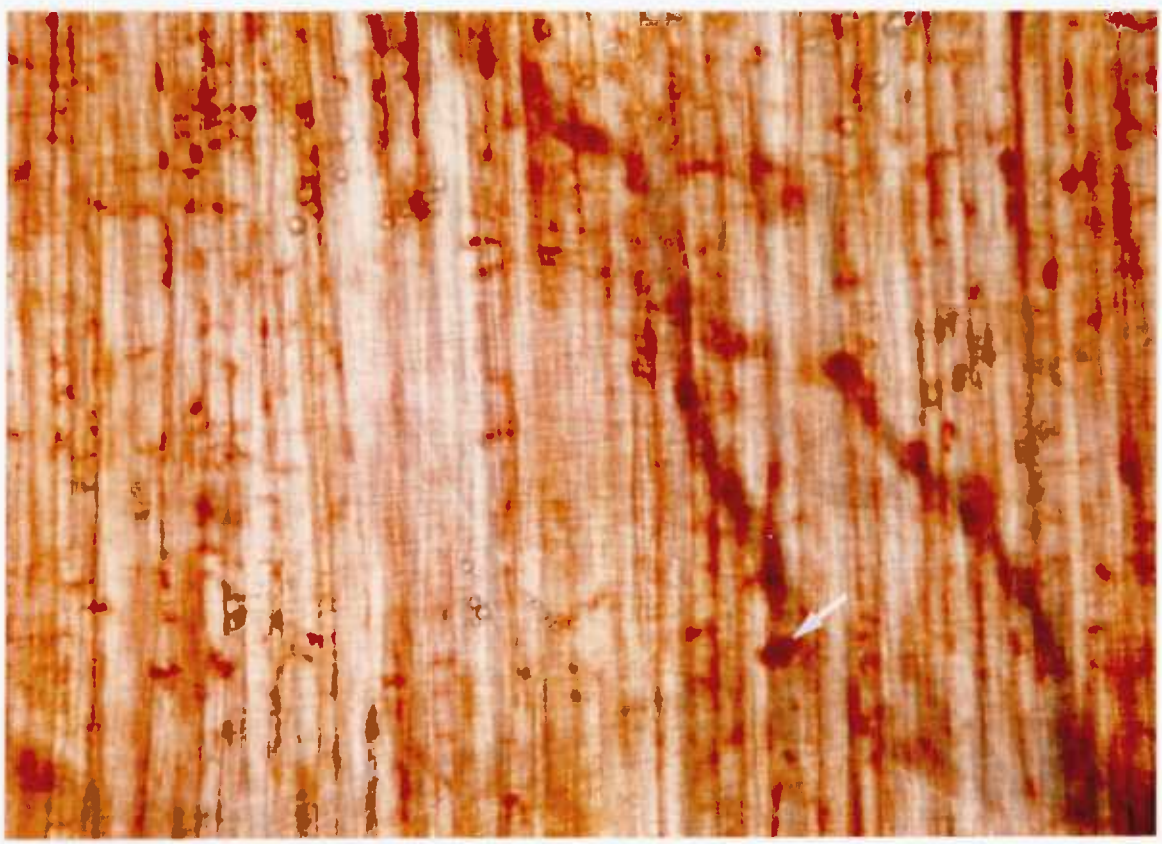


Fig 3·7B

when photographs were taken.

The preparation on its microscope slide was then fixed in formol saline for 4 min: this short fixation period was used to prevent the tissue becoming opaque. The muscle was stained by the use of α-naphthyl acetate-hexazonium para-rosanilin (Barka & Anderson, 1963: Song & Anderson, 1963). The time for staining was 1½ min, and the preparation was washed in distilled water. Finally the muscle on its microscope slide was replaced on the stage and each area was again focused and rephotographed. In early attempts the muscles were over-stained and the entire tissue took on a rosy hue. With repeated trials it became possible to stain the end-plate selectively.

With thick tissues the photographs (Fig. 3.6, Fig. 3.7) show objects which are in one focus, and other objects which are out of focus. However, it can be seen that it is possible with polarizing filters to distinguish small dark objects which gave a characteristic appearance when stained for cholinesterase. The spots identified in the unstained muscle appear to take up the azo dye. This evidence seems to support, along with the microelectrode recordings, the contention that the observed spots could be used to identify the end-plate regions of selected fibres.

Summary of Chapter III

1. The diameters of muscle fibres measured microscopically were found to have a log-normal distribution with median 46μm for rats of 100g (5 rats, 50 - 61 fibres from each diaphragm).

2. When polarizing filters were used with a low-power microscope it was found that dark-coloured spots could be seen on muscle fibres in association with nerve axons.

3. Insertion of microelectrodes into these areas gave miniature end-plate potentials in each of 67 fibres from 5 rats.

4. Fibres were photographed before and after staining for cholinesterase. In some preparations it was shown that the dark areas which had been focussed with polarizing filters became stained by the pararosaniline dye. This gave further support for the identification of end-plates by the use of polarized light.

CHAPTER IV EFFECTS OF POTASSIUM AND OUABAIN IN RAT DIAPHRAGM

The unexcited membrane potential may be maintained by two different mechanisms: the diffusion potential due to the relative permeability to sodium and potassium and the potential directly generated by the sodium-potassium exchange system, the well-known electrogenic pump (Thomas, 1972). To determine the relative contributions of these two mechanisms, and to ensure proper experimental controls for subsequent experiments, the membrane potentials at the end-plate regions were measured in the presence of varying concentrations of potassium and ouabain.

Depolarization by potassium

The end-plate membrane potentials of 25 fibres from each muscle were first measured in a standard Krebs saline containing 5mM potassium (Table 2:1). Then the standard solution was exchanged for an experimental saline containing varying amount of potassium methyl sulphate (Table 2:2) and after 20 min the same 25 sites were re-examined. At each potassium concentration at least 3 muscles were used, making a total of at least 75 fibres at each point.

Fig. 4.1 shows the membrane potential as a function of potassium concentration. With increase of potassium there is as expected a progressive depolarization, and the slope indicates a 52mV change for a 10-fold increase in concentration. The rate of voltage change appears to slow at potassium concentrations of less than 2.5mM potassium and concentrations greater than 60mM potassium.

Resting potentials in absence of potassium

The results obtained by changing from solution with 5mM

or 2.5mM potassium to solution without potassium were variable. By the method of multiple sampling, in which 25 fibres were measured before and after the change-over, it was found that 16 muscles hyperpolarized and 11 muscles depolarized. In these experiments different strips of the left diaphragm were used (antero-medial and postero-lateral). Of the five batches of animals, two batches had diaphragms which all hyperpolarized in potassium-free solution, one batch all depolarized, and the results varied in the other batches of animals.

In several cases continuous records were also obtained from single fibres, and the results were also variable. Fig. 4.2A shows a fibre which hyperpolarized from -68mV to -82mV. Fig. 4.3A and B show fibres which depolarized, in A by 10mV and in B by 22mV. In some cases the fibres which hyperpolarized took 20 min to reach a steady plateau. Fig. 4.2B shows a fibre which depolarized and then hyperpolarized. In 19 individual fibres in which continuous records were obtained, 8 fibres hyperpolarized, 8 depolarized, and 3 fibres gave initial depolarization and subsequent hyperpolarization.

Fig. 4.4 shows results assembled from all the experiments in which solution without potassium was used, with control measurements in standard solution with 5mM potassium. These data have been obtained from two sources, the study on the effect of potassium concentration on membrane potential (Fig. 4.1), and the multiple sampling experiments in potassium-free solutions reported in the following Chapters (see Figs. 5.4, 5.10, 6.5, 6.7). In the 5mM potassium study, 340 fibres from 25 muscles were used. The distribution appears to be Gaussian, with mean -72.2mV and standard deviation 7.7mV.

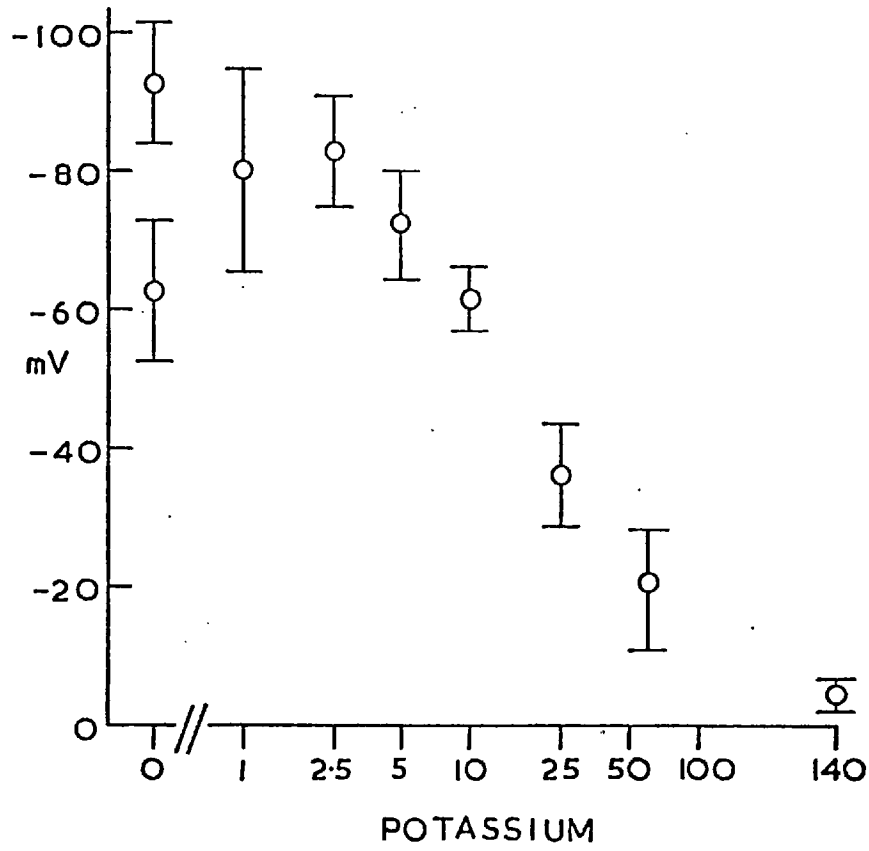


Fig. 4.1. Membrane potential as a function of external potassium, which was increased by addition of potassium methyl sulphate (Table 2.2). Each point is the mean of 75 fibres, except for points at 5mM and at zero potassium, which have been taken from Fig. 4.4 below. The abscissa shows potassium concentration, in mM, on a logarithmic scale. Between 5-60mM the slope is 52mV for a 10-fold change in concentration. In potassium-free solution some fibres polarized and some depolarized, and the two points give means obtained as in Fig. 4.4 (below).

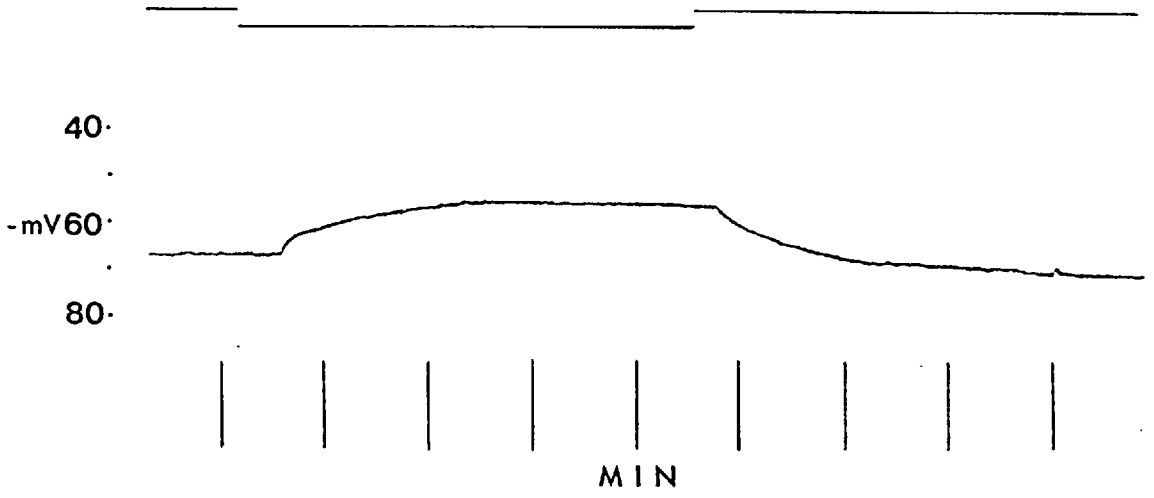


Fig. 4.3 A. Depolarization of end-plate region with potassium-free saline. Break in top line shows change to potassium-free solution and return to standard solution.

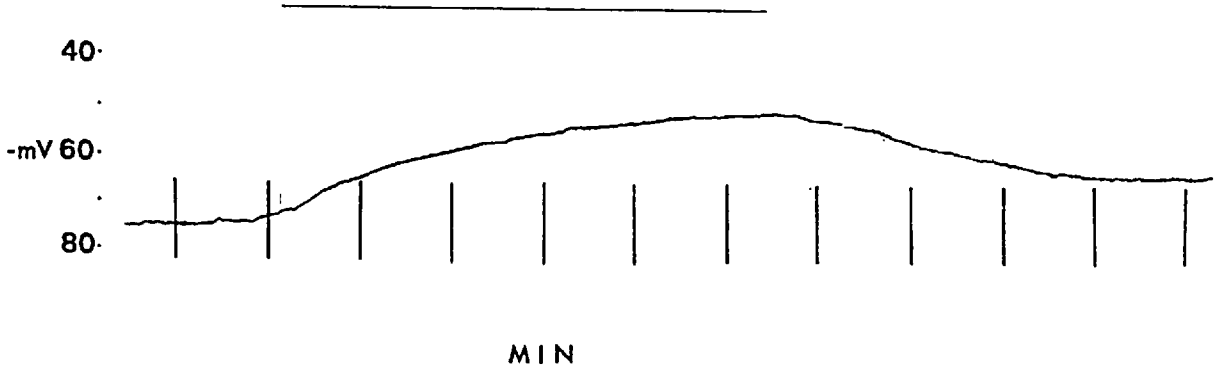


Fig. 4.3 B. As 4.3 A. Top line shows duration of potassium-free solution.

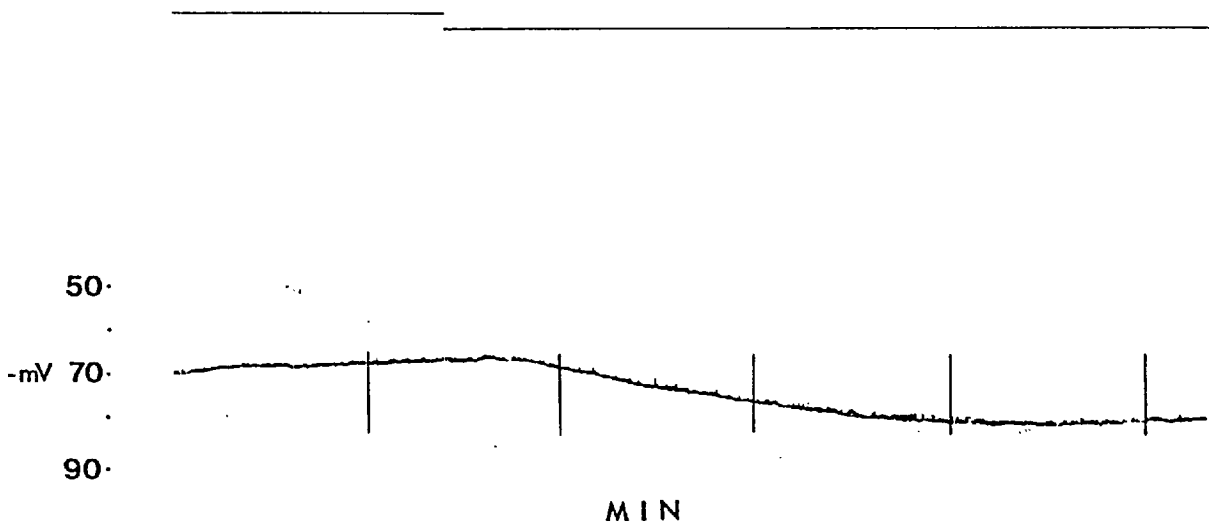


Fig. 4.2 A Hyperpolarization of end-plate region in potassium-free solution. Break in top line shows change to potassium-free solution.

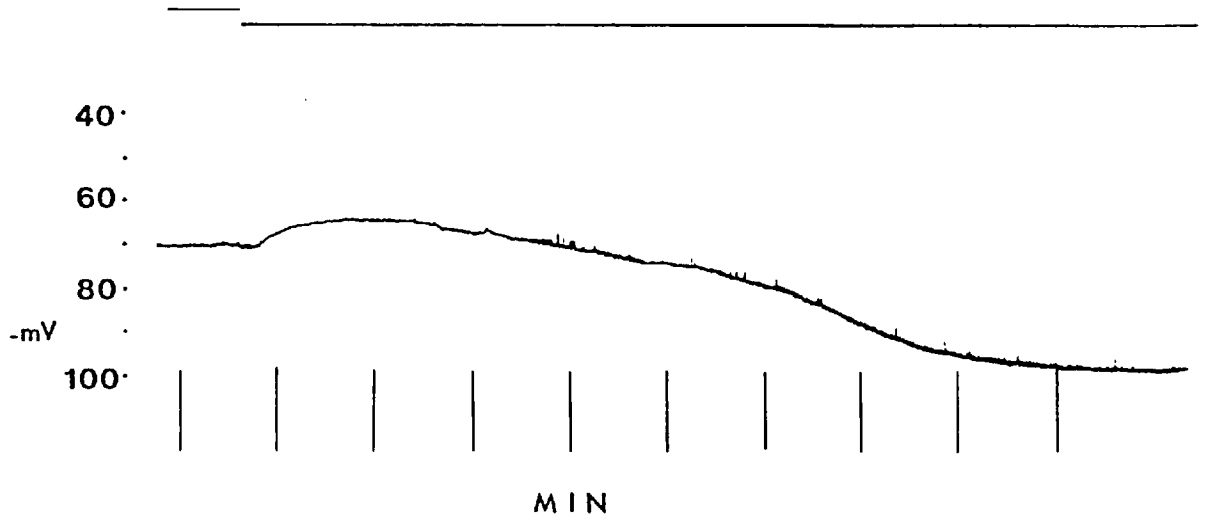


Fig. 4.2 B Mixed response of end-plate region to potassium-free solution. Initial depolarization followed by hyperpolarization. Break in top line shows change to potassium-free solution.

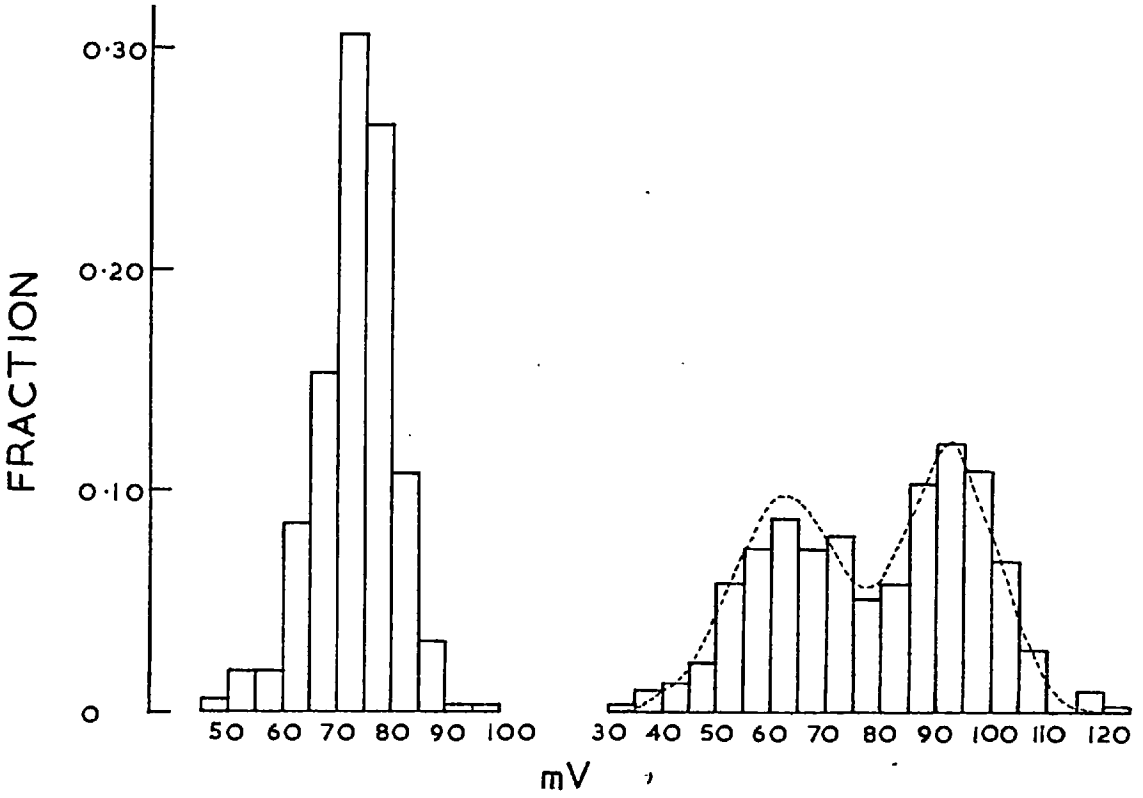


Fig. 4.4. Left graph: histogram to show distribution of membrane potentials in 5.0mM potassium solution: 340 fibres from 25 muscles, 10-25 fibres per muscle. The mean and S.D. are shown in Fig. 4.1 (-72mV $\pm$ 7.7).

Right graph: distribution of membrane potentials in potassium-free bathing solution: 311 fibres from 27 muscles, 10-12 fibres per muscle. The interrupted line gives the best fit for the sum of two Gaussian distributions: the means and S.D. are -61mV $\pm$ 9.9 and -92mV $\pm$ 8.9, and these values are shown in Fig. 4.1.

In potassium-free solution 311 results were collected from 27 muscles. The distribution of resting potentials appeared to be bimodal (Fig. 4.4), and was formed by the sum of two Gaussian distributions. The grand mean was -78mV with standard deviation 18mV. In this series of muscles the use of solution with zero potassium appeared to have revealed a heterogeneity in the response which was not apparent in standard physiological saline.

Computer fit for the sum of two Gaussian curves

The results shown on the right hand side of Fig.4.4 were fitted as the sum of two Gaussian curves with the help of the staff of the Computer Unit, St. Mary's Hospital Medical School (Mr. J. E. Thirlwall). The procedure was taken from that used by Valet, Hofmann and Ruhenstroth-Bauer (1976), who used a computer analysis to demonstrate two erythocyte populations of different size in young guinea-pigs. The original program from the Max-Planck Institute was in FORTRAN II, and it was necessary to transcribe this to FORTRAN IV for use with the University computer. The method was checked by showing that the results given by Valet et al (1976) could be reproduced with the adapted program, and when this had been verified the values in Fig. 4.4 were entered on punched cards and run with initial estimates of the parameters which were obtained by inspection. The final estimates for the two populations were 92.6mV (mean of 50.2 per cent of the total) with standard deviation 8.3mV, and -62.6mV (mean of 49.8 per cent of the total) with standard deviation 10.0mV, and the interrupted line in Fig. 4.4 gives the computed sum. The mean and standard deviation of the two populations are shown in Fig. 4.1 as the values for the resting potential in solution with zero potassium.

Miniature end-plate potentials in potassium-free Krebs

After 20 mins in potassium free Krebs solution the rise time of miniature end-plate potentials was 0.15msec (median of 6, range 0.12 to 0.24msec), and the 50% fall time was 0.33msec (range 0.24 to 0.6msec). In one instance where an adequate polaroid photograph was available, the rise time of the inverted miniature end-plate potential was 0.15msec and the 50% fall time 0.3msec (Fig. 4.5). These times are rapid when compared with results reported previously, and the results are considered in the Discussion below.

Effect of ouabain

The effect of 100μM ouabain on the end-plate region was measured by both continuous single-fibre recording and by the method of repeated sampling. Continuous recordings from 11 different muscles for periods of up to 20 mins gave a median depolarization of 9mV, with a range of 4-11mV (Fig. 4.6). The results obtained by repeated sampling are given in Table 4.1. Prior to treatment, repeated sampling of 125 fibres from 5 muscles had a median membrane potential of -75mV. One hour after the addition of 100μM ouabain, the median membrane potential of 175 fibres was -65mV, and was unchanged after two hours. After 10 mins in ouabain free Krebs solution, the membrane potential at the end-plate was restored to a value of -73mV (Table 4:1).

Concentrations of 1mM ouabain had a greater effect on end-plate membrane potentials (Table 4.2). The median potential of 40 fibres from 4 muscles fell from -69mV to -55mV after 10mins in 1mM. At 30 mins in 1mM ouabain, the median membrane potential remained at -55mV. After 60 mins the potential dropped to -43mV (Table 4.2).

After 20 mins in 100μM ouabain, the rise time of miniature end-plate potentials was 0.3msec (3 muscles, range 0.3 to 0.4msec)

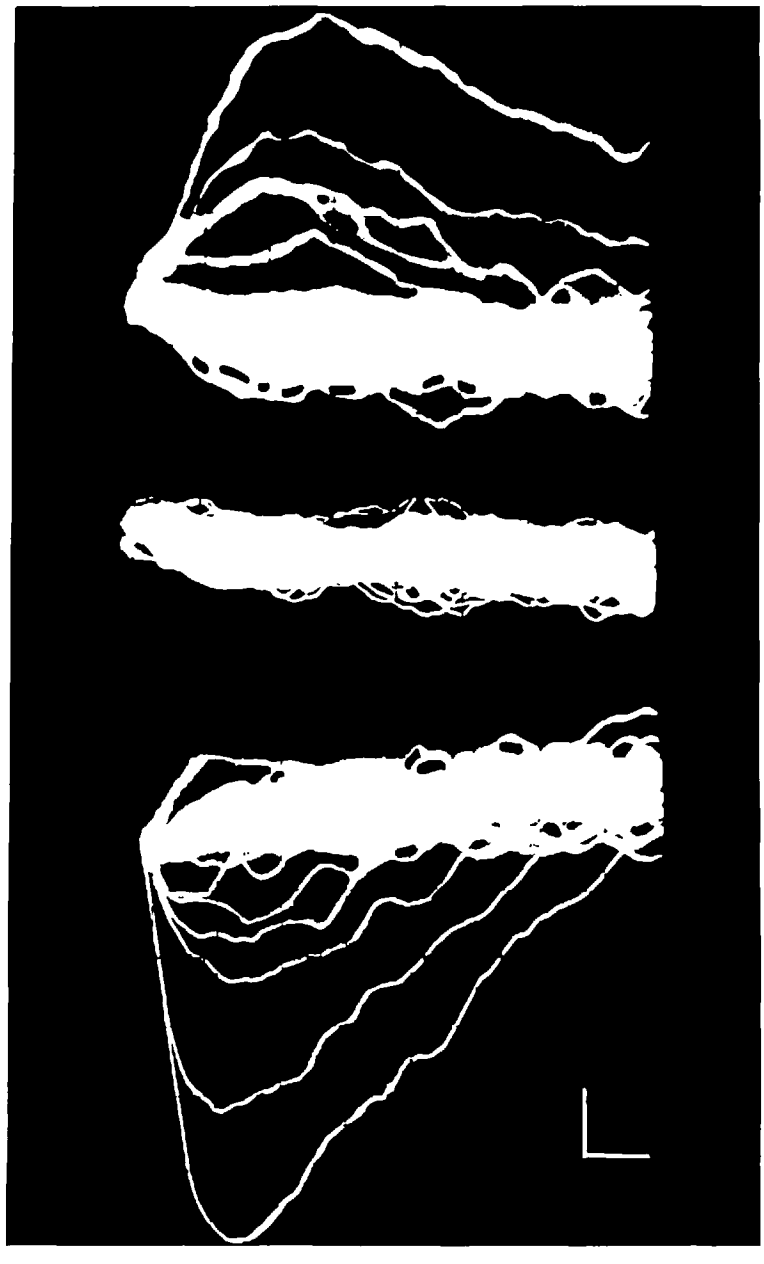


Fig. 4.5

Fig. 4.5 Intracellular and extracellular miniature end-plate potentials recorded from same site in potassium-free solution.

A intracellular recording: rise time 0.2 msec

B electrode above recording site

C extracellular recording: rise time 0.15 msec

Vertical calibration 0.2 mV

Horizontal calibration 0.1 msec.

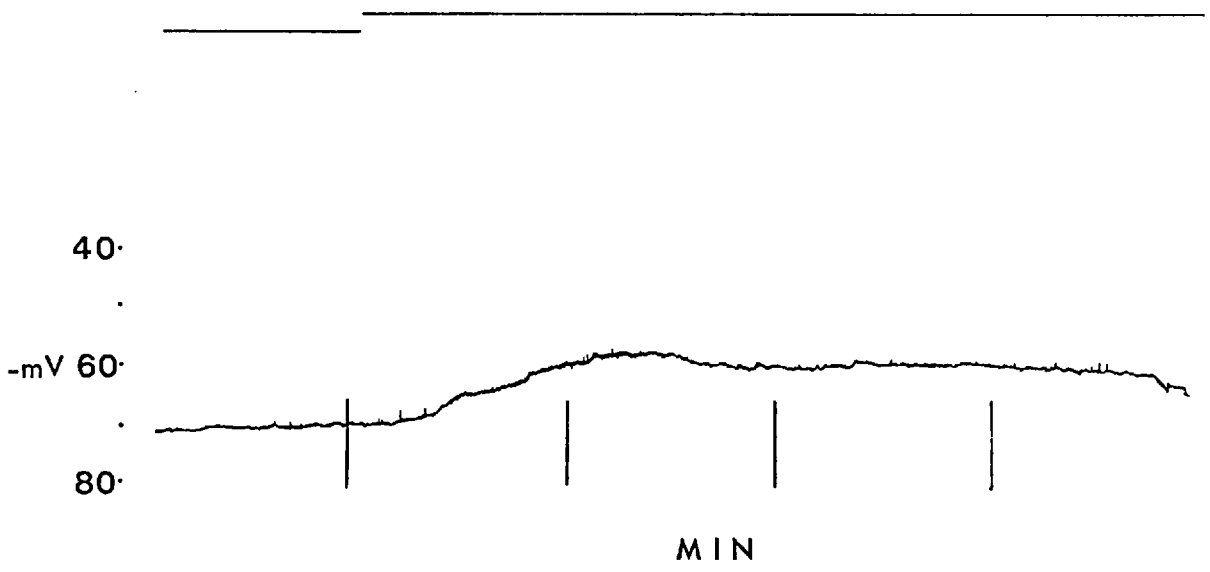


Fig. 4.6 Depolarization of end-plate region with 100μM ouabain. Break in top line shows change to ouabain solution.

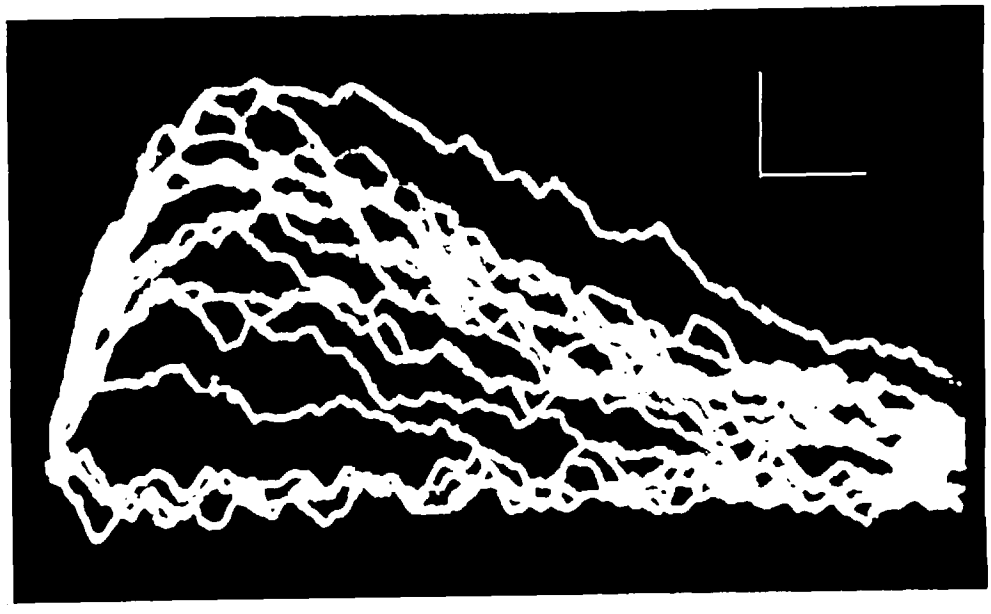


Fig. 4.7 Miniature end-plate potentials with 100μM ouabain. Rise time 0.3 - 0.4 msec. Vertical Calibration 0.2 mV. Horizontal calibration 0.2 msec.

TABLE 4:1 EFFECT OF OUABAIN 100μM ON RAT DIAPHRAGM

	Muscle in Saline mV	After 1 hr Ouabain mV	After 2 hr Ouabain mV	Muscle in Saline mV
	-74	-64	-63	-73
	-76	-67	-68	-73
	-79	-70	-70	-76
	-73	-60	-60	
	-72	-52	-57	
		-67	-67	-75
		-66	-66	-75
No. of fibres	125	175	175	125
Median	-75	-65	-65	-73
95% limits of median	74-75	63-67	64-66	71-74

Controls Median -74mV (250 fibres, 7 muscles, limits 73-75)

Ouabain -65mV (350 " , 7 " , " 64-66)

The resting potentials are given in mV and were measured at the end-plate. Results from 7 rats are shown. Each value is the median of 25 fibres.

TABLE 4:2 EFFECTS OF OUABAIN 1mM ON RAT DIAPHRAGM

	Muscle in Saline	10 min in Ouabain	20 min	30 min	60 min
	mV	mV	mV	mV	mV
	-69	-56	-55	-57	-46
	-71	-55	-54½	-55	-39
	-69	-56	-56	-53	-48
	-68	-52	-59	-52	-40
Median	-69	-55	-56	-55	-43
No. of fibres	40	40	40	40	40
95% limits	-71	-57	-58	-57	-49
of median	-68	-54	-52	-52	-39

The resting potentials are given in mV, and were measured at the end-plate. Results from 4 rats are shown. Each value is the median of 10 fibres.

with a 50% fall time of 0.7msec (range 0.6msec - 0.7msec) (Fig. 4.7). This value is similar to the values of miniature end-plate potentials in 5mM potassium Krebs solution.

Summary

1. The depolarization produced by potassium methyl sulphate was recorded in rat diaphragm. The change was 52mV for a 10-fold increase in concentration.

2. Removal of potassium produced variable results. With single fibre recording at the end-plate most cells became hyperpolarized. Some fibres became depolarized.

3. In solution without potassium the histogram of resting potentials gave a bimodal distribution.

4. In single fibres ouabain 100μM produced depolarization of 9mV (median of 11, range 4-11 mV). By the method of repeated sampling the depolarization was also 9mV (7 muscles, 25 fibres measured at intervals for 2 hr after application).

5. With ouabain 1mM the depolarization was 14mV (4 muscles, 10 fibres measured at intervals for 10-30 min after application).

CHAPTER V CARBACHOL AND SUBERYLDICHOLINE IN RAT DIAPHRAGM

Pilot studies showed some degree of recovery of resting membrane potential in the presence of depolarizing agents. The drugs carbachol and suberyldicholine were selected to investigate this effect in diaphragm muscle of the rat.

Effects of Carbachol (100μM)

Continuous recordings were made in 23 fibres for varying lengths of time. The initial depolarization was from -73mV (95% confidence limits -71mV, -74mV) to -55mV (limits -52mV, -57mV). The shape of the depolarization curve varied considerably (Fig. 5.1).

Fig. 5.2 shows records from two fibres in which the electrode was held in the end-plate for comparatively long periods. All continuous recordings indicated some degree of recovery. In 9 instances the recordings were of sufficient duration to measure the rate of recovery. In most fibres the recovery was sluggish (Fig. 5.2), with occasionally an initial rapid recovery of a few mV which precedes the slow component (see Fig. 5.2, upper trace). In three fibres the recovery was much more rapid with full recovery of potential after 20 mins (Creese, Franklin & Mitchell, 1976). The rate of recovery varied from 0.25mV/min to 6mV/min.

Fig. 5.3 shows results obtained by repeated sampling from 5 - 10 muscles before, immediately after and at various times following application of carbachol 100μM: the results are listed in Table 5.1. The membrane potential was -74mV initially, and immediately after the addition of the drug was -56mV (95% confidence limits -55mV, -58mV). After 15 mins the potential had risen to -64mV and was -73mV after 45 mins. At 60 min, the median membrane potential reached its

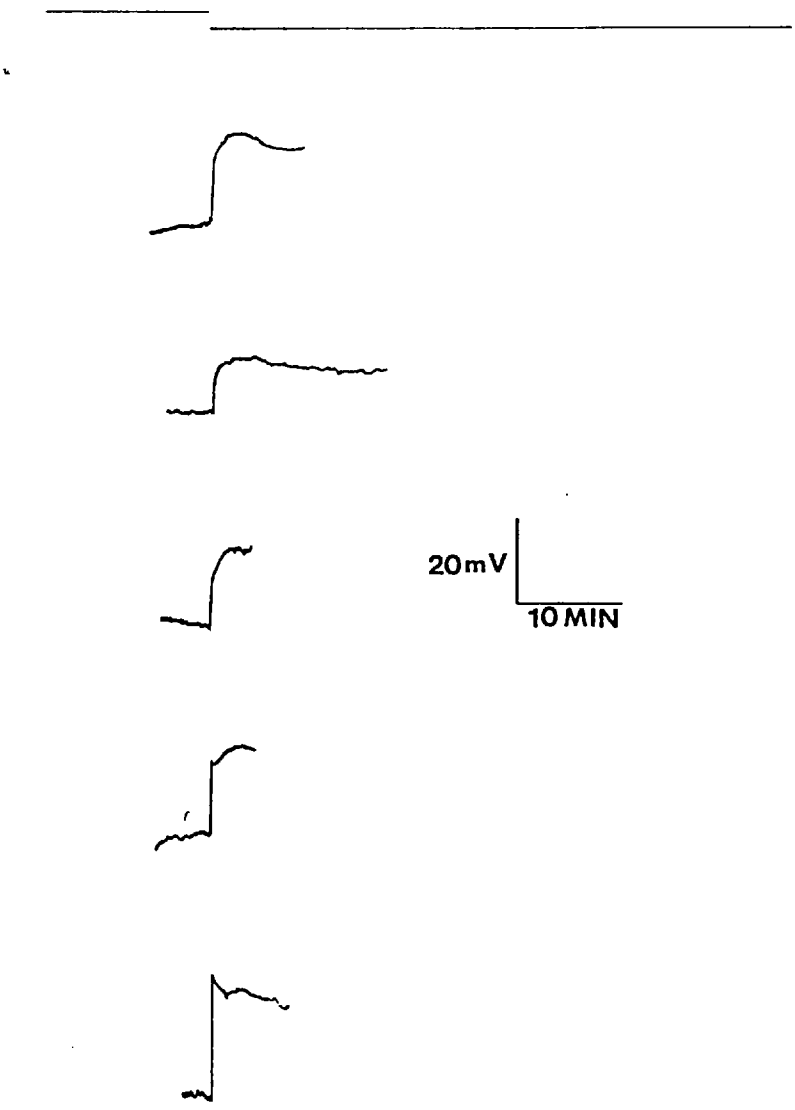


Fig. 5.1. Composite traces. Initial depolarization produced by 100μM carbachol in five rat fibres. Break in top line indicates change to drug solution.

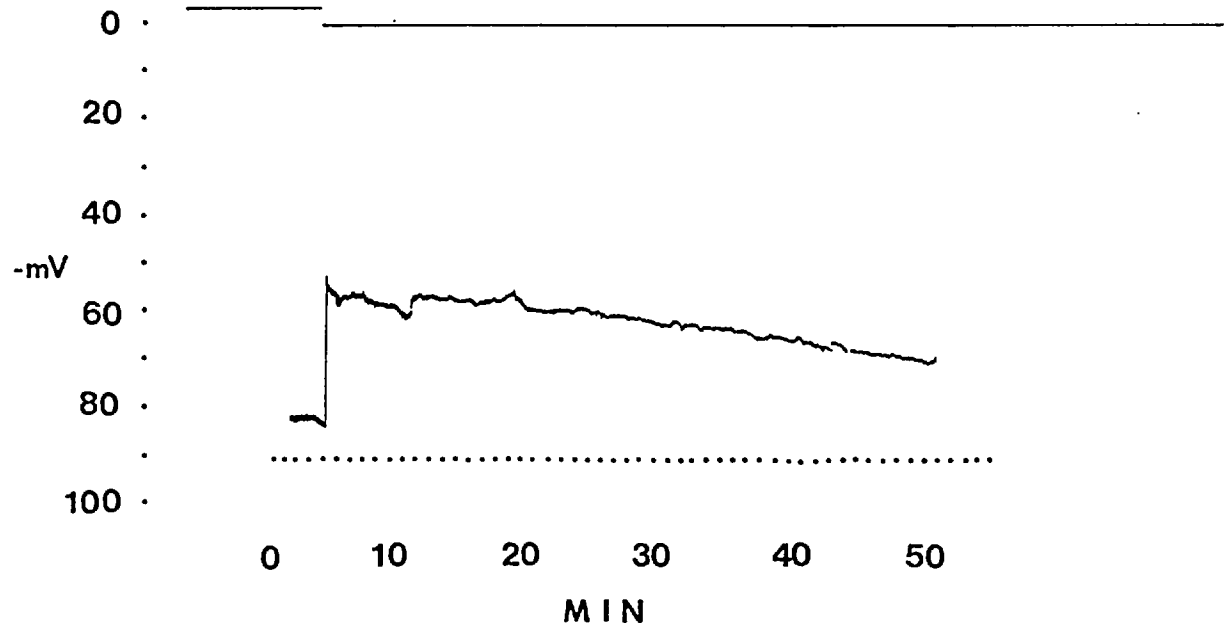
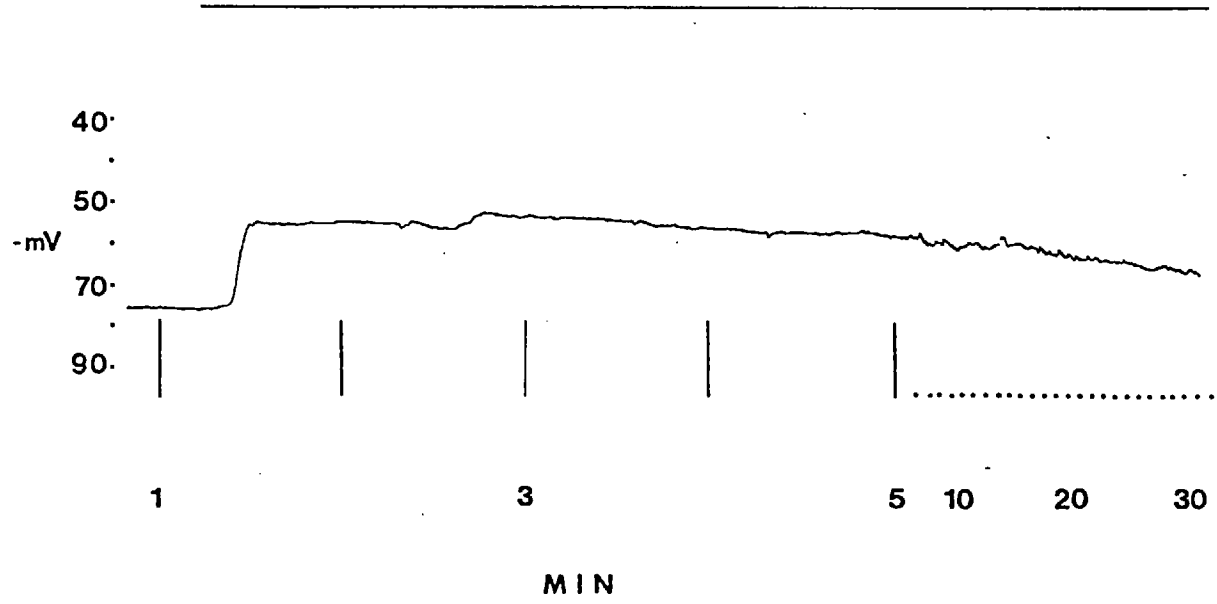


Fig. 5.2. Upper and Lower. Depolarization and partial recovery of potential in 100μM carbachol. Recordings were terminated when electrodes were displaced. Broken top lines indicate change to drug solution. Ordinate in m.V., abscissa in minutes. Vertical lines indicate faster recording speed. Dots, slower recording speed.



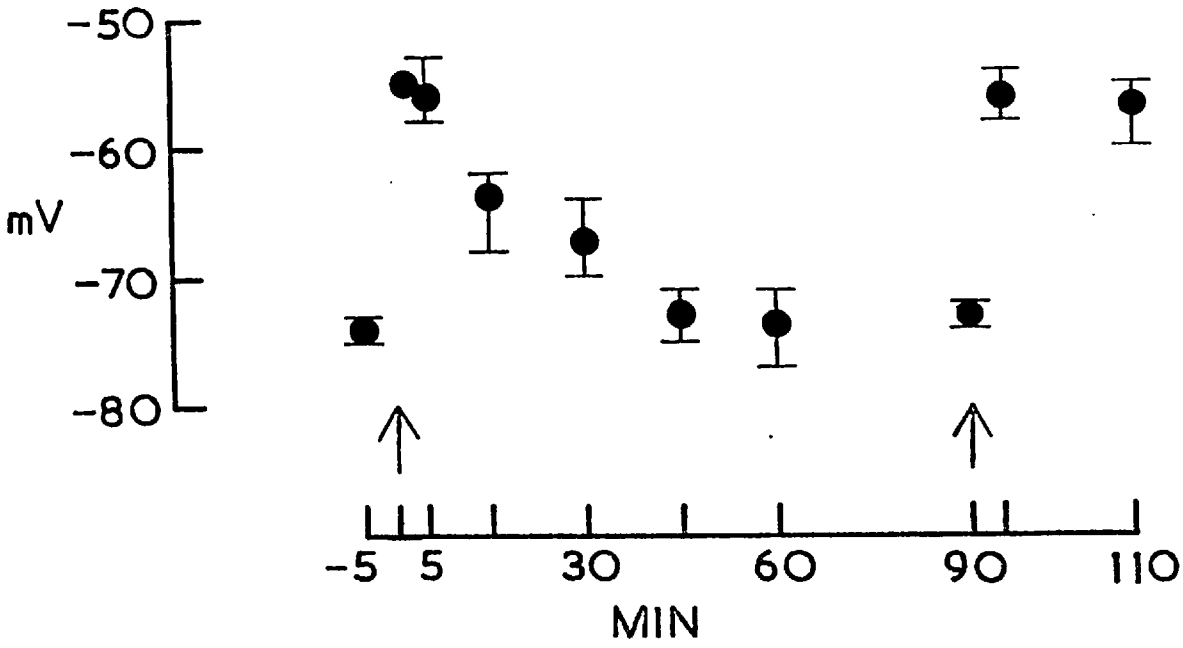


Fig. 5.3. Depolarization produced by 100μM carbachol. Medians of repeated samplings of potential. At first arrow change to solution with drug: the initial depolarization is followed by spontaneous recovery. Bars are 95% confidence limits. Filled circle without bars is the median of 23 continuous recordings. Sample size 54-114 fibres from 5-10 muscles (see Table 5.1). Ordinate is in mV, abscissa is in minutes. Arrows indicate change-over of solutions. At 90 mins, change to solution with 100μM carbachol plus 100μM ouabain: rapid depolarization again occurred.

TABLE 5:1 RESULTS WITH CARBACHOL BY REPEATED SAMPLING

(Cont. Rec. indicates results obtained by continuous recording : membrane potentials in -mV).

FIG 5:3	Standard Krebs	100μM Carbachol							100μM Carbachol +100μM Ouabain	
TIME	-5 min	Cont Rec.	1-5 min	+15 min	+30 min	+45 min	+60 min	+90 min	+15 min	+30 min
MEDIAN	74	55	56	64	67.5	73	74	73	56	57
95% Lower Limit	73	52	53	62	64	71	71	72	54	55
95% Upper Limit	75	57	58	66	70	75	77	74	58	60
n	114	23	54	54	54	48	54	102	60	60

FIG 5:7	Standard Krebs	100μM Carbachol + 100μM Ouabain					100μM Carbachol only	
TIME	-5 min	Cont Rec.	+5 min	+15 min	+30 min	+60 min	+15 min	+30 min
MEDIAN	72	53.5	53	52	52	53	64.5	66.5
95% Lower Limit	71	48	50	51	50	51	62	63
95% Upper Limit	75	59	55	53	54	54	66	70
n	82	12	48	72	72	84	60	60

FIG 5:5	Potassium Free Krebs	100μM Carbachol in Potassium Free Krebs				
TIME	-5 min	Cont. Rec.	+5 min	+30 min	+60 min	+90 min
MEDIAN	89	51	48	49	49	51.5
95% Lower Limit	84	50	44	44	46	44
95% Upper Limit	95	63	52	53	52	55
n	60	6	60	61	60	60

maximum value, -74mV (95% confidence limits -71mV, -77mV). For the first 45 min of application, the rate of recovery was 0.31mV/min. Fig. 5.3 also shows that the fibres depolarized to -56mV when solution containing ouabain 100μM plus carbachol 100μM was used after the muscles had been in contact with carbachol for 90 min, and this is considered below.

100μM Carbachol in the absence of potassium

Continuous recordings of depolarization were made from 6 end-plate regions, and two of these tracings are seen in Fig. 5.4. Prior to the addition of 100μM carbachol, the median end-plate membrane potential in potassium-free Krebs saline was -87mV (range -70mV to -92mV). After the addition of the drug, the median potential was -51mV (range -38mV to -63mV). In all 6 cases the depolarization was maintained, with recovery never exceeding 5mV in the extreme case. In Fig. 5.4, continuous recordings were made from a fibre which hyperpolarized in potassium-free Krebs saline and from one which depolarized in it (see Chapter IV). In Fig. 5.4 (upper) a continuous recording was made for 90 mins in the presence of 100μM carbachol. The muscle was then washed with 5mM potassium Krebs saline. The membrane potential repolarized in 2 min from -62mV to -70mV before the electrode was expelled.

Multiple samplings of the end-plate membrane potentials from 60 fibres in 6 muscles were taken at 5 min prior to and at intervals after the addition of the drug (Fig. 5.5, Table 5.1). In potassium-free Krebs saline, the median potential was -89mV. At +5 min the median potential was -48mV. Again, the depolarization was maintained, and after +90 mins, the median potential was -51mV (95% confidence limits -44mV, -55mV).

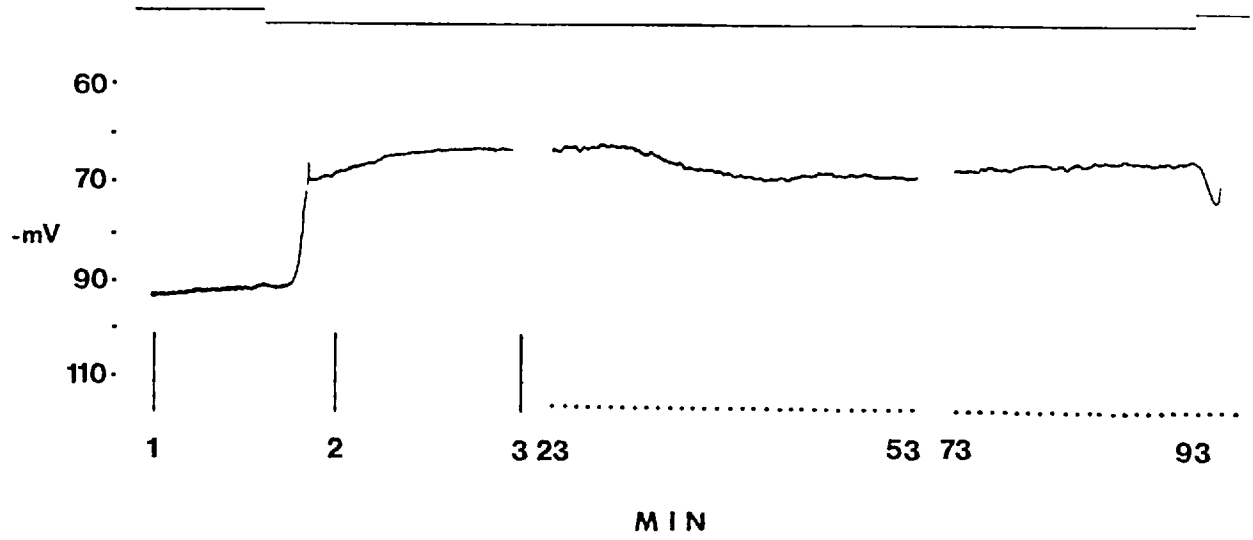


Fig. 5.4. Upper trace. Continuous recording with 100μM carbachol in the absence of potassium. This fibre had hyperpolarized in solution without potassium. Carbachol produced depolarization with little or no evidence of recovery. Vertical lines indicate faster recording speed. Dots, slower recording speed. Top line: (left) indicates change to drug solution. Rt. break indicates change to Krebs' saline with 5mM potassium. Breaks in trace indicate where length of traces were reduced for convenience of presentation. Recording was terminated with the displacement of the electrode.

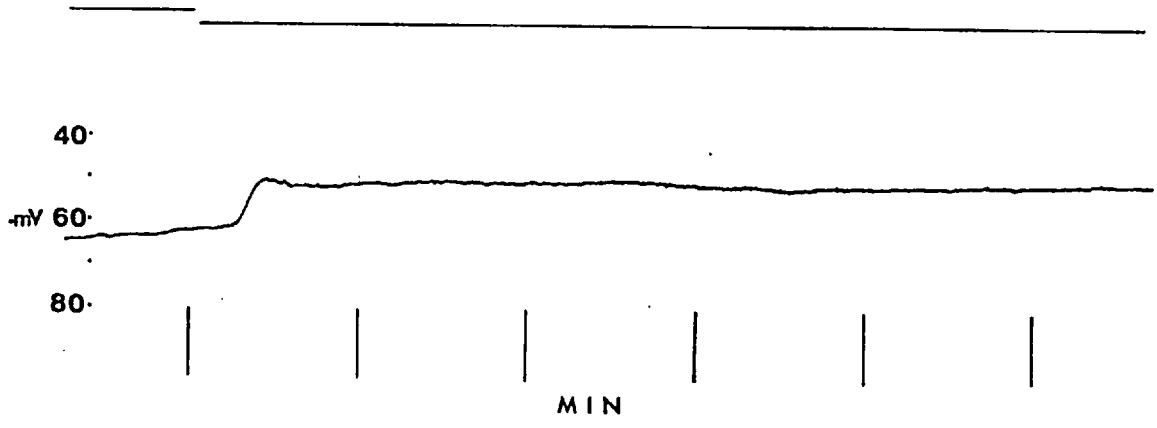


Fig. 5.4. Lower trace. Same as above. This cell had depolarized in potassium-free Krebs saline. Broken top line indicates changeover to drug solution.

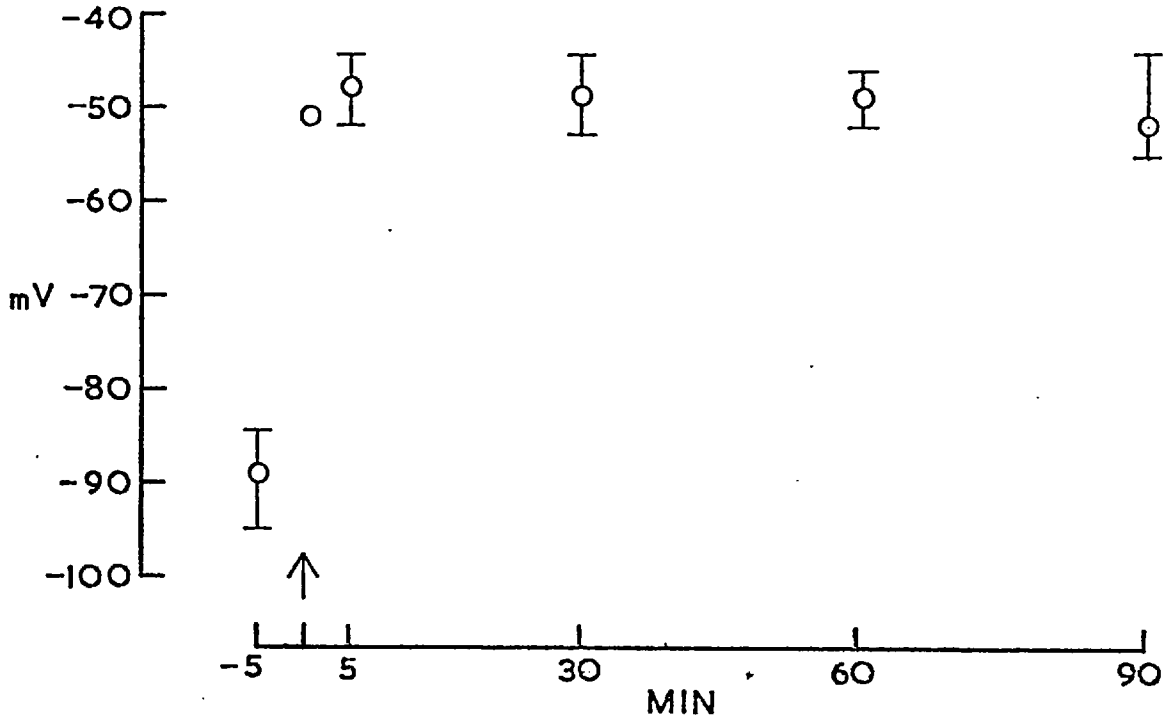


Fig. 5.5. Depolarization produced by 100μM carbachol in potassium-free solution. There is little indication of recovery of potential. Medians of 60 fibres from 6 muscles. Bars represent 95% confidence limits (Table 5.1). Circle without bar gives results obtained by continuous recordings in 6 fibres. Ordinate is in mV. Abscissa is in minutes. Arrow indicates changeover to drug solution.

100μM Carbachol with ouabain

Fig. 5.6 shows continuous recordings of the effects obtained by applying carbachol 100μM and ouabain (100μM). In 12 fibres the initial potential was -73mV (median of 12) and fell to -53.5mV on application of the drugs (limits 42 - 59mV). Fig. 5.6 (lower) shows a single fibre recording where after 60 min the carbachol plus ouabain solution was replaced with Krebs saline containing 100μM carbachol alone. Within 24 mins of the removal of ouabain, the membrane potential had returned to its original level, -82mV, although 100μM carbachol was still present in the bathing solution.

In Fig. 5.7 the method of repeated sampling has been used to show the effects of 100μM carbachol plus ouabain 100μM, and the detailed results are also given in Table 5.1. The initial membrane potential in standard Krebs' solution was -72mV (limits 71, 75mV) and had fallen after 5 min in the presence of the drugs to a value of -53mV (limits -50, -55mV). The depolarization was maintained for 60 min (Fig. 5.7). These results were obtained with 4 muscles. To show the recovery process a separate set of muscles was treated as above for 60 min in solution containing carbachol and ouabain and the potentials were measured. The solution was then changed to carbachol 100μM alone, and the last 2 points in Fig. 5.7 show the recovery process. A substantial measure of recovery was found after a further 30 min. This arrangement was adopted to avoid the necessity of repeated penetration of the fibres, and Table 5.1 lists the values obtained in this composite experiment. It can be seen that the result in Fig. 5.7 is similar to that shown in the continuous recording of Fig. 5.6 (lower).

Depolarizing effect of ouabain after prolonged exposure to 100μM Carbachol

Fig. 5.3 shows (second arrow) that after recovery of the membrane potential in the presence of carbachol 100μM, the addition

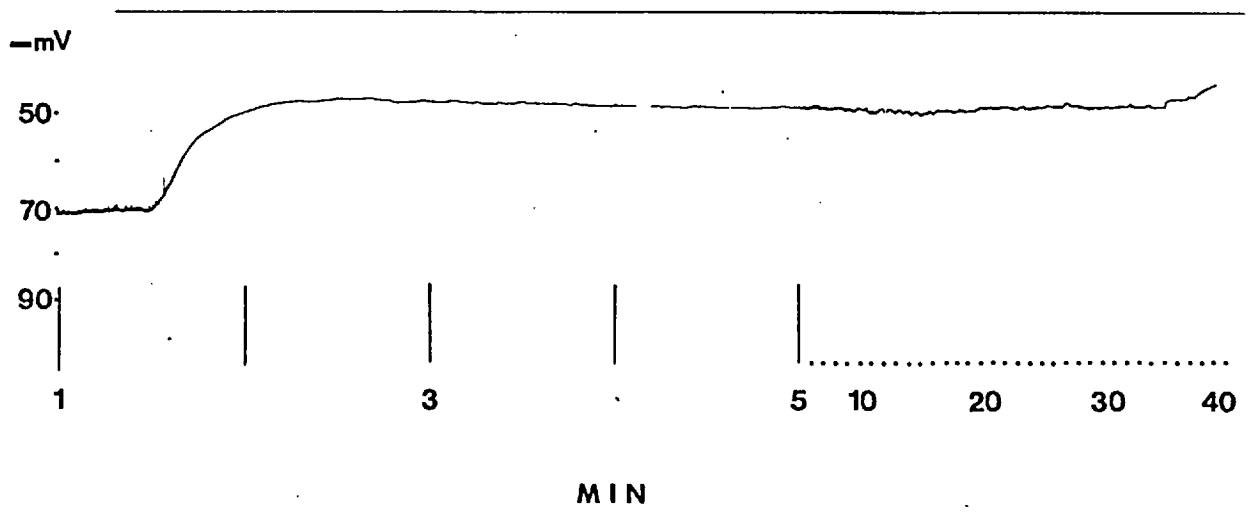


Fig. 5.6. Upper trace. Continuous recordings of effect of 100μM carbachol plus 100μM ouabain. The trace indicates depolarization with no recovery of potentials. Ordinate is in mV. Abscissa is in minutes. Vertical lines indicate faster recording speed. Dots indicate slower recording speed.

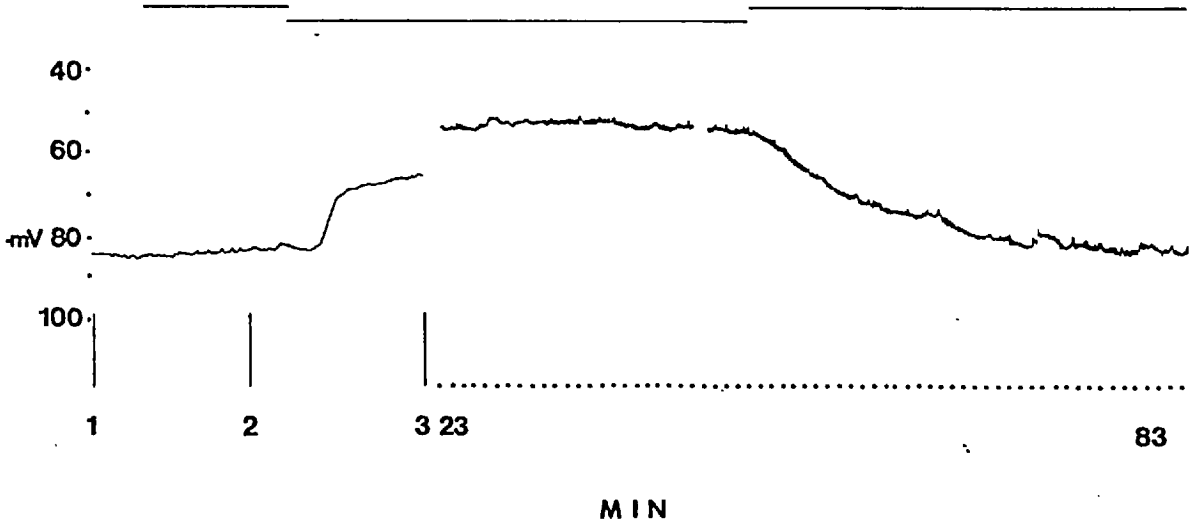


Fig. 5.6. Lower trace. As above: in this fibre the initial rapid depolarization is followed by slower depolarization. At 23 minutes potential was stable. After 60 minutes change-over to solution containing 100μM carbachol alone. Recovery of membrane potential followed within a further 24 minutes. Broken top line: Lt break indicates change over to 100μM carbachol with 100μM ouabain. Rt. break indicates change over to 100μM carbachol alone.

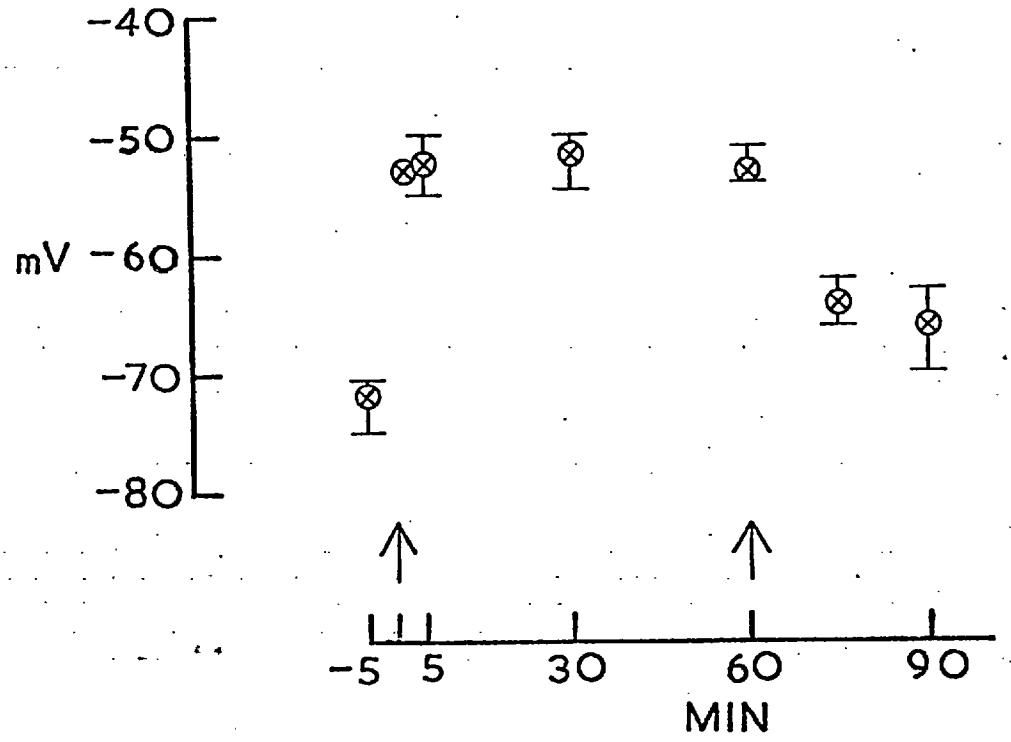


Fig. 5.7. Depolarization produced by 100μM carbachol plus 100μM ouabain. There is little indication of recovery in 60 min. Medians of 24-82 fibres from 4-8 muscles. Bars are the 95% confidence limits (Table 5.1). Crossed circle without bar gives result obtained by continuous recordings in 12 fibres. Ordinate is in mV. Abscissa is in minutes. Left arrow: change to solution containing 100μM carbachol and 100μM ouabain. At 60 min, change to solution containing 100μM carbachol: substantial recovery occurred.

of solution containing carbachol plus ouabain 100μM produced rapid depolarization to -56mV. In Fig. 5.3 the points at 5, 15, 30 and 45 min after the first arrow were given by 5 muscles: the points after the second arrow were given by another 5 muscles which were treated with carbachol for 90 min: the potentials were recorded and then solution with carbachol plus ouabain was added. This arrangement was again adopted to prevent excess damage due to repeated penetration of the fibres, and Table 5.1 gives the composite results from all 10 muscles.

Fig. 5.8 shows a continuous trace of an experiment similar to that shown by the second arrow of Fig. 5.3. The membrane potential at the end-plate region had recovered following prolonged exposure to carbachol 100μM: addition of solution containing carbachol plus ouabain produces prompt depolarization to -57mV. This result, and those of Fig. 5.3 give support to the concept that the recovery of potential in the presence of carbachol is due to operation of an ouabain-sensitive process which is presumably the electrogenic sodium pump.

Effects of Suberyldicholine (100μM)

Results with suberyldicholine were similar to those obtained with carbachol. Continuous recordings (Fig. 5.9) were made in 10 fibres for varying lengths of time prior to the expulsion of the electrode. The median depolarization of the end-plate membrane potential to 100μM suberyldicholine was from -72mV (95% confidence limits -66mV, -73mV) to -54mV (95% confidence limits -51mV, -56mV).

Multiple samplings of 60 end-plate membrane potentials from 5 muscles were made 5 min prior to and +5, +30, +60, +90 and +120 min after the addition of the drug (Fig. 5.10 filled circles: Table 5.2). The median membrane potential before the application of 100μM suberyldicholine

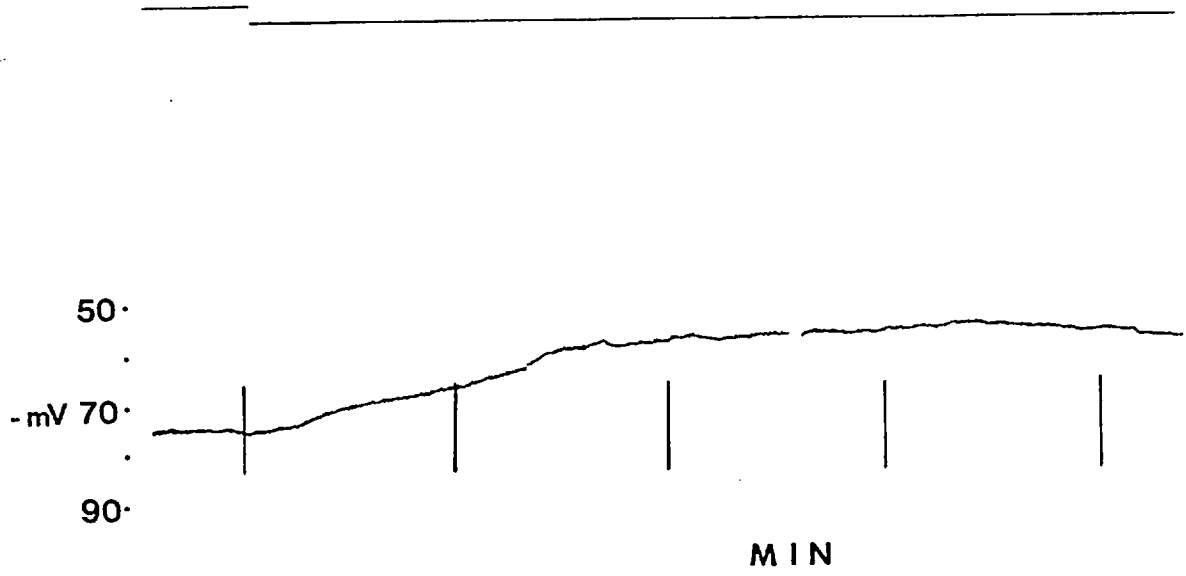


Fig. 5.8. Following recovery of potential in solution containing 100μM carbachol (see Fig. 5.3), a solution containing 100μM carbachol plus 100μM ouabain caused depolarization from -75mV to -57mV. Ordinate in mV, abscissa in minutes. Broken top line indicates change-over to solution with carbachol plus ouabain.

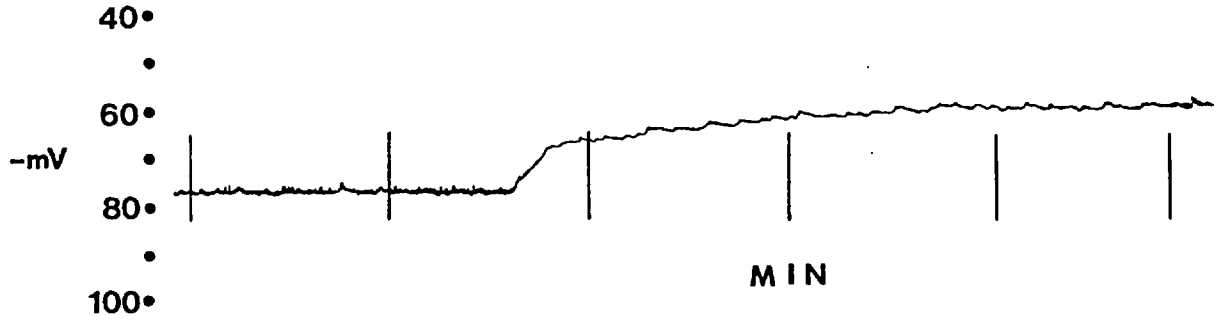
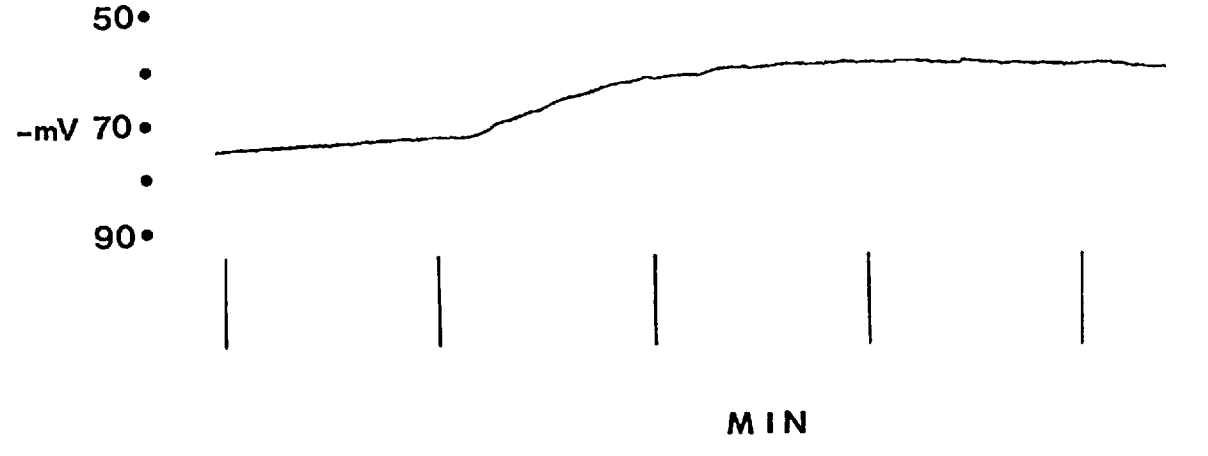


Fig. 5.9. Upper and Lower: Continuous traces of the initial depolarization produced by 100μM suberyldicholine. Break in top line indicates cHangeover to drug solution. Ordinate in mV. Abscissa in minutes.



was -78mV (95% confidence limits -74mV, -80mV). After 5 min the median potential at the end-plate region had fallen to -63mV (95% confidence limits -61mV, -65mV). At 30 min the median membrane potential had risen to -65mV and reached a plateau at 90 min of -73.5mV (95% confidence limits -71mV, -76mV). After 2 hours in the presence of the drug, there was substantial (82%) but incomplete recovery of potential. The rate of recovery, during the first 90 min was 0.22mV/min. The results in Fig. 5.10 have also been listed in Table 5.2.

100μM Suberyldicholine in the absence of potassium

Fig. 5.11 shows a continuous recording of depolarization produced by application of 100μM suberyldicholine in potassium-free saline in which the potential fell from -89mV to -55mV.

Multiple sampling of the end-plate membrane potentials from 47 - 60 fibres (5 muscles) were taken before and at intervals after the addition of the drug (Fig. 5.10, clear circles). In potassium-free Krebs saline, the median potential was -81mV (95% confidence limits -65mV, -90mV). After 5 mins in 100μM suberyldicholine with potassium-free Krebs saline, the median membrane potential in the end-plate region was -50mV (limits -45mV, -52mV). The depolarization of the membrane in the end-plate region continued with no indication of recovery, and after 120 min the membrane potential was -48mV (95% confidence limits -43mV, -55mV).

100μM Suberyldicholine with Ouabain

Continuous recordings were made in 5 fibres for varying lengths of time (Fig. 5.12). The median depolarization of the end-plate membrane potentail to 100μM suberyldicholine with 100μM ouabain was from -74mV (range -70mV to -80mV) to -50mV (range -46mV to -54mV).

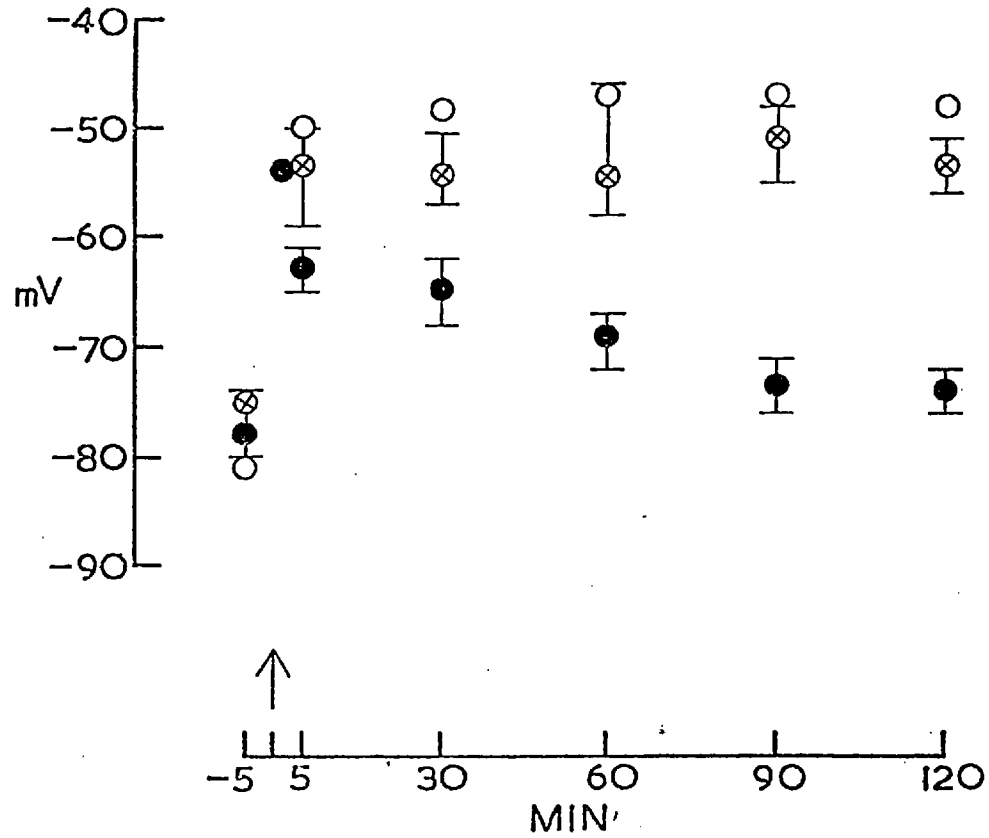


Fig. 5.10. Filled circles show effect of 100μM suberyldicholine. The depolarization is followed by substantial recovery in the presence of the drug. Repeated recordings. Medians of 60 fibres from 5 muscles (Table 5.2). Clear circles show effect of 100μM suberyldicholine in potassium-free solution: recovery does not occur. Crossed circles show effect of 100μM suberyldicholine plus 100μM ouabain: recovery is not seen. Bars represent 95% confidence limits. Bars have been omitted from clear circles. Ordinate is in mV. Abscissa is in minutes. Arrow indicates change over to drug solution.

TABLE 5:2 RESULTS WITH SUBERYLDICHOLINE BY REPEATED SAMPLING

(Cont. Rec. indicates results obtained by continuous recording: membrane potentials, in-mV).

FIG 5:10	Standard Krebs	100μM Suberyldicholine					
TIME	-5 min	Cont. Rec.	+5 min	+30 min	+60 min	+90 min	+120 min
MEDIAN	78	54	63	65	69	73.5	74
95% Lower Limit	74	51	61	62	67	71	72
95% Upper Limit	80	56	65	68	72	76	76
n	60	10	60	60	60	60	60

FIG 5:12	Standard Krebs	100μM Suberyldicholine + 100μM Ouabain					
TIME	-5 min	Cont. Rec.	+5 min	+30 min	+60 min	+90 min	+120 min
MEDIAN	75	50	53.5	54.5	54.5	51	53.5
95% Lower Limit	71	54	50	51	46	48	51
95% Upper Limit	78	46	59	57	58	55	56
n	60	5	60	60	60	60	60

FIG 5:11	Potassium Free Krebs	100μM Suberyldicholine in Potassium Free Krebs					
TIME	-5 min	Cont. Rec.	+5 min	+30 min	+60 min	+90 min	+120 min
MEDIAN	81	55	50	48.5	47	47	48
95% Lower Limit	65	41	45	45	43	43	43
95% Upper Limit	90	57	52	51	53	9.9	55
n	60	3	47	60	60	60	59

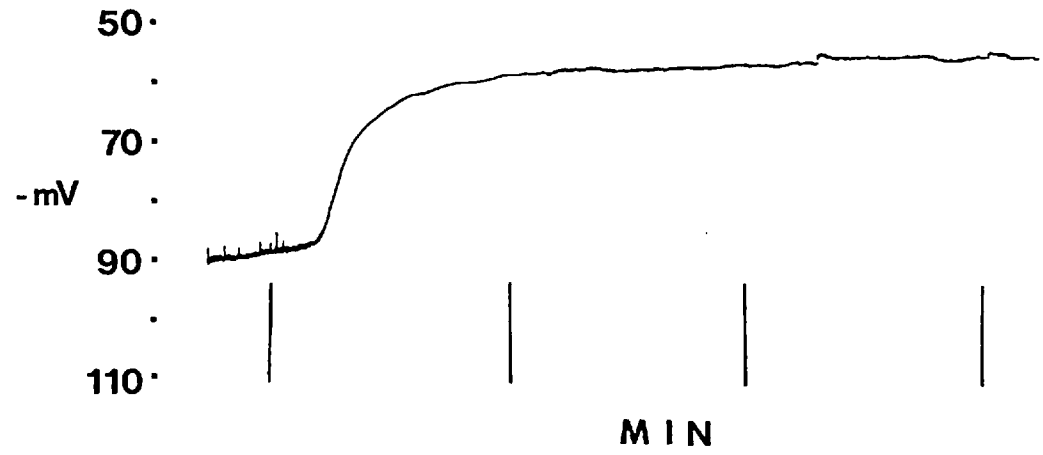


Fig. 5.11. Depolarization produced by 100μM suberyldicholine in the absence of potassium. Miniature end-plate potentials are particularly well marked prior to the addition of the drug. Broken top line indicates changeover to drug solution. Ordinate is in mV. Abscissa is in minutes.

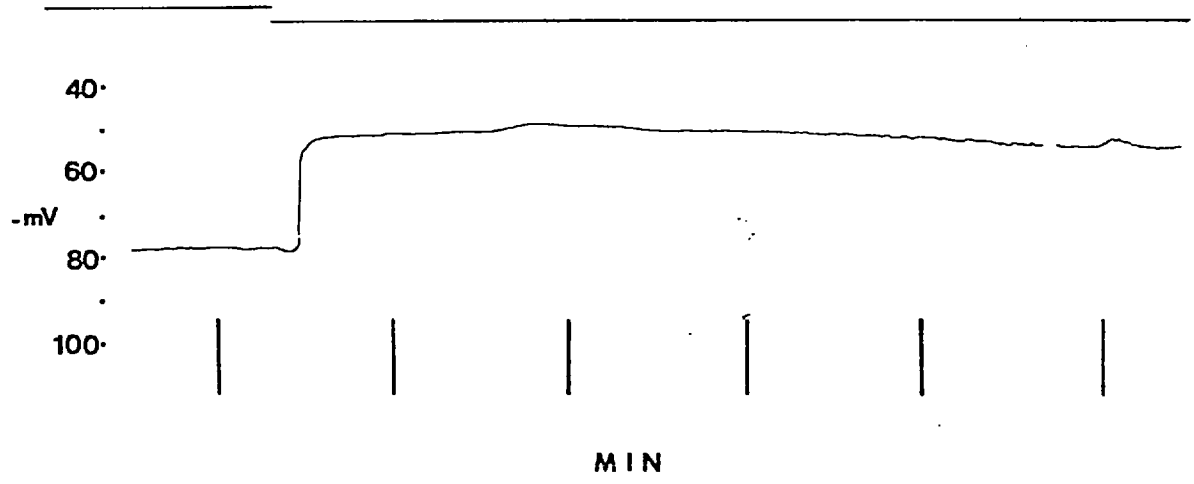
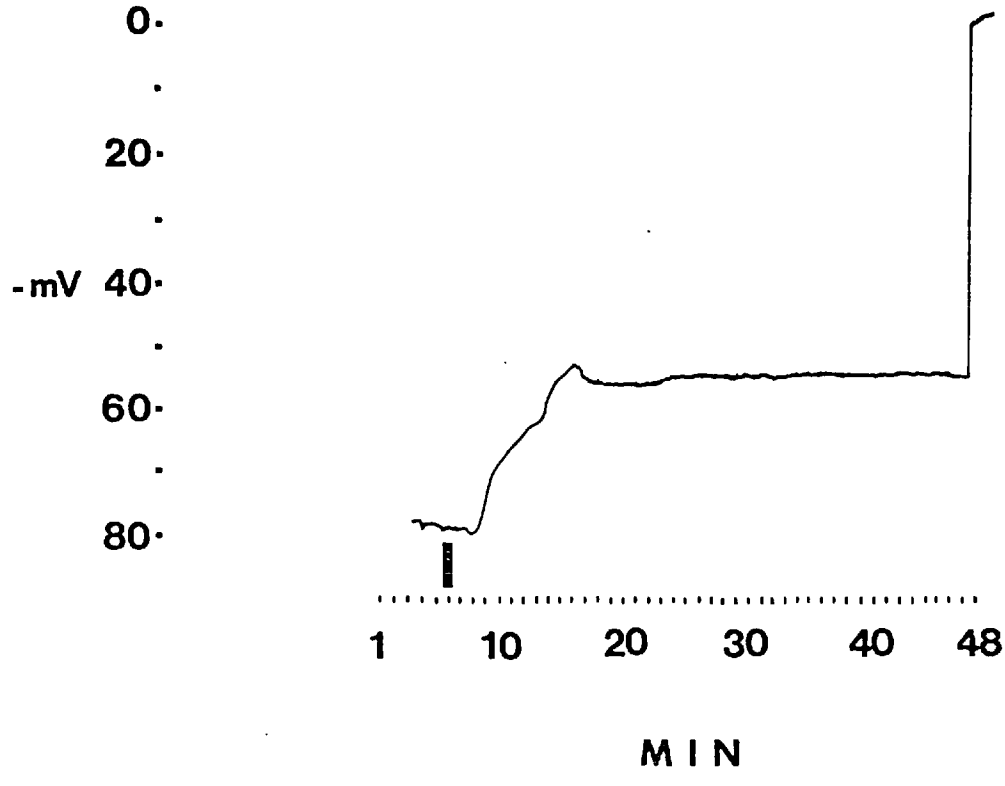


Fig. 5.12. Continuous recording of depolarization produced by 100μM suberyldicholine plus 100μM ouabain. No recovery of potential is indicated in either case. Ordinate in mV. Abscissa in minutes.

Upper trace: Rapid recording of depolarization. Broken top line indicates changeover to drug-containing solution.

Lower trace: Slow recording speed. Removal of electrode at end of experiment indicated virtually no electrical drift. Bar above abscissa indicates changeover to drug-containing solution.



The record in Fig. 5.12 (lower) was obtained with the muscle mounted vertically. The initial depolarization to -53mV is followed by an apparent small recovery of a few mV, and the potential was subsequently stable. This was the maximum "recovery" seen in the presence of ouabain. At 48 min the electrode came out of the cell, and the voltage dropped to near zero.

Multiple sampling from the end-plate region of 60 fibres in 5 muscles were taken before and after the addition of drugs (Fig. 5.10, crosses). The potential was initially -75mV (95% confidence limits -71mV, -78mV). After 5 min, the membrane potential at the end-plate region had depolarized to -53.5mV (95% confidence limits -50mV, -59mV) and there was very little indication of recovery for 120 min (Fig. 5.10, Table 2.2).

Summary of Chapter V.

1. Carbachol 100μM produced depolarization of end-plates in rat diaphragm of 17mV (median of 23 fibres, 95% limits 15-19mV). By the method of repeated sampling the fibres depolarized from -74mV to -56mV (median of at least 54 fibres in each case, in 5 muscles).

2. With repeated sampling, recovery of membrane potential occurred in the presence of the drug and was complete in 60 min. Individual fibres showed much variation in the rate of recovery, and in some fibres the recovery was much more rapid.

3. In the absence of potassium, carbachol 100μM produced depolarization of 34mV (median of 6, range 11-48mV). With repeated sampling the effect of carbachol 100μM was to reduce the membrane potential to -48mV (median of 60 fibres) and the end-plates remained depolarized in the presence of the drug, without significant evidence of recovery, for 90 min.

4. When muscles were exposed to ouabain (200μM) in addition to carbachol (100μM) the end-plates remained depolarized without recovery for 60 min. The effect of ouabain was reversible: withdrawal of ouabain (in the presence of carbachol) led to substantial recovery during the following 60 mins.

5. End-plates whose membrane potentials had recovered in the presence of carbachol became rapidly depolarized when ouabain (100μM) was added in addition to the carbachol.

6. Suberyldicholine (100μM) produced depolarization of end-plates in rat diaphragm of 18mV (median of 10, range 8 - 26mV). With repeated sampling, substantial recovery of resting potential occurred in the presence of the drug and recovery progressed during 90 min of exposure.

7. There was no recovery from the effects of suberyldicholine in the absence of potassium, and in the presence of ouabain (100μM).

8. It was concluded that the spontaneous recovery of membrane potential in the presence of carbachol and of suberyldicholine is a process which is sensitive to potassium and to ouabain.

CHAPTER VI DECAMETHONIUM ON RAT DIAPHRAGM

Decamethonium is of interest in that it behaves as a partial agonist in frog muscle (Parsons, 1969); rat muscle is notoriously resistant to the neuro-muscular blocking actions of decamethonium (Zaimis & Head, 1976); and decamethonium is known to cross the post-synaptic membrane of rat muscle, possibly through sodium channels (Creese, Franklin & Mitchell, 1977). The entry as measured by the rate of clearance rises to a plateau, and falls at very high concentrations in the mM range (Creese and England, 1970). To determine whether the electrical response also varied, a concentration effect curve was constructed, and the effect of ouabain and potassium free solutions on maximal and supra maximal concentrations of decamethonium were investigated.

Concentration-effect Curve

The measurements of drug response were taken as the difference between membrane potential prior to its addition and the maximum depolarization following its application. Figs. 6.1, 6.2 and 6.3 show effects produced by 1μM, 2μM, 3μM, 100μM and 3mM, and the results are also listed in Table 6.1.

Due to the small sample size used (n = 5 - 7) and the diversity of the animals' origins, a predictably wide range of responses was observed (Fig. 6.1 - 6.3). Fig. 6.4 gives the mean values plotted against the logarithm of the concentration.

The mean values of depolarization were converted to probits using the effect of 100μM as the maximal response. With the three points at 1, 2 and 3 μM the regression of empirical probits on log concentration was 3.17 probit units/log unit (see Table 6.2), and the

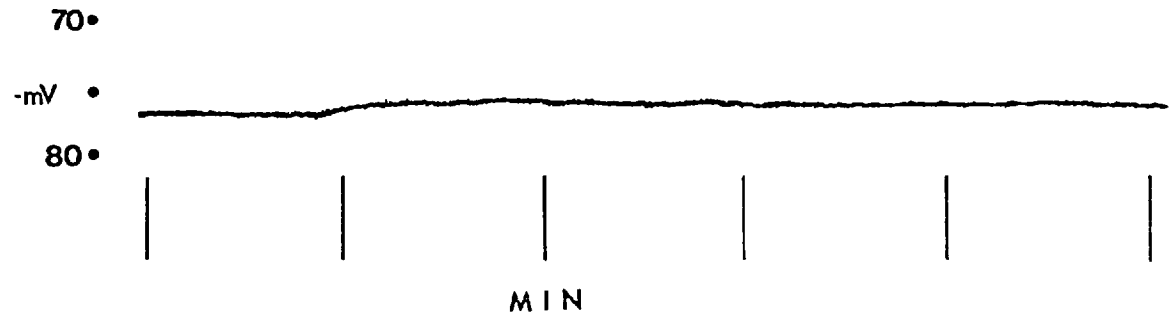
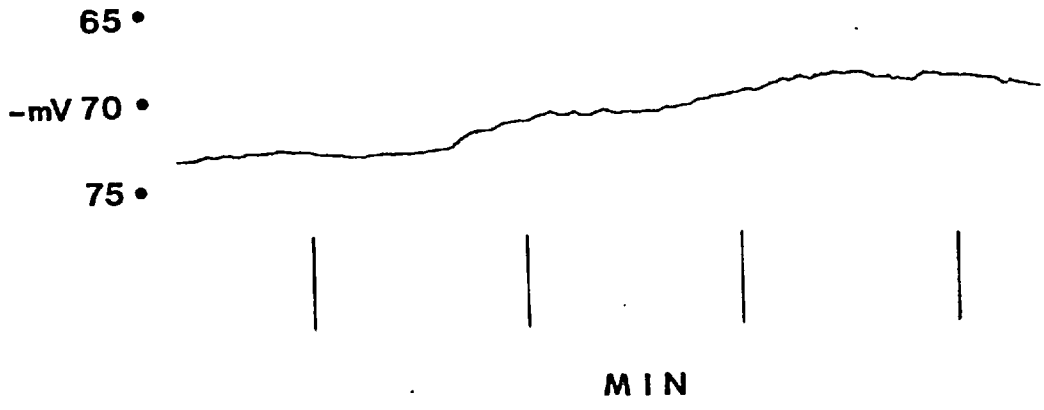


Fig. 6.1. Upper: Continuous recording. Depolarization to 1μM decamethonium. Broken top line indicates changeover to drug solution. Ordinate in mV. Abscissa in minutes.

Lower: Continuous recording. Depolarization to 2μM decamethonium. Broken top line indicates changeover to drug solution. Ordinate in mV. Abscissa in minutes.



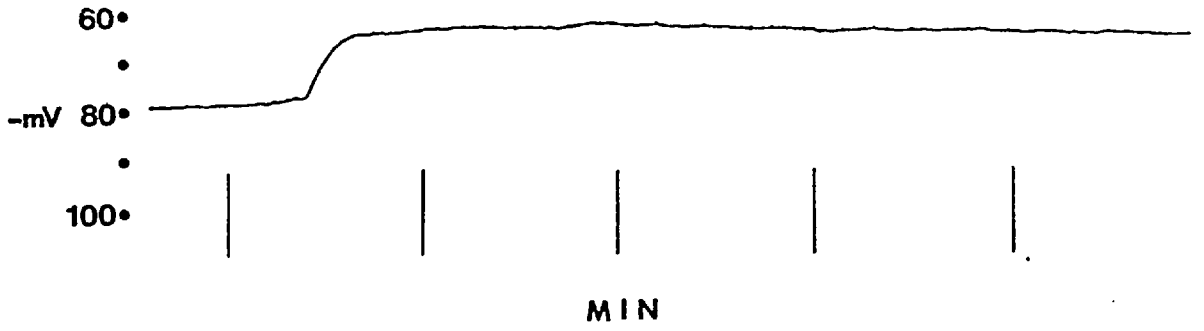
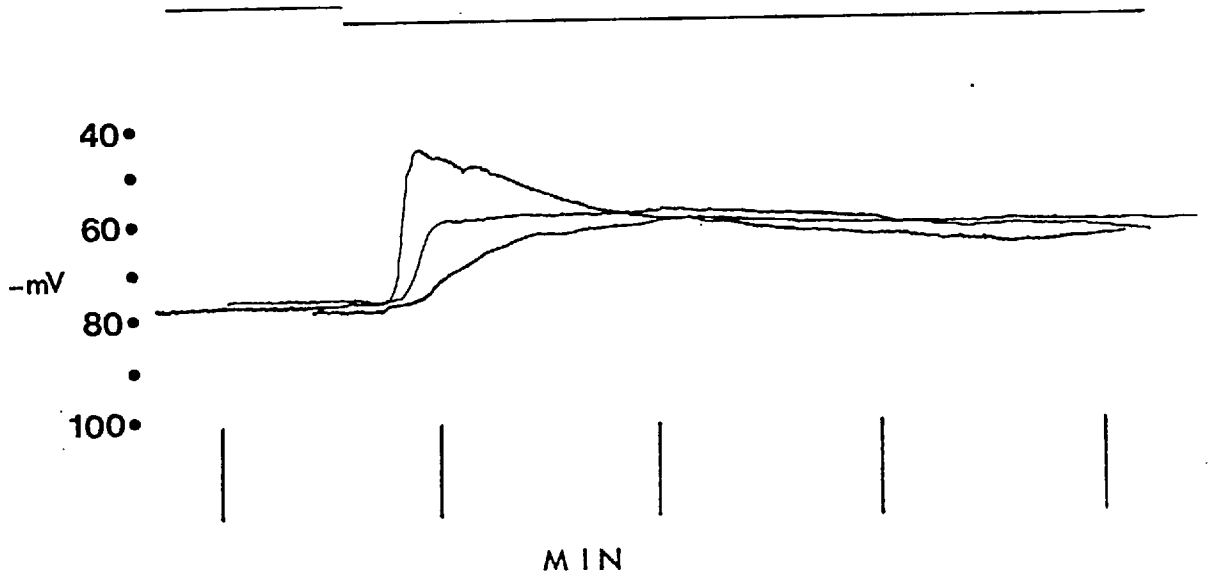


Fig. 6.2. Upper: Continuous recordings. Depolarization to 3μM decamethonium. Broken top line indicates changeover to drug solution. Ordinate in mV. Abscissa in minutes.

Lower: 3 superimposed continuous recordings. Depolarization to 100μM decamethonium. Note variation in the initial response at this concentration. Broken top line indicates changeover to drug solution. Ordinate in mV. Abscissa in minutes.



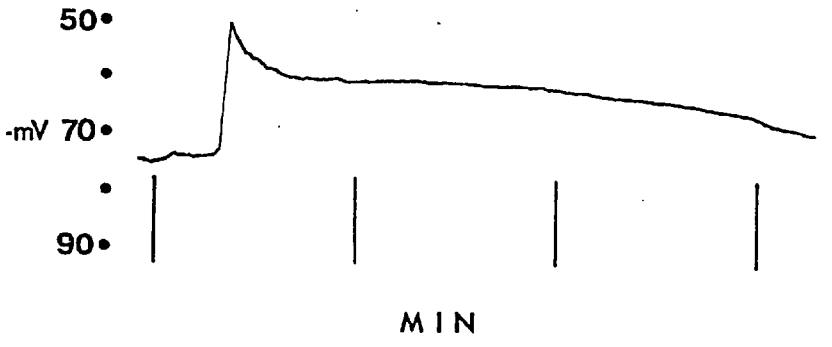


Fig. 6.3. Upper and Lower: Continuous recordings. Depolarization to 3mM decamethonium. Note variation in rate of depolarization and recovery of potential. Cell in upper trace had initially a higher membrane potential than cell in lower trace. Broken top lines indicate changeover to drug solution. Ordinate in mV. Abscissa in minutes.

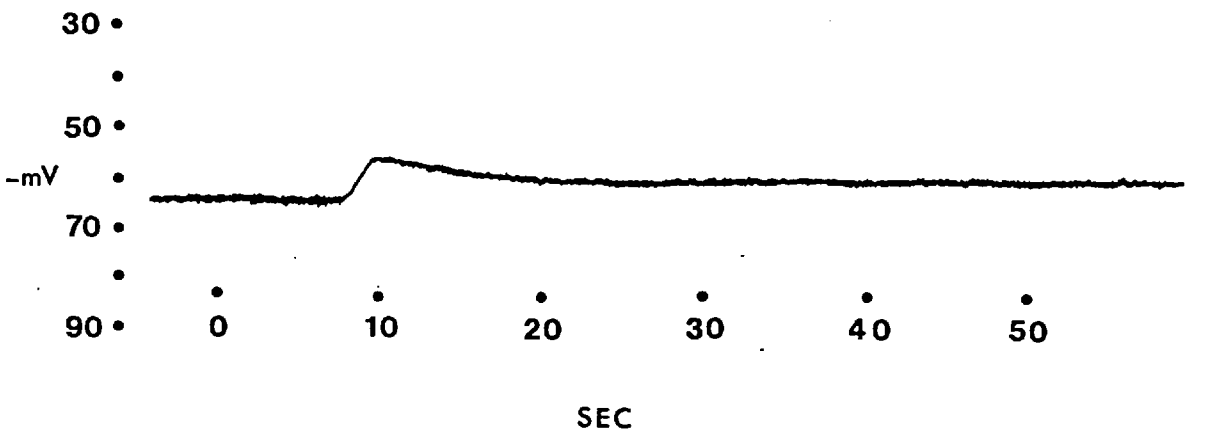


Fig. 6.4. Depolarization produced by different concentrations of decamethonium at end-plate region of rat diaphragm. Filled circles give means from 5-7 continuous recordings, being the difference between resting potential and maximum depolarization. Bars are standard errors. The drug concentration is represented on a logarithmic scale.

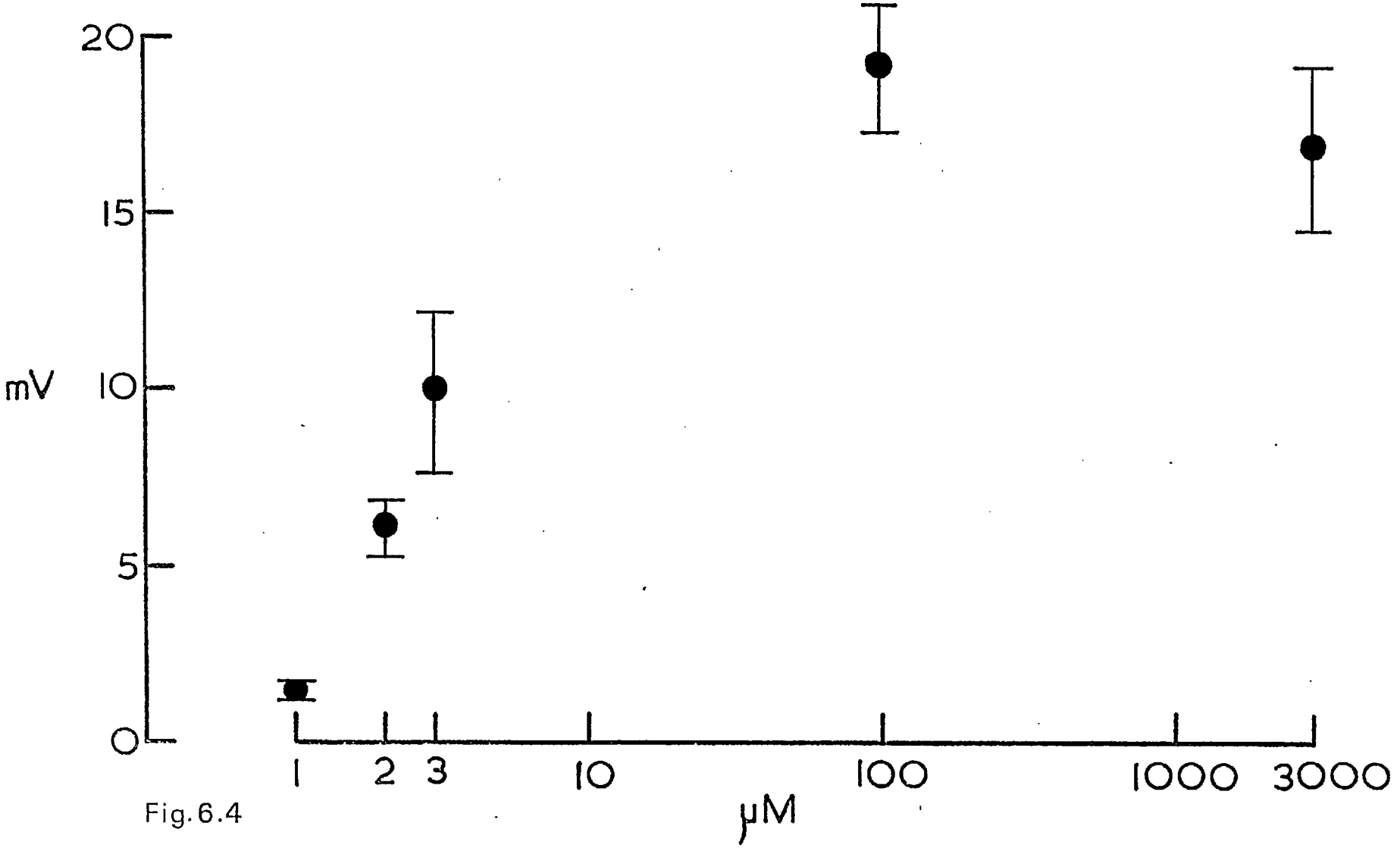


Fig. 6.4

TABLE 6:1 RESPONSES TO DECAMETHONIUM IN RAT DIAPHRAGM

Conc^{n}. μM	Range mV	n	Mean mV	S.E.M.	S.D./$\bar{x}$
1	1-2	5	1.4	0.13	0.38
2	4-8	5	6.2	0.75	0.26
3	4-18	7	10.0	2.04	0.54
100	15-28	7	19.3	1.68	0.23
3000	10-26	6	17.0	2.18	0.31

The maximum response was taken as 19.3 mV (See Fig. 6:4).

TABLE 6:2 CONCENTRATION - EFFECT CURVE OF DECAMETHONIUM IN RAT DIAPHRAGM

CONC.	LOG CONC. x	n	nx	nx^2	MEAN DEPOL (mv)	FRACTION	EMPIRICAL PROBIT	EXPECTED PROBIT	SLOPE b	DEVIATIONS ABOUT REGRESSION s^2
1μM	0	5	0	0	1.4	0.0726	3.5434	3.554		
2μM	0.301	5	1.505	0.453005	6.2	0.3215	4.5365	4.507	3.1663	0.00131193
3μM	0.4771	7	3.397	1.593371	10.0	0.5185	5.0464	5.065		
100μM					19.29					
Sum		17	4.8447	2.046376						

$b = S_{xy}/S_{xx} = 3.1663$, $b^2 = 10.02546$: Hill slope = b/1.4 = 2.3 : $\bar{x} = S(x)/S(n) = 0.2850$, Antilog = 1.93μM

MED $= x_m = x_3 - \frac{5.065-5}{3.1663} = 0.4771 - 0.0205 = 0.4566$: Antilog = 2.86μM. N = 3, $s^2_{y.x} = s^2/(N-2) = 0.00131193$.

$t = 12.8$, $t^2 = 163.83$. $g = \frac{t^2 s^2_{y.x}}{b^2 S_{xx}} = 0.026$, and $\tilde{m} - \bar{x} = \frac{1}{1-g}\left[(x_m - \bar{x}) \pm \frac{t.s_{y.x}}{b}\sqrt{\frac{1-g}{S(n)} + \frac{(x_m - \bar{x})^2}{S_{xx}}}\right]$.

By Fieller's theorem, $\tilde{m} = 0.2850 + 1.02669\left[0.1716 \pm 0.14643\sqrt{0.05729 + 0.044232}\right]$ and 95% confidence limits of x_m are 0.5090, 0.4133 with antilogs 2.59, 3.23μM.

Nomenclature for S_{xy}, S_{xx} as Finney, 1964. Other symbols as Goldstein, 1964.

The slope b and the sum of squares about regression were obtained by use of a desk calculator (Creese, Franklin & Mitchell, 1977).

The Hill slope is taken as probit slope/1.4 (Gaddum, 1957).

slope and the deviations about regression were obtained by use of a desk calculator (Creese, Franklin, & Mitchell, 1977). The median effective dose, obtained by standard methods described by Goldstein (1964) was 2.9μM, with 95% limits estimated as 2.6, 3.2μM. The slope of the Hill plot, which was taken as probit slope/1.4 (Gaddum, 1957) was 2.3. In this initial estimate the unweighted rather than weighted regression has been used, and no correction has been attempted for non-linearity of conductance with change of voltage (Ginsborg & Jenkinson, 1976).

The results in this small series are in general similar to other dose-response curves obtained on skeletal muscle. The Hill slope contains a non-integer value, and at low drug concentrations the concentration-effect curve is non-linear on an arithmetic scale. The estimate of 3μM for the median effective concentration is similar to the value of approximately 4μM found by entry of labelled decamethonium (Creese & England, 1970), and the half-maximal concentration provides some justification for the use of 100μM to achieve maximal response.

Effects of Decamethonium (100μM)

Collected results similar to those shown in Fig. 6.2 (lower) indicate that the membrane at the end-plate became depolarized by decamthonium (100μM) to -57mV (median of 13 fibres, 95% limits -55, -62mV). This is similar to values found with carbachol and with suberyldicholine, and show that these three compounds produce a similar degree of depolarization in rat muscle.

Spontaneous recovery could be demonstrated in the presence of the drug, but two distinctive features were found. In some cases there was an initial rapid phase of recovery during the first min (see Fig. 6.2, lower). There was also a very slow phase of recovery taking

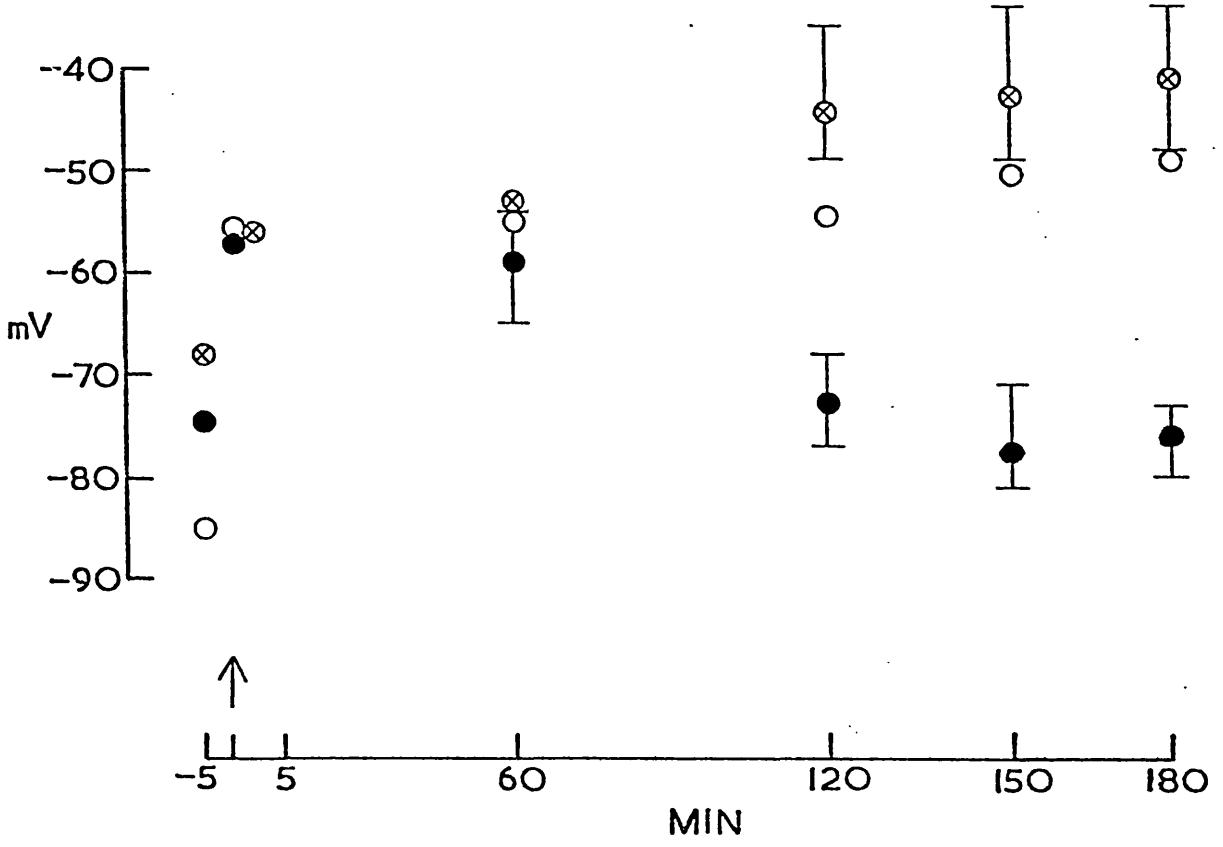


Fig. 6.5. Effect of decamethonium 100μM, obtained by repeated sampling. Filled circles: depolarization produced by decamethonium, and slow recovery: 36 fibres from 6 muscles (Table 6.3). Crossed circles: effect of 100μM decamethonium plus ouabain 200μM: the depolarization is not followed by recovery: 42-48 fibres from 4 muscles. Clear circles: decamethonium 100μM in potassium-free solution: depolarization with no recovery of membrane potential: 60 fibres in 5 muscles. Bars represent 95% confidence limits. Initial depolarization to decamethonium alone and in potassium-free Krebs obtained from continuous recordings in 13 and 6 muscles respectively. Confidence limits are deleted on some points for clarity of presentation. Ordinate in -mV. Abscissa in minutes. Arrow indicates changeover to drug solution.

up to two hours for completion.

Multiple sampling of the end-plate membrane potential was made just prior to and +60, +120, +150 and +180 mins after the addition of the drug (Fig. 6.5, Table 6.3). Following application of 100μM decamethonium, the membrane depolarized. Fig. 6.5 shows the subsequent slow rise in potential. After 60 mins, the potential was -59mV. At 120 min, the median potential was -73mV. Maximum potential was observed at 150 mins, the median potential in the end-plate region being -77.5mV (limits -71mV, -81mV). At 180 min the median potential was -76mV (limits -73mV, -80mV), so that complete recovery had occurred.

100μM Decamethonium in the absence of potassium

Continuous recordings were made of the end-plate membrane potentials from 6 muscles prior to and during the application of the drug in potassium-free saline. In Fig. 6.6 the membrane potential changed from -94mV to -41mV. In 6 fibres the depolarization ranged from 30 to 53mV, with median 41mV. Four of the fibres showed an initial rapid phase of recovery during the first min, as in Fig. 6.6.

Multiple samplings were made prior to and +60, +120, +150 and +180 mins after the changeover to the drug solution (Fig. 6.5: clear circles). After 60 mins the median end-plate membrane potential had depolarized from -85mV (95% confidence limits -78mV, -88mV) to -55mV (limits -55mV, -58mV) in 60 fibres from 5 muscles. Subsequently the potential slowly declined, and at +180 mins, the median potential was -49mV (95% confience limits -43mV, -54mV).

100μM Decamethonium with ouabain

Multiple samplings were made of the end-plate membrane potentials from 42 to 48 fibres in 4 muscles prior to, and at intervals

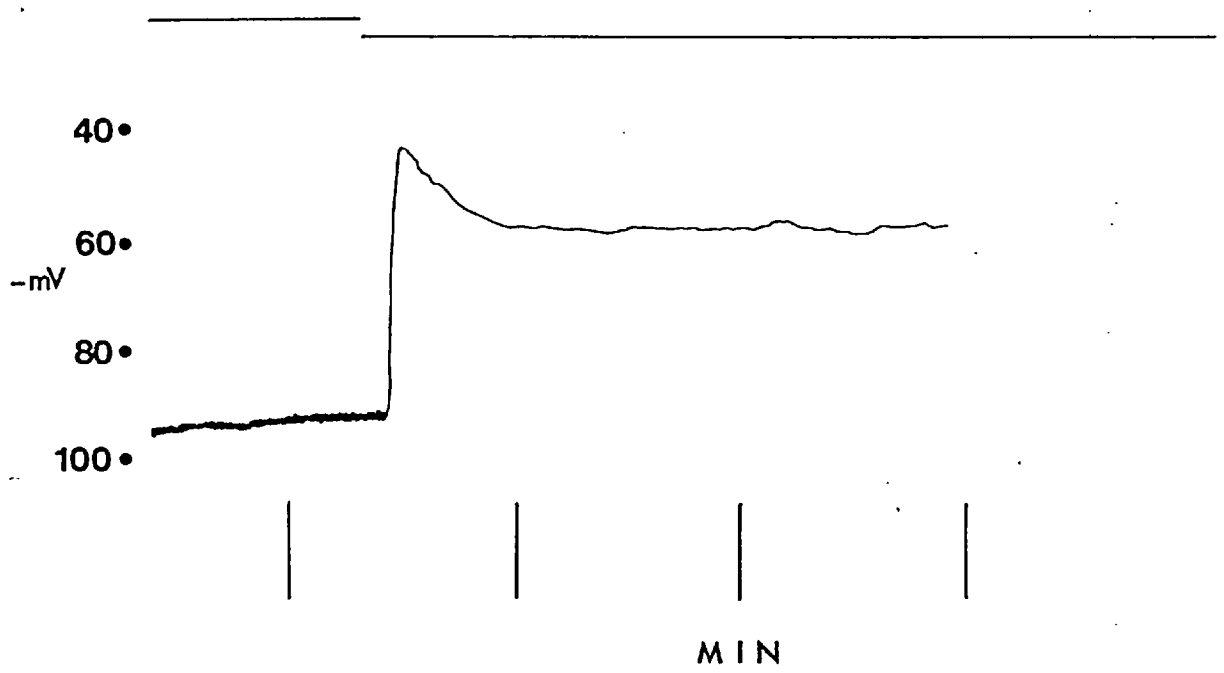


Fig. 6.6. Initial depolarization to 100μM decamethonium in potassium free solution. Continuous reçording. Note limited initial recovery of potential. Broken top line indicates turnover to drug solution. Ordinate in mV. Abscissa in minutes.

after the addition of 100μM decamethonium with 200μM ouabain (Fig. 6.5 crosses: Table 6.3). The membrane depolarized from -68mV (95% confidence limits -66mV, -69mV) to -53mV (limits -50mV, -56mV). The depolarization was maintained; at 120 min the value was -44.5mV and at +180 mins, the median potential was -41mV (limits -34mV, -48mV). It is seen from the results in Fig. 6.5 that the slow recovery with 100μM decamethonium is abolished in the presence of ouabain and in the absence of potassium.

Effects of 3mM Decamethonium

The depolarization produced by 3mM decamethonium is shown in Fig. 6.3. The results were variable, and the median value was comparable to that produced by 100μM decamethonium (Table 6.1). Most of the fibres gave an initial rapid partial recovery during the first min as in Fig. 6.3 (upper).

Repeated samplings were made just prior to and 5, 20, 30 and 60 mins after the application of the drug. The initial values of the potential was -72mV (Fig. 6.7, filled circles: Table 6.3). After 5 mins in the drug, the potential in the end-plate region was -75mV. After 30 min and 60 min, the potentials were respectively -72mV (95% confidence limits -70mV, -75mV), and -70.5mV (limits -67mV, -75mV). The recovery of the membrane potential was very much more rapid than was the case with 100μM decamethonium (Fig. 6.5, Fig. 6.7).

3mM Decamethonium with ouabain

Fig. 6.7 (crosses) shows the effects of 3mM decamethonium plus 200μM ouabain for 60 min, and the results are also listed in Table 6.3. At 5 min the membrane potential was -58mV (36 fibres from 6 muscles). It can be seen from Fig. 6.7 that some degree of repolarization occurred in spite of the presence of ouabain, and at

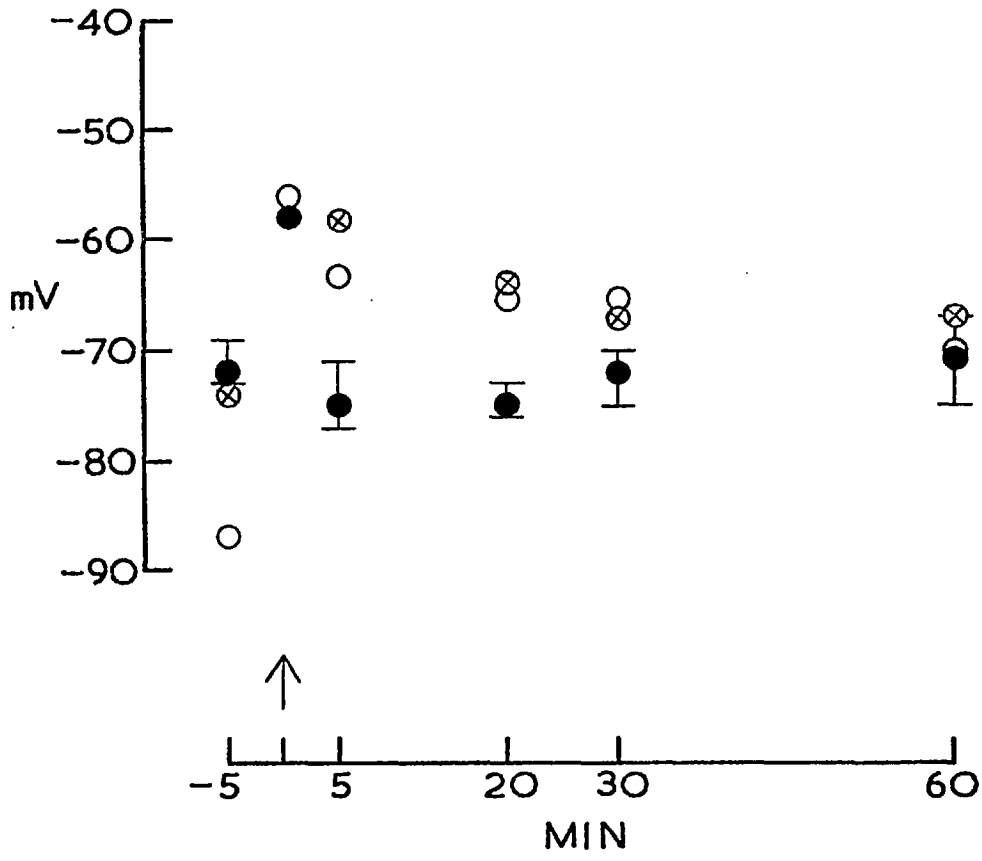


Fig. 6.7. Effect of decamethonium 3mM, obtained by repeated sampling. Filled circles: decamethonium alone, showing rapid recovery following initial depolarization in 36 fibres from 6 muscles. Crossed circles: effect of decamethonium 3mM plus ouabain 200μM: the initial depolarization is followed by partial recovery in 60 minutes: 48-60 fibres from 5 muscles. Clear circles: decamethonium 3mM in potassium-free solution: depolarization followed by some recovery. Bars represent 95% confidence limits.

30 min the membrane potential was -67mV. A Mann-Whitney U-test between values at 5 min and at 30 min gave x^*as 4.6 ($P<0.01$), so that the repolarization was not likely to have been fortuitous. These results are different from those found with 100μM decamethonium and ouabain (Fig. 6.5).

3mM Decamethonium in potassium-free Krebs saline

Continuous recordings were made of the end-plate membrane potentials in 7 fibres during the change from potassium-free Krebs saline to the same type of solution containing 3mM decamethonium (Fig. 6.8). The median depolarization was from -91mV (range -81mV to -105mV) to -56mV (range -48mV to -66mV). Most of the fibres showed an initial rapid partial recovery as is seen in Fig. 6.8.

Repeated sampling of end-plate membrane potentials from 48 to 60 fibres in 5 muscles were made in the two solutions (Fig. 6.7: clear circles: Table 6.3). Depolarization was from -87mV to -63.5mV 5 mins after the exchange of solutions. At 20 min, the median potential was -65mV and at 60 min the median potential was -70mV (95% confidence limits -66mV, -75mV). After 60 min a residual depolarization of 17mV remained as compared with the initial value.

Two rates of recovery with decamethonium

Recovery of potential, following depolarization, appears to occur in two phases. The first phase occurs within one or two min of the application of the depolarizing drug (Fig. 6.2 lower, 6.3 upper, 6.6, 6.8). There is also a slower recovery which may require up to 2 hours to complete (Fig. 6.5).

The occurrence of the rapid phase of recovery may be dependent upon both the concentration of the drug and the constitution of the

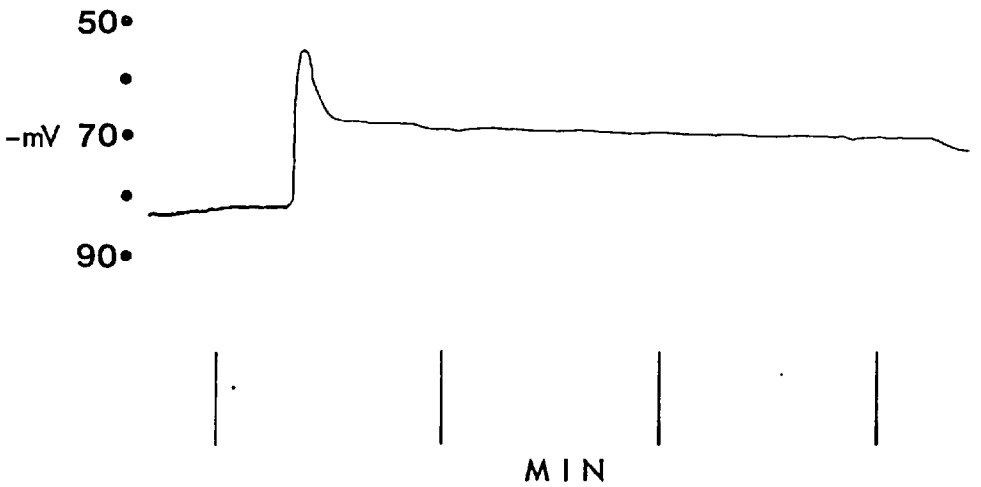


Fig. 6.8. Initial depolarization to 3mM decamethonium in potassium free solution. Continuous recording. Note depolarization and rapid initial recovery. Broken top line indicates turnover to drug solution. Ordinate in mV. Abscissa in minutes.

TABLE 6:3 RESULTS WITH DECAMETHONIUM BY REPEATED SAMPLING

(Cont. rec. indicates results obtained by continuous recording. : membrane potential in -mV)

FIG 6:5	Standard Krebs	100μM Decamethonium				
TIME	-5 min	Cont. Rec.	+60 min	+120 min	+150 min	+180 min
MEDIAN	74.5	57	59	73	77.5	76
95% Lower Limit	72	62	54	68	71	73
95% Upper Limit	77	55	65	77	81	80
n	36	13	36	36	36	36

FIG 6:5	Standard Krebs	100μM Decamethonium +200μM Ouabain			
TIME	-5 min	+60 min	+120 min	+150 min	+180 min
MEDIAN	68	53	44.5	43	41
95% Lower Limit	66	50	36	34	34
95% Upper Limit	69	56	49	49	48
n	42	43	48	48	48

FIG 6:5	Potassium Free Krebs	100μM Decamethonium in Potassium Free Krebs				
TIME	-5 min	Cont. Rec.	+60 min	+120 min	+150 min	+180 min
MEDIAN	85	56.5	55	54.5	50.5	49
95% Lower Limit	78	41	55	54.5	45	43
95% Upper Limit	88	60	58	58	54	54
n	60	6	60	60	60	60

FIG 6:7	Standard Krebs	3mM Decamethonium				
TIME	-5 min	Cont. Rec.	+5 min	+20 min	+30 min	+60 min
MEDIAN	72	58	75	75	72	70.5
95% Lower Limit	69	53	71	73	70	67
95% Upper Limit	73	61	77	76	75	75
n	54	12	36	36	36	36

FIG 6:7	Standard Krebs	3mM Decamethonium + 200μM Ouabain			
TIME	-5 min	+5 min	+20 min	+30 min	+60 min
MEDIAN	74	58	64	67	67
95% Lower Limit	72	56	63	65	66
95% Upper Limited	76	63	68	69	69
n	42	36	36	47	42

FIG 6:7	Potassium Free Krebs	3mM Decamethonium in Potassium Free Krebs				
TIME	-5 min	Cont. Rec.	+5 min	+20 min	+30 min	+60 min
MEDIAN	87	56	63.5	65	65.5	70
95% Lower Limit	73	48	60	62	63	66
95% Upper Limit	92	66	66	68	71	75
n	59	7	48	60	60	60

Krebs saline. The rapid recovery never occurred with drug concentrations less than 100μM. In 100μM decamethonium, one recording had this fast phase. At 3mM, 5 out of 6 exhibited an even more rapid initial recovery phase. When potassium free Krebs was used, this initial phase occurred 4 out of 6 times in 100μM decamethonium, and 6 out of 7 times in 3mM. The most exaggerated forms occurred in 3mM decamethonium with potassium-free Krebs saline (Fig. 6.8).

Summary of Chapter VI

1. Decamethonium (100μM) produced depolarization from -76 to -57mV (13 fibres). With lower concentratins the half-maximal concentration was estimated as 2.9μM.

2. Spontaneous recovery of potential occurred by two processes. Some fibres showed an intial rapid partial repolarization during the first min of application of the drug. With 100μM decamethonium there was also a slow spontaneous recovery of potential which took over 2 hours for full recovery.

3. When ouabain (200μM) was added as well as decamethonium 100μM there was depolarization without recovery.

4. In the absence of potassium decamethonium 100μM produced depolarization with no recovery of membrane potential from 5 -180 min of exposure.

5. With decamethonium 3mM the initial depolarization was followed by complete recovery of membrane potential, and it was found with repeated sampling that substantial recovery took place after the first 5 min of exposure in the presence of ouabain. Recovery was also observed in potassium-free solution.

6. It was concluded that high concentrations of decamethonium can show spontaneous recovery by a process which is not prevented by ouabain.

CHAPTER VII DECAMETHONIUM IN DENERVATED GUINEA-PIG DIAPHRAGM

Experiments described in previous chapters support the hypothesis that one mechanism of recovery from depolarizing drugs may be an electrogenic sodium potassium pump. To test this hypothesis further, an alternative preparation was sought. The denervated guinea-pig diaphragm has particular appeal. Aside from denervation, the preparation could be treated in the same manner as the rat diaphragm. Yet the muscles come from a different, though related species, and denervation furthers their dissimilarities.

Resting potentials in normal and denervated muscle

The membrane potentials of denervated guinea-pig diaphragm was compared to end-plate membrane potentials from innervated guinea-pigs. Both sets of guinea-pigs were of the same weight (150-180g), sex (male), and the investigations were performed on succeeding weeks The membrane potentials from 6 denervated muscles was -60mV (median of 150 fibres, 95% confidence limits -56mV, -60mV). The membrane potentials from end-plates in 5 innervated muscles was -76mV (median of 125 fibres, limits -75, -77mV). The fall in resting potential which accompanied denervation is similar to that described by Lullman (1958) and others.

Miniature end-plate potentials in innervated guinea-pig diaphragm

The miniature end-plate potentials from 3 sites in 3 muscles were examined by polaroid photographs of the storage oscilloscope (Fig. 7.1). The median rise time was 0.32 msec., range 0.3 to 0.4 msec. The 50% fall time was 1.0 msec. in the two cases where adequate photographs were available. These potentials are comparable to those seen in rat diaphragm (Fig. 3.5).

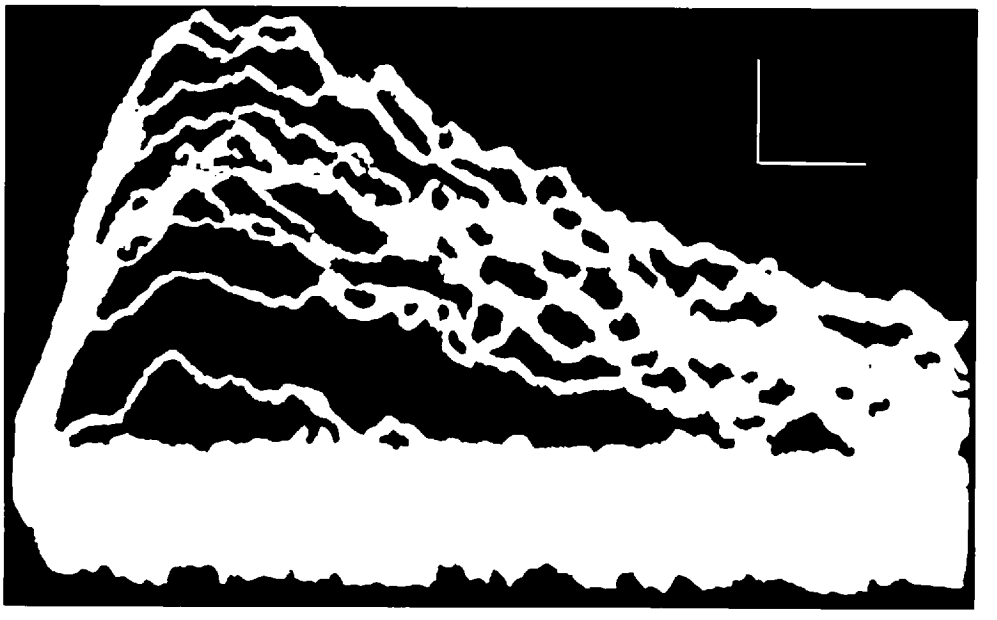


Fig. 7.1. Miniature end-plate potentials from guinea-pig diaphragm muscle. Time to peak voltage 0.3 msec. Calibration: vertical 0.2 mV, horizontal 0.2 msec.

Effect of 100μM decamthonium in denervated guinea-pig diaphragm

Many denervated muscles of the guinea-pig showed spontaneous fibrillation potentials in vitro similar to those described by Smith & Thesleff (1976), and these are described below.

Fig. 7.2 shows the effect of 100μM decamthonium on denervated guinea-pig muscle. In 7 fibres the depolarization was 32mV (median of 7, range 23 to 41mV), from a pre-treatment potential of -65mV (median of 7, range -55 to -69mV). The lower trace in Fig. 7.2 shows depolarization to -9mV, with a rapid initial recovery during the first min, as seen with decamthonium in rat muscle (Chapter 6).

The action of 100μM decamthonium was also examined by repeated penetration of 53 to 72 fibres from 6 muscles (Fig. 7.3., filled circles). The samples were taken at 5 min prior to the addition of the drug, and +5, +60, +90, +120 and +180 min in its presence. The pre-treatment potential was -59mV (median of 72, 95% confidence limits -57mV, -61mV). After 5 min, the potential of 54 fibres was -45.5mV (limits -41mV, -53mV). The fibres continued to repolarize during the following hour or more and by 90 min the membrane potential was -64mV (median of 72 fibres, limits -63mV, -65mV). This small hyperpolarization occurred in all 6 muscles, and is statistically significant ($P<0.01$, Mann-Whitney U-test for non-parametric data, Goldstein 1964).

The hyperpolarization was followed by a slow decline in potential. After 120 min the median membrane potential was -61mV. A final set of penetrations was made at 180 min and indicated little change, median -62mV (limits -59mV, -64mV).

Use of solution without potassium

To prevent contamination by leakage of potassium from the

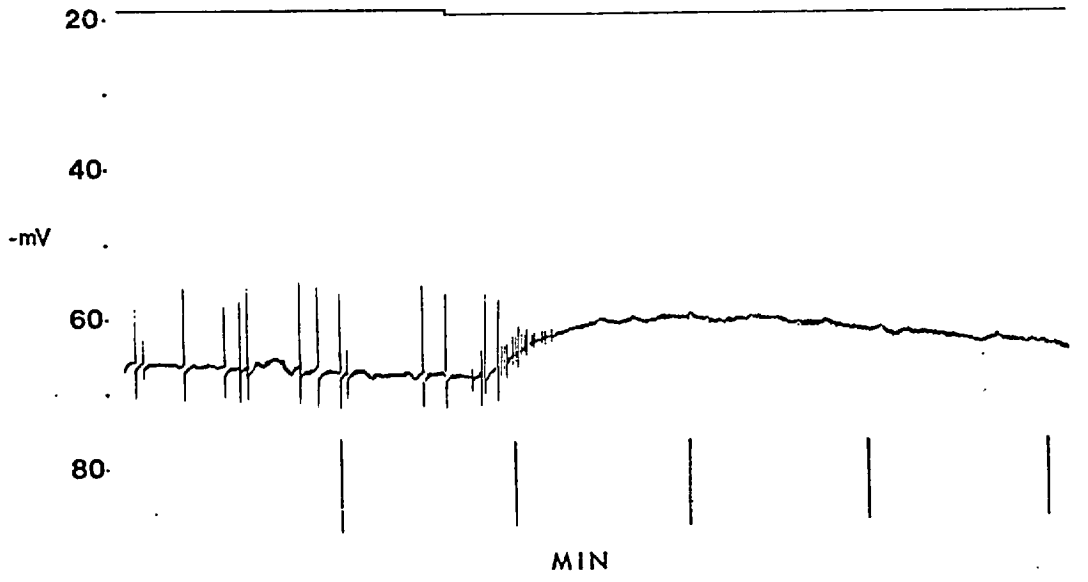
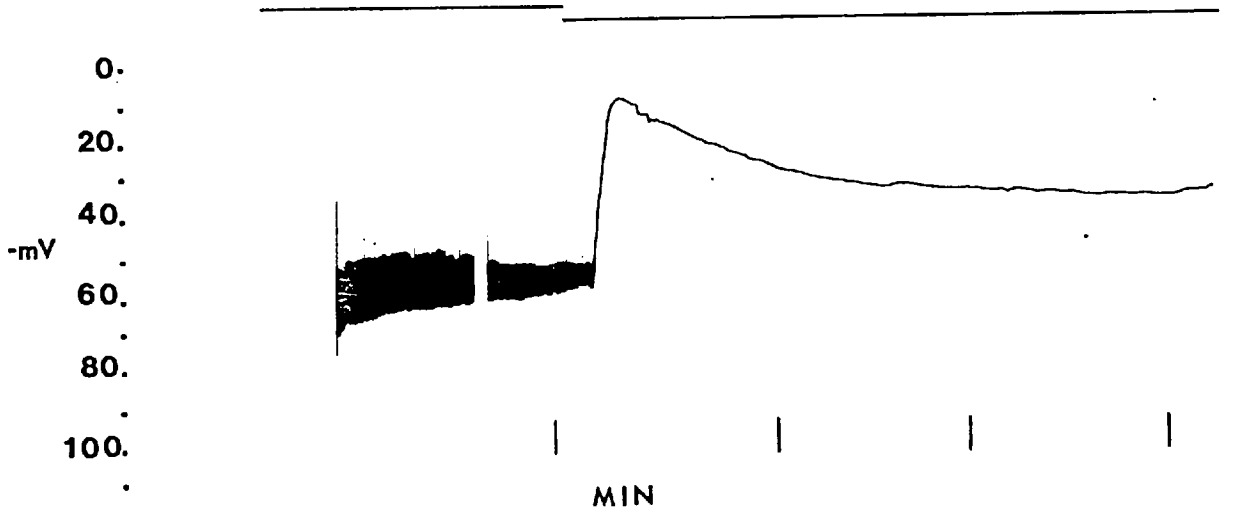


Fig. 7.2. Upper and Lower. Depolarization produced by 100μM decamethonium in denervated guinea-pig diaphragm. Note marked difference in the amount of depolarization, and number of spike potentials. In the lower trace these potentials are no longer individually distinguishable. Ordinate is in mV. Abscissa is in minutes. Broken line indicates changeover to drug solution.



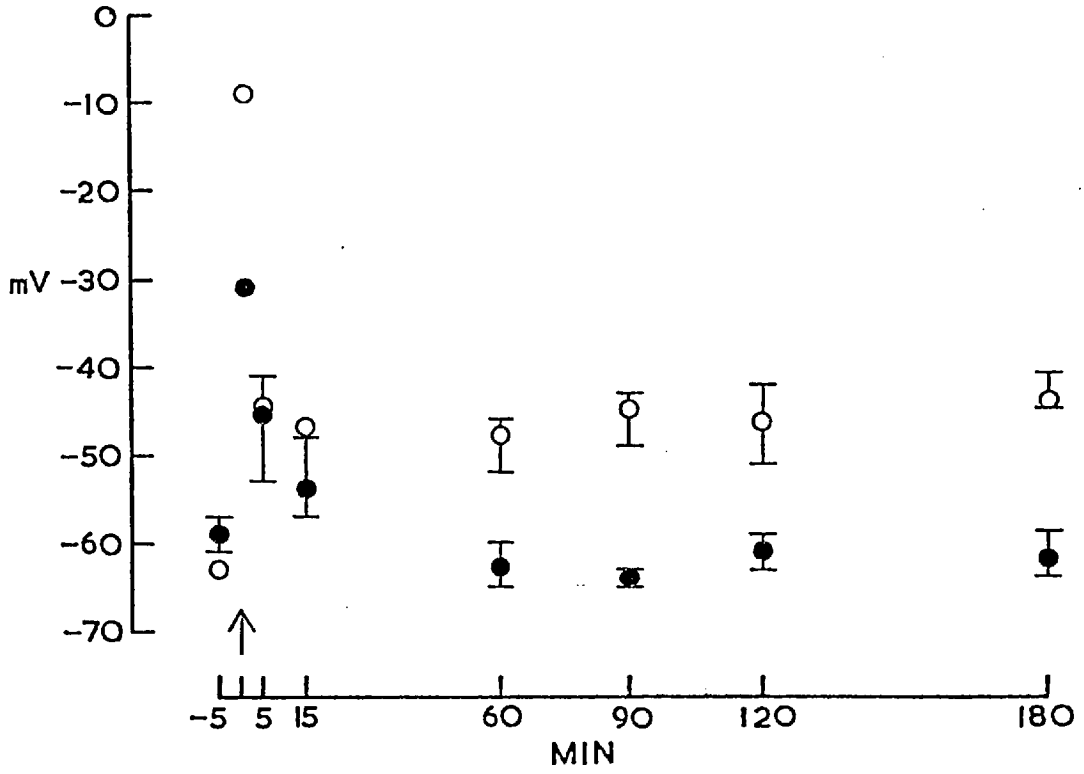


Fig. 7.3. Repeated sampling in denervated guinea-pig diaphragm. Filled circles indicate effects of decamethonium 100μM: the initial depolarization is followed by recovery of potential within 60 min, and some hyperpolarization at 90 min. Median of 53-72 fibres from 6 muscles: initial depolarization obtained by continuous recording from 7 fibres. Clear circles: effect of decamethonium 100μM in potassium-free solution: the large initial depolarization is followed by initial rapid recovery which does not continue after 15 min: medians of 60 fibres from 5 muscles: initial depolarization gives results obtained by continuous recording in 6 fibres. Bars represent 95% confidence limits. Bars at some points eliminated for clarity of presentation. Ordinate is in mV: abscissa in min. Arrow indicates time of changeover of solution.

muscle, potassium free Krebs salines were not re-circulated. The solutions were oxygenated for at least 60 min prior to use and were sent to waste immediately after exposure to the preparation.

The effect of potassium free Krebs on the membrane potential was measured by repeated penetration of 72 fibres from 6 muscles 5 min prior to, and 20 min following the change from 5μM potassium Krebs saline. Fig. 7.4 shows the effect of the change-over on the individual fibres, and some degree of hyperpolarization was found in each case. Twelve measurements were taken from each muscle in the usual solution and in potassium-free saline, and from the median values each muscle showed hyperpolarization ranging from 2.5mV to 7.5mV. In solution with 5mM K^+, the membrane potential was -60mV (median of 60, 95% confidence limits -57mV, -63mV). In potassium-free Krebs saline, the potential was -64mV (median of 60, limits -61mV, -67mV).

Spontaneous depolarizaing potentials

Spontaneous depolarizing potentials were observed in most muscles, but not in all fibres. They were found in the usual saline and also in potassium free saline. In one instance (Fig. 7.4 lower) the onset of the depolarizing potentials were observed after the exchange to potassium-free Krebs saline. The spikes occurred in bursts (Fig. 7.5). They varied in height from a few mV to 80mV. The larger potentials had over-shoots with spikes reaching +15 to +20mV. The duration of the spikes also varied. In Fig. 7.5b the duration at 50% of the spike height was 0.8 msec.

100μM decamethonium in potassium free Krebs saline

Potassium free Krebs saline enhanced depolarization due to 100μM decamethonium, increased initial and prevented long-term recovery. Continuous recordings were made from 6 muscles (Fig. 7.6). The

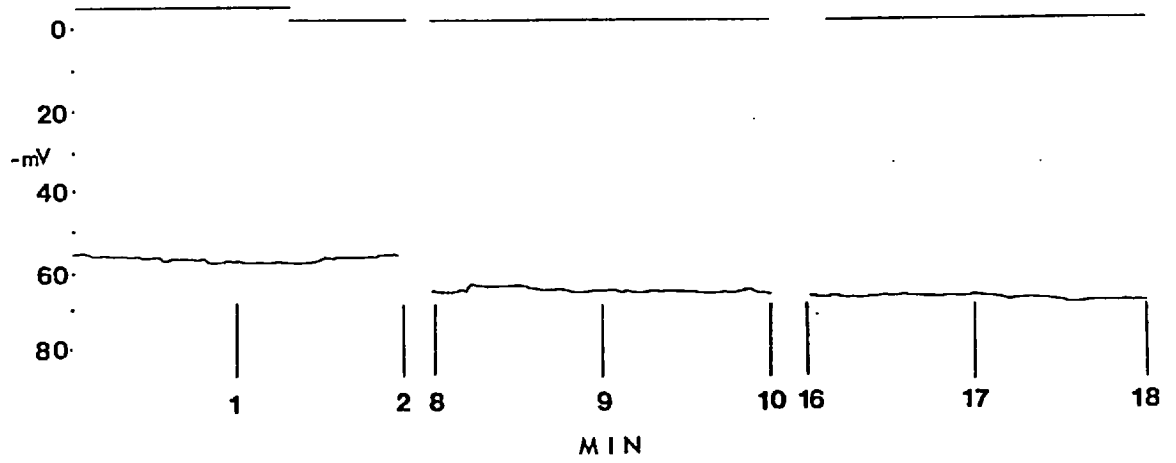


Fig. 7.4. Upper and Lower. Denervated guinea-pig diaphragm. Effect of potassium free saline. Both traces show slow onset of hyperpolarization. In lower trace note onset of spike potentials after 5 min in potassium-free solution. Break in traces indicate where length has been reduced for clarity of presentation. Broken top lines indicate changeover to potassium-free solution. Ordinate in mV: abscissa in min.

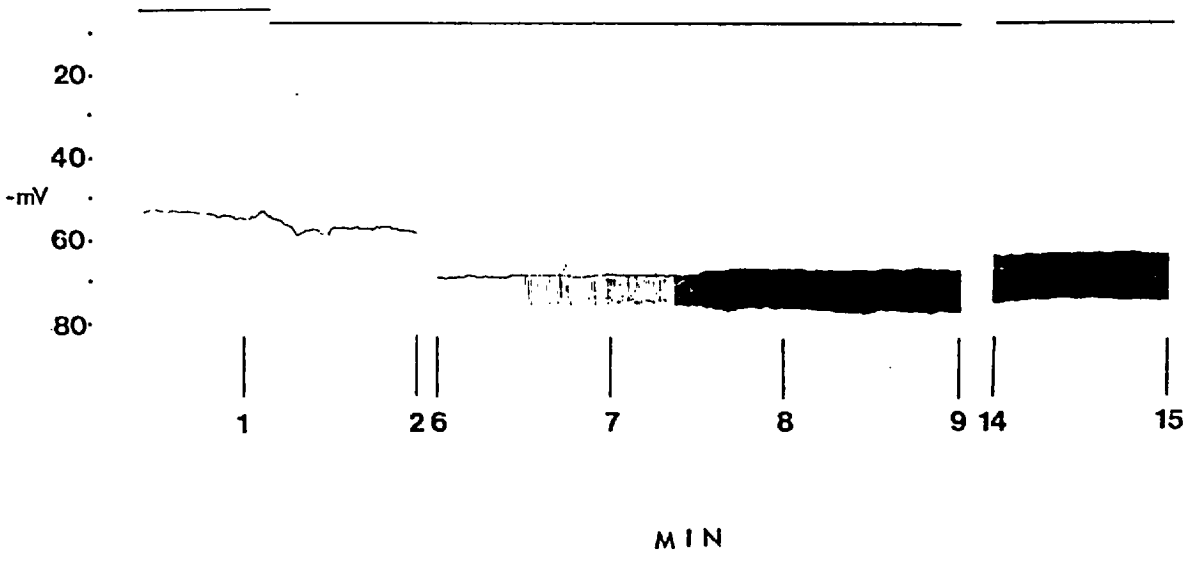
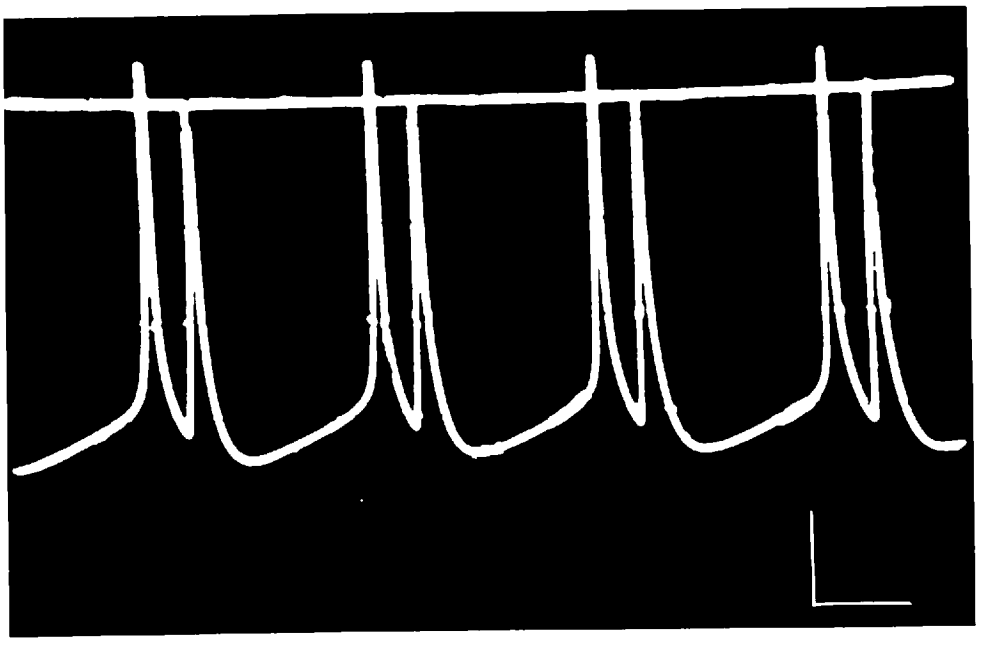


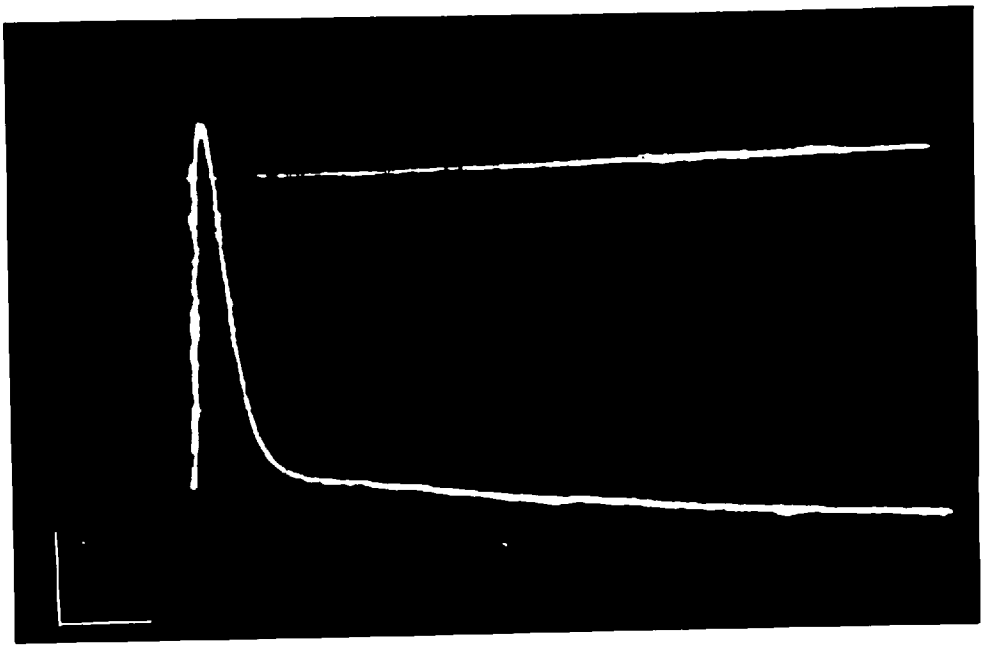
Fig. 7.5. Spontaneous potentials in denervated guinea-pig diaphragm recorded with internal electrodes.

A. Short bursts of potentials, with after-potentials. Horizontal line indicates zero potential. Calibration: vertical 20mV horizontal 20 msec.

B. Individual spike potential after 20 minutes in potassium-free solution. The width of the spike at 50% of the depolarization was 0.8 msec. Horizontal line indicates zero potential. Calibration: vertical 20mV, horizontal 2 msec.

C. Small depolarizations in potassium-free Krebs recorded at two different amplifications. Calibration for the greater amplification: vertical 1mV, horizontal 2 msec.





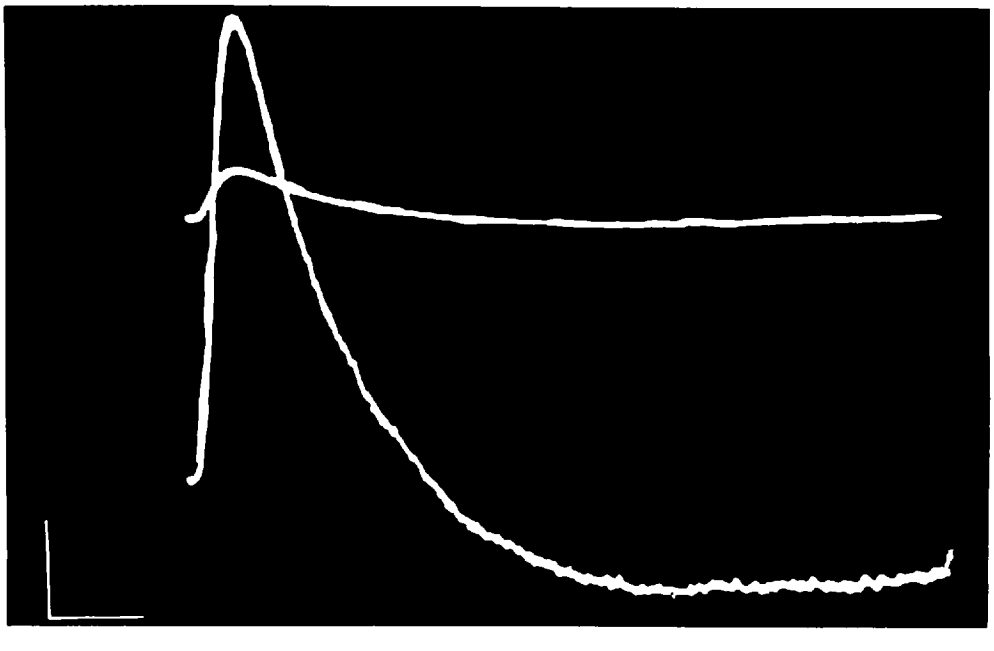


Fig. 7.5

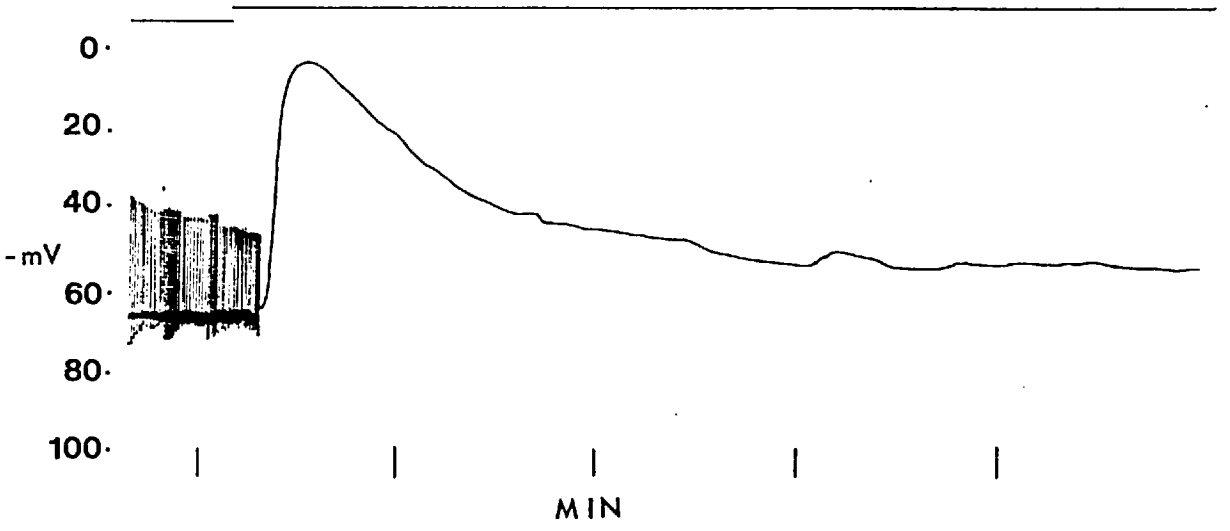
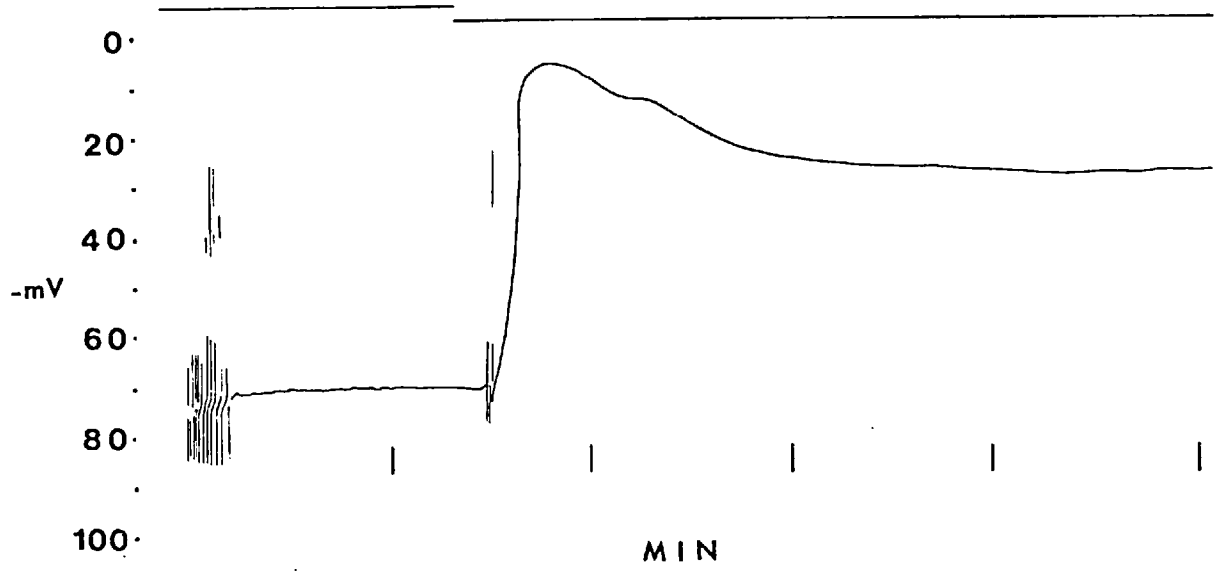


Fig. 7.6. Upper and Lower. Effect of decamethonium 100μM on denervated guinea-pig diaphragm in absence of potassium. The depolarization is followed by initial rapid recovery of variable extent. Spike potentials are present in both recordings. Broken top line indicates changeover to drug solution. Ordinate in mV. Abscissa in minutes.



addition of 100μM decamthonium produced prompt depolarization (range -1mV to -31MV, median -9mV). Rapid initial recovery occurred (Fig. 7.6), and after exposure of 4 min the median potential was -38mV (range -27, -50mV).

The long term effects of 100μM decamthonium in potassium free Krebs saline were measured by repeated sampling (Fig. 7.3, clear circles). The membrane potential of 60 fibres in 5 muscles were measured at 5 min prior to, and at intervals after the addition of the drug. The initial potential was -63mV. After 5 min in the drug solution, the potential was -44.5mV (median of 60, 95% confidence limits -42mV, -49mV). The potential showed very little recovery after 5 min. At 60 min the median membrane potential was -48mV (limits -46mV, -52mV), and at 180 min the potential was -44mV (limits -45mV, -41mV). It can be seen from Fig. 7.3 that the recovery which occurred in standard saline from 5 - 90 min was largely abolished in potassium-free saline.

100μM decamthonium with ouabain

The response of denervated guinea-pig diaphragm to 100μM decamthonium with 100μM ouabain was examined by both continuous recordings and repeated sampling.

Continuous recordings indicate that the amount of depolarization was variable. In two traces (Fig. 7.7), the depolarizations were from -59mV to -49mV and from -56mV to -20mV. The upper record (Fig. 7.7) indicates that the potential was -27mV at 50 min. The failure to show recovery of potential was confirmed by repeated sampling. After 3 hrs in 100μM decamethonium and 100μM ouabain, the median membrane potentials of 125 fibres from 5 muscles was -16mV (95% confidence limits -13mV, -16mV).

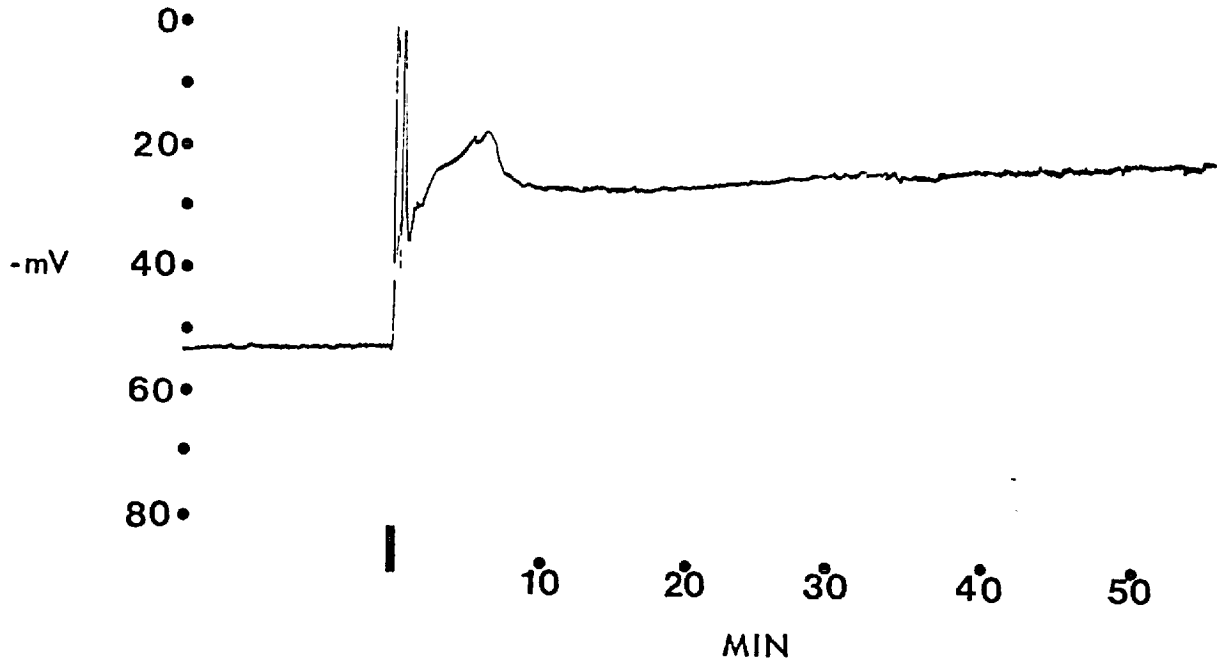


Fig. 7.7. Depolarization to 100μM decamethonium and 100μM ouabain is denervated guinea-pig. Upper trace: depolarization is well maintained. Initial recording may be due to displacement and return of electrodes. Ordinate in mV, abscissa in min. Bar indicates changeover to drug solution.

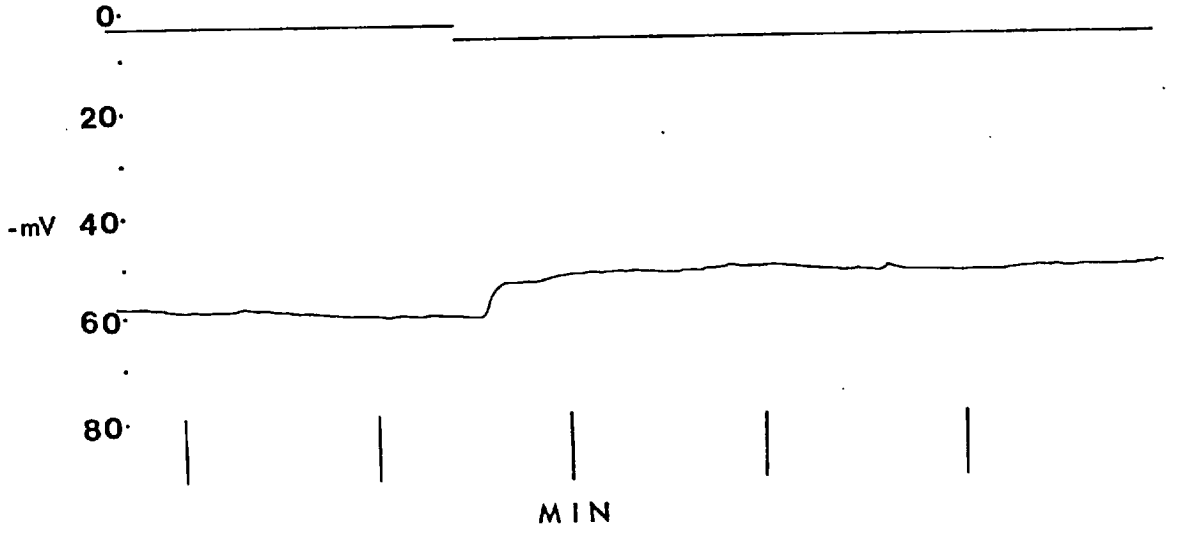


Fig. 7.7. Lower trace: as above. Initial depolarization is small in this fibre. Top broken line indicates changeover to drug solution.

Summary Chapter VII

1. Resting potentials in normal diaphragm of the guinea-pig were -76mV (125 fibres from 5 muscles, 95% limits -75, -77mV). In muscle denervated 14-24 days the values were -60mV (150 fibres from 6 muscles, limits -56, -60).

2. In denervated muscles the decamethonium 100μM produced initial depolarization of 31mV (range 10-47mV in 11 fibres), and some initial recovery of potential. This was followed by spontaneous recovery of membrane potential in the presence of the drug, with hyperpolarization at 90 min.

3. In solution without potassium, decamethonium 100μM produced initial depolarization of 49mV (6 fibres, range 36-61mV), with some initial recovery. The prolonged phase of recovery was not found and the fibres remained depolarized.

4. Recovery of the membrane potential was not found when ouabain 100μM was added with decamethonium 100μM.

5. The results give some further support to the concept that the recovery of potential in the presence of decamethonium is partly attributable to the action of an ionic pump.

CHAPTER VIII DISCUSSION

Important points which derive from the results are considered in the final chapter.

Identification of the end-plate region

Liley (1956) noted the paucity of"active spots"where inverted miniature end-plate potentials could be recorded in rat diaphragm muscle. Auerbach and Betz (1971) noted that the fine terminations of the phrenic nerve in the same preparation were not an adequate guide for precise location of end-plate regions. The visual identification of end-plates in living preparations has been achieved by use of Nomarski interference microscopy (Kuffler and Yoshikami 1975; Dreyer, Muller, Peper and Sterz, 1976). But due to the thickness of the tissue, this technique has not proved feasible on diaphragm muscle (Dreyer et al., 1976). The ability to identify end-plate regions by visual means was needed in the present series of experiments for long-term recordings by repeated sampling at a time when end-plate potentials had been eliminated by the action of depolarizing drugs.

Dark spots appear on the finer filaments of the phrenic nerves innervating the rat diaphragm. These were first observed when polarizing filters were used. When the muscles were mounted in the vertical bath, nerve axons appeared to course through the tissue and end on a slight black pin-head on the muscle. When electrodes were inserted at these spots, miniature end-plate potentials with very fast rise times were found. Later it was found that these spots could also be identified on horizontally mounted preparations, and a more detailed investigation with cholinesterase staining was undertaken. Spots which had been identified with polarizing filters as likely end-plate regions were repeatedly demonstrated to contain cholinesterase (Fig. 3.6, 3.7).

Intracellular recordings from 67 of these spots in 5 rats with the microelectrodes carefully positioned showed all to be associated with miniature end-plate potentials and most with inverted miniature end-plate potentials, characteristic of "active spots." It was therefore concluded that whatever the nature of the structures identified by polarizing filters, the method was a practical guide to the location of end-plate regions in rat diaphragm.

The physical explanation for the use of polarizing filters in distinguishing some end-plate regions, or structures associated with these regions is uncertain. These muscles had a thickness of several hundred micra and at no time did the spots appear as white dots against a dark background. Muscle fibres contain anisotropic and isotropic bands (A and I bands), and the appearances seen in living muscle by the use of polarizing filters may be analogous to the effects produced when thin sections of minerals (usually 30μm) are viewed in the polarizing microscope (see Hurlbut & Klein, 1977). Under crossed polarizers anisotropic minerals display cleavage planes and also distinct colours and patterns which change in varying the orientation and thickness of the sample, and similar appearances were seen in diaphragm muscle. In composite minerals grains of differing composition may be distinguished by the use of polarizers, and Cox, Price and Harte (1974, p.98) have described how certain grains which are isotropic may appear black with almost no transmission of light when embedded in an anisotropic matrix. In skeletal muscle the filaments present a succession of anisotropic bands while the end-plate region is free of filaments: the isotropic end-plate region embedded in the muscle fibre presumably acts like the isotropic grains which have been studied by mineralogists. Whatever the

physical explanation for their differentiation, the spots were a practical aid in locating end-plate regions and they could not be observed without the aid of both a polarizer and an analyzer filter.

Miniature end-plate potentials

The rise time of focally recorded rat diaphragm miniature end-plate potentials was 0.45 m sec (median of 12 active sites, range 0.3 m sec to 1.5 m sec). This value is consistent with that obtained by Head (1975) in cat tenuissimus muscle, and similar values were obtained in rat muscle with each of the amplifiers which were used. However, this value does differ from the 1 - 2 m sec rise-time previously reported in rat diaphragm (Liley, 1956; Miledi, 1960). Auerbach & Betz (1971) point out that recordings made some distance from the end-plate will produce distortion due to the cable properties of the fibres, and they found in practice that end-plates in rat diaphragm could not be routinely located by following the small nerve branches on the surface of the muscle. It is possible that the methods used in the present study, which were developed after much trial, give a more precise means of localisation.

When potassium-free Krebs saline was used there was a consistent and marked reduction in the rise time of the miniature end-potentials to 0.15 m sec (median of 6 active sites, range 0.1 m sec to 0.24 m sec). Since all recordings were made in hyperpolarizing fibres, it is not possible to determine whether this effect is due to the lack of extracellular potassium or the increased membrane potential. However it can be demonstrated that the increased rate of depolarization is not due to the elimination of sodium pump activity since 100μM ouabain had little effect of the shape of miniature end-plate potentials (Fig. 4.7).

Spontaneous depolarizing potentials

Fibrillation potentials have been defined as spontaneous action potentials appearing in chronically denervated mammalian skeletal muscle. These potentials are considered to occur in vitro only when the muscle is mounted in organ culture or adequately perfused on both sides in bathing fluid (Belmar & Eyzaguirre, 1966; Smith & Thesleff, 1976). According to Grampp, Harris and Thesleff (1972) fibrillation potentials can be distinguished from action potentials in denervated muscle in that the former are sensitive to tetrodotoxin while the latter are not. In a few cases the records show that depolarizing potentials were present with 0.1μM tetrodotoxin, but this test was not employed in all cases.

In the studies on denervated guinea-pig diaphragm, it was observed in the horizontal bath that most muscles exhibited spontaneous depolarizing potentials. No previous reference to such events in this preparation have been noted. Further in this particular bath only the superficial surface had been considered to be adequately perfused.

These spontaneous depolarizations have several characteristics associated with fibrillation potentials. Unlike action potentials, the spikes of these potentials do not always overshoot and ~~have~~ a positive voltage; they were only observed in muscles which had been denervated 2 to 3 weeks; and they had the general appearance associated with fibrillation potentials (Smith & Thesleff, 1976). However, they were not associated with a particular area of the muscle, whereas fibrillation potentials have been (Belmar and Eyzaguirre, 1966). Further no experiments were conducted to distinguish them from degenerate action potentials induced by injury potentials or excessive stretching of the

muscle fibres.

Prolonged Recording

When these experiments were planned it seemed unlikely that continuous recordings from single fibres could be made for long periods of time. The alternative method was to measure the membrane potentials from many fibres at pre-selected intervals. This also had the advantage of ensuring that all fibres types were sampled, rather than just the thicker cells from which continuous recordings are much easier. The major difficulty with multiple sampling is to ensure that the majority of recordings are taken from chemosensitive areas. While this is relatively easy in denervated muscle no method had previously been developed to perform this task in innervated diaphragm muscle. In the course of measuring fibres diameters with polarizing lenses, small spots were observed from which miniature end-plate potentials could always be recorded. The phenomenon was investigated and subsequently exploited as an aid in the multiple sampling experiments.

Multiple sampling does not obviate the need for continuous recording, although it lessens the risk of obtaining biased results. Multiple sampling cannot be used to follow the initial effects of drug action, and the traces from a single fibre appear to give reliable results of the changeover. Thus both methods of gathering data were used and compared where possible.

Effect of potassium-free solution

A proportion of the resting potential of muscle fibres may be contributed by an electrogenic sodium-potassium pump. There are two methods by which this contribution may be unmasked. One may either add a glycoside such as ouabain to the bathing solution, or one may

remove the potassium from it. The potential that then remains is presumably due to a diffusion potential, and any depolarization which is produced is evidence of a direct contribution of the pump (Thomas 1972).

Either method has associated with it certain additional effects which might make analysis difficult. Some studies indicate that 100μM ouabain increases the sodium permeability (Rogus and Zierler, 1973; Robbins, 1977). Robbins reported that 1mM ouabain was required to prevent the active exchange of sodium and potassium. If the direct contribution of the pump to the membrane potential is eliminated, potassium-free Krebs' saline might then be expected to produce a hyperpolarization due to the increased electrochemical gradient. The rate of onset for this hyperpolarization would be governed by the degree of anomalous rectification (Hodgkin and Horowicz, 1959). The unwanted effects of the two methods used to block the pump are quite different. Increased sodium permeability induced by ouabain might be expected to continue to depolarize the cell after eliminating the electrogenic component. Potassium-free solution would eventually be expected to hyperpolarize all fibres, and might mask small electrogenic effects.

In the experiments reported above all the fibres depolarized by about 10mV when 100μM ouabain was applied. When 1mM ouabain was used there was a steady loss of potential which may be due to increased sodium permeability or to loss of potassium. In 5mM potassium Krebs' saline, the membrane potentials appeared to be symmetrically distributed with a mean of -72mV. When the standard Krebs solution was replaced by potassium-free saline, the membrane potentials became bimodally distributed, the means of the two populations being -63mV and -93mV (Fig. 4.4). This appears to show that rat diaphragm contains two types of fibres.

Since both ouabain and potassium-free solution depolarized some fibres by about 10mV, it is concluded that in these fibres a proportion of the resting potential is contributed by an electrogenic sodium-potassium pump. This value of 10mV is in agreement with the potential predicted by Thomas (1972) for a resting membrane with active exchange of sodium for potassium and a pump ratio of 3:2.

In potassium-free Krebs' saline Hall, Hilton and West (1972) found that rat diaphragm muscle hyperpolarized. Den Hartog and Mooij (1976) used chloride-free saline and found that removal of potassium produced an immediate depolarization. This difference in results may possibly be due to the different fibres sampled by each investigator. The proportion of each type may vary. The amount of hyperpolarization observed would be expected to increase with time. In the study by Hall et al (1972), membrane potentials were measured at 10 min intervals which may have masked the depolarizing action of potassium free solution.

Robbins (1977) concluded that the sodium pump does not directly contribute to the resting potential in the rat extensor digitorum longus muscle. Akaike (1975) found an electrogenic pump effect in sodium-loaded rat soleus muscle. The rat extensor digitorum is a fast muscle and the soleus is a slow muscle. The bimodal distribution of membrane potentials in potassium-free Krebs' saline may be due to the rat diaphragm muscle containing both types of fibres (Padykula and Gauthier, 1970).

Limited depolarization in rat diaphragm muscle

Application of carbachol to the end-plate region of frog sartorius muscle causes a depolarization to -17mV (Johnson and Parsons 1972). Zaimis and Head 1976) report that 1μM decamethonium causes a depolarization to -30mV in cat muscle. Decamethonium (100μM) causes a similar depolarization in denervated guinea-pig diaphragm muscle (Fig. 7.3) and in some cases peak depolarization reached -9mV (Fig. 7.2 lower).

However, in rat diaphragm muscle the extent of depolarization is limited. In 2.5mM potassium both solution, 100μM decamethonium caused a depolarization to -60mV (Thesleff, 1955b, 1956). In the present study in which 5mM potassium was used, all three transmitter analogues caused depolarization which was close to -56mV (Fig. 5.3, 5.10, 6.5). The extent of depolarization has been shown to be limited and this confirms Thesleff's observations.

There are several possible explanations for this limited response. The end-plate equilibrium potential in frog muscle is -15mV (see Ginsborg and Jenkinson 1976) and it may be, although unlikely, that the value in rat diaphragm is much more negative. Magazanik and Potapova (1969) found an equilibrium potential of -15mV in the extrajunctional receptors of denervated rat diaphragm. Ritchie and Fambrough (1975) found a similar value for acetylcholine receptors in cultured rat myotubes. Ginsborg and Hirst (1972) considered that an end-plate equilibrium potential of -15mV was consistent with the observed mean height of miniature end-plate potentials in rat diaphragm muscle. Mallart and Trautmann

(1973) did find that in denervated frog muscle, the equilibrium potential of extrajunctional receptors was -35mV. However, this latter finding has yet to be confirmed (Ginsborg and Jenkinson, 1976) and should be treated with caution.

Another possible explanation for the limited depolarization is that there may be relatively few receptors in rat diaphragm muscle. Liley (1956) noted that there were fewer active spots in this preparation than in frog sartorius muscle. This was confirmed by Auerbach and Betz (1971) who noted the relative difficulty of finding miniature end-plate potentials in rat diaphragm. Alph-bungarotoxin binding studies have indicated that there may be more end-plate receptors in frog sartorius than in rat diaphragm muscle. However, there is a wide variation in results reported by different authors, and a firm conclusion cannot as yet be reached (Ginsborg and Jenkinson, 1976).

A third possibility is that due to some peculiar geometry of the end-plate region, bath applied depolarizing drugs may not reach the receptor region rapidly enough to prevent the onset of inactivating processes, so that equilibrium with the drug would not be reached. The inactivating processes might include a decrease in the voltage-sensitive sodium conductance, or an increase in the voltage sensitive potassium conductance (see Aidley 1971 for reviews). The electrogenic sodium pump described elsewhere may reduce a drug's depolarization effect. The rapid desensitization which can be shown by iontophoresis studies might prevent full depolarization (Katz and Thesleff 1957).

Spontaneous recovery of potential - two processes

Following depolarization due to the application of transmitter analogues, complete recovery occurred in the presence of 100μM carbachol (Fig. 5.2, 5.3), and decamethonium (Fig. 6.5), and substantial recovery in 100μM suberyldicholine (Fig. 5.10). In denervated guinea-pig diaphragm muscle the recovery was such that there was a small, transient, but consistently observed hyperpolarization which occurred after 90 min exposure to decamethonium.

Repolarization appeared to occur in two phases: a limited rapid recovery which occurred within the first few minutes of the drugs' application, and a slow recovery which might, in some cases, require hours to complete (Fig. 5.3; 6.5; 7.3). The rapid phase was not always present. It was most frequently observed when decamethonium was used (Fig. 6.2 lower), and occasionally occurred when carbachol was applied (Fig. 5.1 lower). This rapid recovery however was never observed when suberyldicholine was used.

The rapid phase also appears to be sensitive to the concentration of the drug and to the ionic constitution of the bath solutions. This phase was never seen in decamethonium concentrations below 100μM (Fig. 6.1; 6.2 upper). When 100μM or 3mM decamethonium was applied in the absence of potassium, the initial rapid phase of recovery was present and appeared to be enhanced with increased drug concentration (Fig. 6.6; 6.8). The rapid phase of recovery resembles the effects of iontophoretically applied drugs. With this technique complete desensitization can occur within a minute of the drug's application (Katz and Thesleff, 1957). Like the initial

rapid phase of recovery, the desensitization produced by iontophoresis also depends upon the amount of the drug applied (Magazanik and Vyskocil 1975).

The rapid phase occurred most frequently with decamethonium and this may be related to the same mechanism which causes it to act as a partial agonist on frog sartorius muscle (del Castillo and Katz, 1957). Or it may be related to the proposal that some types of desensitization are due to occlusion of the ionic channels which are activated by the receptors (Creese, Franklin and Mitchell, 1976).

Another possible explanation for the rapid phase of recovery is that it is due to dissociation of agonist from receptors (Rang and Ritter, 1970). According to these authors this will be true for any receptor model of drug action. Hence the rapid phase of recovery observed under the above conditions would be related to the establishment of equilibrium. The chief difficulty with such an explanation is that the initial phase, when it occurs, requires 1 to 3 min for completion, which is probably too slow for such a process.

The initial rapid recovery of potential might also be related to the process of accommodation in which depolarization in some tissues leads to increased potassium and decreased sodium conductance (Aidley, 1971). This might be expected to cause the greatest recovery of membrane potential when the driving force for potassium is increased, and this prediction is consistent with the effects observed in the absence of potassium. However accommodation cannot easily account for the initial effects produced by different drugs or concentrations.

If a third receptor state, the desensitized receptor, exists in frog muscle, and also in rat and denervated guinea-pig diaphragm muscle, then various qualities could be ascribed to such a receptor. In the rodent preparation, it could be more sensitive to one drug than another, or it could be more effective under one set of ionic conditions than another, and this might account for part of the recovery of membrane potential.

It may be that desensitization observed with iontophoresis is related to the initial recovery observed with 100μM carbachol, and 100μM and 3mM decamethonium. It is not possible in the present study to determine the cause of this initial rapid partial recovery. It may be that more than one of the processes discussed here are involved in both types of desensitization.

When 100μM drug concentrations were used, the second phase of recovery could be prevented by the addition of ouabain, or by the exclusion of potassium. Further when the second phase of recovery is complete, the addition of ouabain will cause the membrane potential to return to its depolarized state (Fig. 5.8). In denervated guinea-pig hyperpolarization occurs following recovery (Fig. 7.3). In innervated rat diaphragm muscle, a portion of the membrane potential in some fibres appears to be maintained by an electrogenic sodium pump (Fig. 4.1; table 4.1). All this evidence is consistent with the hypothesis that the recovery of membrane potential in the presence of moderate drug concentrations is due to an electrogenic pump.

As reviewed in the introduction, decamethonium in μM concentrations produces a depolarizing neuromuscular block in some muscles and a block which changes from depolarizing to competitive (curare-like) in other muscles (Jewell and Zaimis, 1954; Jenden, 1955). It would be pleasant to think that the recovery of potential by means of an electrogenic pump might account for the transition of one type of block into the other. A reduction in temperature might be expected to slow the sodium pump, and it has been demonstrated that cooling prevents the change of a depolarizing block to a competitive block (Bigland, Goetzee, Maclagan and Zaimis, 1958). The characteristics associated with a "dual block" are consistent with the hypothesis that the recovery of membrane potential is due to an electrogenic sodium pump. The difference then between a muscle which responds to depolarizing drugs by producing a dual block and those which produce a depolarizing block would be due to the nature of their sodium-potassium pumps.

When 3mM decamethonium is applied, full recovery of potential occurred in the presence of ouabain and substantial recovery occurred in potassium-free solution (Fig. 6.7). Creese and England (1970) found that high concentrations of decamethonium produced a decrease in the uptake as measured by the clearance. They further noted that the permeability of decamethonium as measured by the clearance was similar to that of sodium. It may be concluded that a likely mechanism for recovery of potential in the presence of decamethonium in the mM range is due to blocking of sodium channels by the charged organic cation, by competition between sodium and the drug for sites in the ionic channels. In the absence of potassium complete recovery

of potential with 3mM decamethonium did not occur in 1 hour (Fig.6.7). This might be attributable to the reduction in potassium conductance of the extrajunctional electrosensitive channels which could occur with zero potassium,and this prccess could delay the recovery by a mechanism which is similar to that of anomalous rectification (Hodgkin & Horowitz, 1959).

The hypothesis for channel block differs somewhat from occlusion theories proposed by Magazanik and Vyskocil (1970) and by Adams (1975). In the first model, occlusion is due to the absorption of calcium or magnesium ions onto the walls of the ionic channels. Adams proposes on the other hand, that quaternary ammonium ions enter and block the ionic channel. In the proposed model there is a saturable flux across the membrane which rises with concentration to a steady rate and which competes with sodium ions for sites in the ionic channels.

In this monograph it is proposed that in addition to mechanisms which are found in other preparations, there may exist two processes which might account for the phenomenon of desensitization in innervated rat diaphragm muscle. In the 100μM range, desensitization to transmitter analogues could be due to a potassium and ouabain-sensitive sodium pump. Recovery of potential in the presence of decamethonium in the mM range is considered to be due to competition between the drug and sodium in ionic channels (Creese, Franklin & Mitchell, 1976).

REFERENCES

Adams, P. R. (1974). The mechanism by which amylobarbitone and thiopentone block the end-plate response to nictonic agonists. J.Physiol. 241, 41-42P.

Adams, P. R. (1975). A study of desensitization using voltage clamp. Pflügers. Arch. Ges. Physiol. 360, 135-144.

Aidley, D. J. (1971). The Physiology of Excitable Cells. Cambridge: Cambridge University Press.

Akaike, N. (1975). Activation of electrogenic sodium pump in mammalian skeletal muscle by external cations. Pflügers Arch. 355(4), 281-90.

Albuquerque, E. & McIsaac, R. J. (1970). Fast and slow mammalian muscles after denervation. Expl. Neurol. 26, 183-202.

Anderson, C.R. & Stevens, C.F. (1973). Voltage clamp analysis of acetylcholine. J. Physiol. (Lond). 235, 655-691.

Auerbach, A. & Betz, W. Does curare affect transmitter release? J. Physiol. (Lond). 213, 691-705.

Axelson, J. & Thesleff, S. (1958). The "desensitizing" effect of acetylcholine on the mammalian motor end-plate. Acta. Physiol. Scand. 43, 15-26.

Bates, J., Rowlands, S. & Sirs, J. A. (1973). Influence of diffusion on dispersion of indicators in blood flow. J. Appl. Physiol. 34, 866-872.

Belmar, J. & Eyzaguirre, C. (1966). Pacemaker site of fibrillation potentials in denervated mammalian muscle. J. Neurophysiol. 29, 425-441.

Beranek, R. & Vyskocil, F. (1967). The action of tubocurarine and atropine on the normal and denervated rat diaphragm. J. Physiol. 188, 53-66.

Bigland, B., Goetzee, B., Maclagan, J. & Zaimis, E. (1958). The effect of lowered muscle temperature on the action of neuromuscular blocking drugs. J. Physiol. 141, 425-434.

Bigland, B. & Zaimis, E. (1958). Factors influencing limb temperature during experiments in skeletal muscle. J. Physiol. 141, 420-424.

Bray, J.J., Hawken, M.J., Hubbard, J.I., Pockett, S. & Wilson, L. (1976). The membrane potential of rat diaphragm muscle fibres and the effect of denervation. J. Physiol. 255, 651-667.

Brockes, J.P. & Hall, Z.W. (1975). Acetylcholine receptors in normal and denervated rat diaphragm muscle II. Comparison of junctional and extrajunctional receptors. Biochemistry. 14, 2100-2106.

Buller, A. J. (1972). The neural control of some characteristics of skeletal muscle. In Modern Trends in Physiology No. 1. Ed. Downman, C. B. B. London: Butterworth & Co.

Burgen, A. S. V. (1966). The drug-receptor complex. J. Pharm. Pharmac. 18, 137-149.

Burgen, A. S. V. & Mitchell, J. F. (1968). Gaddum's Pharmacology 6th Edn. London: Oxford University Press.

Burns, B. D. & Paton, W. D. M. (1951). Depolarization of the motor end-plate by decamethonium and acetylcholine. J. Physiol. 115, 41-73.

Chang, C. C., Chen, T. F. & Chuang, S. T. (1973). N, O-di and N, N,O-tri [^{3}H] acetyl α-bungarotoxins as specific labelling agents of cholinergic receptors. Br. J. Pharmac. 47, 147-160.

Close, R. (1967). Properties of motor units in fast and slow skeletal muscles of the rat. J. Physiol. 193, 45-55.

Colquhoun, D. (1971). Lectures on Biostatistics. Oxford: Clarendon Press.

Colquhoun, D. (1975). Mechanisms of drug action at the voluntary muscle end-plate. Ann. Rev. Pharmacol. 15, 307-325.

Cookson, J.C. & Paton, W. D. (1969). Mechanisms of neuromuscular block. A review article. Anaesthesia. 24(3), 395-416.

Cox, K. G., Price, N. B. & Harte, B. (1974). The Practical Study of Minerals and Rocks, Revised Edition. London: McGraw-Hill Book Co. (U.K.) Ltd.

Creese, R., El-Shafie, A. L. & Vrbova, G. (1968). Sodium movements in denervated muscle and the effects of antimycin A. J. Physiol. 197, 279-294.

Creese, R. & England, J. M. (1970). Decamethonium in depolarized muscle and the effects of tubocurarine. J. Physiol. 210, 345-361.

Creese, R., Franklin, G. I. & Mitchell, L. D. (1976). Two mechanisms for spontaneous recovery from depolarizing drugs in rat muscle. Nature (Lond). 261, 416-417.

Creese, R., Franklin, G. I. & Mitchell, L. D. (1977). Sodium entry in rat diaphragm induced by depolarizing drugs. J. Physiol. 272, 295-316.

Creese, R. & Northover, J. (1961). Maintenance of isolated diaphragm with normal sodium content. J. Physiol. 155, 343-357.

Creese, R., Taylor, D. B. & Case, R. (1971). Labelled decamethonium in denervated skeletal muscle. J. Pharmac. Exp. Ther. 176, 418-422.

Del Castillo, J. & Katz, B. (1955). On the localization of acetylcholine receptors. J. Physiol. 128, 157-181.

Del Castillo, J. & Katz, B. (1956). Localization of active spots within the neuromuscular junction of the frog. J. Physiol. 132, 630-649.

Del Castillo, J. & Katz, B. (1957). A study of currare action with an electrical micro-method. Pro. Roy. Soc. B, 146, 339-356.

Den Hertog, A. & Mooij, J. J. (1976). Potassium induced potential changes in rat diaphragm muscle. Pflügers Arch. 362(1), 69-79.

Dreyer, F., Muller, K. D., Peper, K. & Sterz, R. (1976). The M. Omohyoideus of the mouse as a convenient mammalian muscle preparation. Pflügers Arch. 367, 115-122.

Dreyer, F., Walther, C. & Peper, K. (1976). Junctional and extra-junctional acetylcholine receptors in normal and denervated frog muscle fibres. Pflügers Arch. 366, 1-9.

Dubowitz, V. & Pearse, A. G. E. (1960). A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle. Histochemie. 2, 105-117.

Elmquist, D. & Thesleff, S. (1962). Ideas regarding receptor desensitization at the motor end-plate. Rev. Canada. Biol. 21, 229-234.

Fambrough, D. M., Drachman, D. B. & Satyamurti, S. (1973). Neuromuscular junction in myasthenia gravis: decreased acetylcholine receptors. Science. 182, 293-295.

Fambrough, D. M. & Hartzell, H. C. (1972). Acetylcholine receptors: number and distribution at nueormuscular junctions. Science, 176, 189-191.

Fatt, P. (1950). The electromotive action of acetylcholine at the motor end-plate. J. Physiol. (Lond.). 111, 408-422.

Gaddum, J. H. (1957). Drug antagonism. Pharmacological Reviews. 9, 211-218.

Gauthier, G. F. & Padykula, H. A. (1966). Cytological studies on fibre types in skeletal muscle. A comparative study of the mammalian diaphragm. J. Cell. Biol. 28, 333-354.

Geddes, L. A. (1972). Electrodes and the Measurement of Bioelectrical Events. London: Wiley-Interscience.

Ginsborg, B. L. & Hirst, G. D. S. (1972). The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. J. Physiol. (Lond). 224, 629-645.

Ginsborg, B. L. & Jenkinson, D. J. (1976). Transmission of impulses from nerve to muscle. In Handbook of Experimental Pharmacology Vol. 42. Neuromuscular Junction. Ed. Zaimis, E. New York: Springer-Verlag.

Glynn, I. M. & Karlish, S. J. D. (1975). The sodium pump. Ann. Rev. Physiol. 37, 13-55.

Goldstein, A. (1964). Biostatistics: An introductory text. London: Collier-Macmillan Ltd.

Goodman, L. S. & Gilman, A. (1975). The Pharmacological Basis of Therapeutics. 5th Edn. London: Bailliere Tindall.

Grampp, W., Harris, J. B. & Thesleff, S. (1972). Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221, 743-754.

Günther, P. G. (1952). Die Morphologischen Grundlagen der Bewegungs und Halteleistung (Tetanus und Tonus) des Zwerchfells. Acta Anat. 14, 54-64.

Hall, A. E., Hilton, E. L. & West, D. C. (1972). Neuromuscular transmission in potassium-free Krebs solution. J. Physiol. 226, ~~45-46P.~~ 95P-96P

Hartzell, H. C. & Fambrough, D. M. (1972). Acetylcholine receptors: distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. J. Gen. Physiol. 60, 248-262.

Hartzell, H. C., Kuffler, S. W. & Yoshikami, D. (1975). Postsynaptic potentiation: interaction between quanta of acetylcholine at the skeletal neuromuscular synapse. J. Physiol. 251, 427-463.

Head, S.D. (1975).

Hess, A. & Pillar, G. (1963). Slow fibres in the extraocular muscles of the cat. J. Physiol. 169, 780-798.

Hodgkin, A. L. & Horowicz, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. J. Physiol. (Lond). 148, 127-160.

Hurlbut, C. S. & Klein, C. (1977). Manual of Mineralogy (after James Dana) (Ed. 19), 195-216. New York: John Wiley Co.

Jenden, D. J. (1955). The effect of drugs upon neuromuscular transmission in the isolated guinea pig diaphragm. J. Pharm. Exp. Ther. 114, 398-408.

Jenkinson, D. H. (1960). The antagonism between tubocurarine and substances which depolarized the motor end-plate. J. Physiol. 152, 309-324.

Jenkinson, D. H. & Terrar, D. A. (1973). Influence of chloride ions on changes in membrane potential during prolonged application of carbachol to frog skeletal muscle. Brit. J. Pharmacol. 47, 363-376.

Jewell, P. A. & Zaimis, E. J. (1954). A differentiation between red and white muscle in the cat based on responses to neuromuscular blocking agents. J. Physiol. 124, 417-428.

Johnson, E. W. & Parsons, R. L. (1972). Characteristics of postjunctional carbamycholine receptor activation and inhibition. Amer. J. Physiol. 222, 793-799.

Katz, B. (1966). Nerve, Muscle and Synapse. New York: McGraw-Hill.

Katz, B. & Miledi, R. (1977). The reversal potential at the desensitized end-plate. Proc. R. Soc. Lond. B. 199, 329-334.

Katz, B. & Thesleff, S. (1957). A study of the "desensitization" produced by acetylcholine at the motor end-plate. J. Physiol. 138, 63-80.

Klaus, W., Lullmann, H. & Muscholl, E. (1960). Der Kalium-Flux des Normalen und Denervierten Rattenzwerchfells. Pflügers Arch. Ges. Physiol. 271, 761-775.

Krüger, P. (1949). Die Innervation der Tetanischen und Tonishcen Fasern der Quergestreifen Skeletmuskulatur der Wirbeltiere. Anat. Anz. 97, 169-175.

Krüger, P. & Günther, P. G. (1955). Fasern mit 'Fibrillenstruktur' und Fasern mit 'Felderstruktur' in der Quergestreiften Skeletmuskulatur der Säuger und des Menshen. Z. Ges. Anat. I.Z. Anat. Entw-Gesch. 118, 313-323.

Kuba, K. & Koketsu, K. (1976). Decrease of Na^+ conductance during desensitization of the frog end-plate. Nature (Lond). 262, 504-505.

Kuffler, S. W. & Yoshikami, D. (1975). The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscle: iontophoretic mapping in the micron range. J. Physiol. 244, 703-730.

Lavallee, M., Schanne, O.F. & Herbert, T. (1969). Glass Microelectrodes. London: Wiley-Interscience.

Li, C. (1960). Mechanism of fibrillation potentials in denervated mammalian skeletal muscle. Science. 132, 1889-1890.

Liley, A. W. (1956). An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. 132, 650-666.

Lüllmann, H. (1958) Uber die Konstanz des Memranpotentials bei spontanen Anderungen der Ionengradienten am Normalen und Denervierten Rattenzwerchfell. Pflügers Arch. Ges. Physiol. 267, 188-199.

Magazanik, L. G. & Potapova, T. V. (1969). Equilibrium potentials of the extraysnaptic membrane of denervated muscle with change in the extracellular ionic medium. Biofizika. 14, 658-661.

Magazanik, L. G. & Vyskocil, F. (1970). Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. J. Physiol. 210, 507-518.

Magazanik, L. G. & Vyskocil, F. (1973). Desensitization at the motor end-plate. In Rang, H. P. (Ed): Drug Receptors. 105-119. Macmillan.

Magazanik, L. G. & Vyskocil, F. (1975). The effect of temperature on desensitization kinetics at the post-synaptic membrane of the frog muscle fibre. J. Physiol. 249, 285-300.

Mallart, A. & Trautmann, A. (1973). Ionic properties of the neuromuscular junction of the frog: effects of denervation and pH. J. Physiol. 234, 553-567.

Manthey, A. A. (1966). The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. J. Gen. Physiol. 49, 963-976.

Manthey, A. A. (1970). Further studies of the effect of calcium on the time course of the action of carbamylcholine at the neuromuscular junction. J. Gen. Physiol. 56, 407-419.

Manthey, A. A. (1972). The antagonistic effects of calcium and potassium on the time course of action of carbamylcholine at the neuromuscular junction. J. Membrane Biol. 9, 319-340.

Martin, A. R. (1955). A further study of the statistical composition of the end-plate potential. J. Physiol. 130, 114-122.

Miledi, R. (1960). Junctional and extra-junctionl acetylcholine receptors in skeletal muscle fibres. J. Physiol. 151, 24-30.

Miledi, R. & Potter, L. T. (1971). Acetylcholine receptors in muscle fibres. Nature (Lond). 233, 599-603.

Muscholl, E. (1957). Elektrophysiologische Untersuchung der Einzelnen Faseranteile des Isolierten Rattenzwerchfelles. Pflügers Archiv. 264, 467-483.

McArdle, J. J. & Albuquerque, E. (1975). Effects of ouabain on denervated and dystrophic muscles of the mouse. Expl. Neurol. 47, 353-356.

Nastuk, W. L. (1971). Mechanisms of neuromuscular blockade. Ann. N.Y. Acad. Sci. 183, 171-182.

Nastuk, W. L. & Liu, J. H. (1966). Muscle postjunctional membrane: changes in chemosensitivity produced by calcium. Science. 154, 266-267.

Nastuk, W. L., Manthey, A. A. & Gissen, A. J. (1966). Activation and inactivation of postjunctional membrane receptors. Ann. N.Y. Acad. Sci. 137, 999-1014.

Nastuk, R. L. & Parsons, W. L. (1970). Factors in the inactivation of postjunctional membrane. J. Gen. Physiol. 56, 218-249.

Nedergaard, O. A. & Taylor, D. B. (1966). The uptake by skeletal muscle of tritium labelled decamethonium and dimethyltubocurarine. Experientia. 22, 521-522.

Padykula, H. A. & Gauthier, G. F. (1970). The ultrastructure of the neuromuscular junction of mammalian red, white and intermediate skeletal muscle fibres. J. Cell. Biol. 46, 27-41.

Parsons, R. L. (1969). Changes in postjunctional receptors with decamethonium and carbamylcholine. Am. J. Physiol. 217, 805-811.

Paton, W. D. M. (1961). A theory of drug action based on the rate of drug-receptor combination. Pro. Roy. Soc. B. 154, 21-69.

Peper, K., Dreyer, F. & Muller, K. D. (1975). Analysis of cooperativity of drug-receptor interaction by quantitative iontophoresis of frog motor end-plates. Cold Spring Harbour Symp. Quant. Biol. 40, 187-92.

Peper, K. & McMahan, U.J. (1972). Distribution of acetylcholine receptors in the vicinity of nerve terminals on skeletal muscle of the frog. Proc. Roy. Soc. B. 181, 431-440.

Rang, H. P. (1971). Drug receptors and their function. Nature (Lond). 231, 91-96.

Rang, H. P. & Ritter, J. M. (1970). On the mechanism of desensitization at cholinergic receptors. Molec. Pharmacol. 6, 357-382.

Ritchie, J. M. & Fambrough, D. M. (19). Acetylcholine receptors in cultured rat myotubes. J. Gen. Physiol. 65, 751-767.

Robbins, N. (1977). Cation movements in normal and short term denervated rat fast twitch muscle. J. Physiol. 271, 605-624.

Rogus, E. & Zierler, K. L. (1973). Sodium and water contents of sarcoplasm and sarcoplasmic reticulum in rat skeletal muscle: effects of anisotonic media, ouabain and external sodium. J. Physiol. 233, 227-270.

Smith, J. W. & Thesleff, S. (1976). Spontaneous activity in denervated mouse. J. Physiol. 257, 171-186.

Streter, F. A. & Woo, G. (1963). Cell water, sodium and potassium in red and white mammalian muscles. Am. J. Physiol. 205, 1290-1294.

Stewart, D. M. (1955). Changes in protein composition of muscles of the rat in hypertrophy and atrophy. Biochem. J. 59, 553-558.

Takeuchi, N. (1963). Effect of calcium on the conductance change of the end-plate membrane during the action of transmitter. J. Physiol. 167, 141-155.

Taylor, D. B. & Nedergaard, O. A. (1965). Relation between structure and action of quaternary ammonium neuromuscular blocking agents. Physiol. Rev. 45, 523-554.

Thesleff, S. (1955a). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. Acta. Physiol. Scand. 34, 218-231.

Thesleff, S. (1955b). The effects of acetylcholine, decamethonium and succinylcholine on the neuromuscular transmission in the rat. Acta. Physiol. Scand. 34, 386-392.

Thesleff, S. (1956). A further analysis of the neuromuscular block caused by acetylcholine. Acta. Physiol. Scand. 37, 330-334.

Thesleff, S. & Ward, M.R. (1975). Studies on the mechanism of fibrillation potentials in denervated muscle. J. Physiol. 244, 313-323.

Thomas, R. C. (1972). Electrogenic sodium pump in nerve and muscle cells. Physiol. Rev. 52, 563-594.

Valet, G., Hofmann, H. & Ruhenstroth-Bauer, G. (1976). The computer analysis of volume distribution curves. Demonstration of two erythrocyte populations of different size in the young guinea-pig and analysis of the mechanism of immune lysis of cells by antibody and complement. J. Histochem. Cytochem. 24, 231-246.

Vyskocil, F. & Magazanik, L.G. (1972). The desensitization of postjunctional muscle membrane after intracellular application of membrane stabilizers and snake venom polypeptides. Brain Res. 48, 417-419.

Waud, D.R. (1968). Pharmacological receptors. Pharmacol. Rev. 20, 49-88.

Waud, D. R. (1969). On the measurement of the affinity of partial agonists for receptors. J. Pharmacol. Exp. Ther. 170, 117-122.

Yellin, H. (1974). Changes in fiber types of the hypertophying denervated hemidiaphragm. Expl. Neurol. 42, 412-428.

Zaimis, E. (1976). Handbook of Experimental Pharmacology Vol. 42 Neuromuscular Junction. New York: Springer-Verlag.

Zaimis, E. (1954). Transmission and block at the motor end-plate and in autonomic ganglia. Pharmac. Rev. 6, 53-57.

Zaimis, E. (1953). Motor end-plate differences as a determining factor in the mode of action of neuromuscular blocking substances. J. Physiol. 122, 238-251.

Zaimis, E. & Head, S. (1976). Depolarizing neuromuscular blocking drugs. In Handbook of Experimental Pharmacology Vol. 42. Neuromuscular Junction. Ed. Zaimis, E. New York: Springer-Verlag.

Zolovick, A. J., Norman, R. L. & Fedde, M. R. (1970). Membrane constants of muscle fibers of rat diaphragm. Am. J. Physiol. 219, 654-657.

J. Physiol. (1977), 272, *pp.* 295–316
With 11 *text-figures*
Printed in Great Britain

SODIUM ENTRY IN RAT DIAPHRAGM INDUCED BY DEPOLARIZING DRUGS

BY R. CREESE, G. I. FRANKLIN* AND L. D. MITCHELL

From the Department of Physiology,
St Mary's Hospital Medical School, London W2 1PG

(*Received 2 December 1976*)

SUMMARY

1. An increased uptake of labelled sodium was found in the end-plate region of rat diaphragm following brief exposure to solution containing ^{24}Na plus carbachol (100 μM), with a wash in inactive saline. Tetrodotoxin (0·1 μM) was also present. Comparable results were obtained with decamethonium and suberyldicholine.

2. With carbachol (100 μM) the influx of labelled sodium at the end-plate region was increased by a factor of at least three as compared with that at the end of the fibres.

3. After entry the labelled sodium spread along the fibres with an apparent diffusion coefficient which was half that expected in the external solution.

4. The dose–response curve for the effect of carbachol gave a half-maximal value of 72 μM.

5. In muscles depolarized by potassium methyl sulphate the effect of carbachol on the entry of sodium was reduced although demonstrable.

6. The entry of labelled sodium at the end-plate was maintained during prolonged exposure to carbachol (100 μM) or decamethonium (100 μM).

7. The rate of entry of ^{24}Na obtained with carbachol, after corrections for the wash, was estimated as $1{\cdot}5 \times 10^3$ ions channel^{-1} sec^{-1}, measured over a period of 15 sec.

8. Labelled decamethonium and labelled carbachol also accumulated at the end-plate region. After extrapolation to allow for the effects of the wash the entry of decamethonium when expressed as a clearance (pl. mg^{-1} sec^{-1}) was comparable to that of sodium, as expected if decamethonium and sodium enter through the same channels.

* Present address: Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1.

INTRODUCTION

Acetylcholine and other depolarizing substances are believed to act on skeletal muscle by inducing channels to open for the passage of sodium and other cations (Takeuchi & Takeuchi, 1960; Ginsborg & Jenkinson, 1976). If the current is mainly carried by sodium ions then it should be possible to record this process by the use of labelled isotopes. Methods have been found to detect the extra influx of sodium at the end-plate region (Creese & Mitchell, 1975; Creese, Franklin, Humphrey & Mitchell, 1976), and this has enabled the chemosensitive properties of the end-plate to be studied in terms of the movement of labelled sodium. In the case of decamethonium it is known that there is also an entry of the organic cation in the region of the end-plate (Creese & Maclagan, 1970), and in some cases labelled decamethonium has been used so that the permeabilities to sodium and decamethonium may be compared.

METHODS

Solutions. The physiological saline was that used by Creese & Northover (1961), with, 5 mM-K^+, at 38 °C, gassed with a mixture of 95 % (v/v) oxygen and 5 % (v/v) carbon dioxide. Solutions containing high concentrations of carbachol (up to 100 mM) were prepared by substitution of carbachol chloride in place of sodium chloride.

Solutions with potassium ion greater than 5 mM were prepared by the substitution of $KMeSO_4$ for sodium chloride (Creese & England, 1970). For depolarization of the muscle a solution was used with the following composition (mM): K^+ 141, Na^+ 6, Ca^{2+} 2·5, $MeSO_4^-$ 141, Cl^- 8, HCO_3^- 3: this was gassed with oxygen.

Handling of tissues. Diaphragm muscles from rats of 100 g were rapidly dissected and mounted on holders (Creese & Northover, 1961) and immersed in tubes which contained 10 ml. solution. Tubes and holders were boiled before use. Muscles on their holders were briefly exposed (usually 15–30 sec) to solution containing ^{24}Na plus depolarizing drug (and usually tetrodotoxin 0·1 μM), then washed (usually 3–6 min) in a succession of tubes of inactive solution, the tubes being changed every 30 sec. The diaphragms were then removed, attached to a metal strip, frozen on solid carbon dioxide and stored in a sealed plastic cover at −20 °C. After an interval of 2–24 hr the muscle was again placed in contact with a block of solid carbon dioxide, sliced at intervals of 1 mm from tendon to rib, weighed and the slices were transferred to vials containing 0·5 ml. M-potassium hydroxide in methanol. The slices were dissolved at 70 °C (Creese & England, 1970).

Labelled compounds. $^{24}NaCl$ was obtained from Radiochemical Centre, Amersham, Buckinghamshire. 2·4 mc (2·0 ml. NaCl 0·9 g/dl.) was usually added to give physiological saline with 0·1 mc $ml.^{-1}$. Tubes containing 8 ml. radioactive solution were used for exposure of diaphragm muscles, and the tubes were positioned in a container which was jacketed for control of temperature and surrounded by 15 cm lead for protection.

[^{3}H-methyl]decamethonium chloride (mol. wt. 329) was supplied by the Radiochemical Centre with specific activity 559 mc/m-mole. The aqueous solution (1 mc $ml.^{-1}$, 1·8 mM) was frozen for storage.

Carbamyl(methyl-[^{14}C]choline chloride) was supplied by the Radiochemical Centre with specific activity 13·1 mc/m-mole. The freeze-dried compound was

dissolved in sodium chloride (0·9 g/dl.) to make a solution of 19 mM carbachol, and frozen for storage. The radiochemical purity was checked by ascending paper chromatograms (5 μl. of 19 mM) in a mixture of *n*-butanol : acetic acid : water : ethanol (8 : 1 : 3 : 2), and developed with iodine vapour. The peak of radioactivity corresponded with the spot of unlabelled carbachol, without indication of decomposition products.

Radioactivity. The vials were counted by liquid scintillation with separate channels for the different isotopes, the efficiencies being 27 % for ^{3}H, 12 % for ^{14}C and 40 % for ^{24}Na. Backgrounds were 8 counts min^{-1} for ^{3}H, 30 counts min^{-1} for ^{14}C and 50 counts min^{-1} for ^{24}Na. Corrections were made for background and, in the case of ^{24}Na, for decay. The solutions were counted by addition of 0·01–0·1 ml. to 0·5 ml. methanolic potassium hydroxide in a vial. For experiments involving both ^{24}Na and [^{3}H]decamethonium the ^{24}Na was first counted and, after 10 days, [^{3}H]decamethonium: by this time the count due to ^{24}Na was negligible. In most cases the results were expressed initially as a clearance, being (counts min^{-1} mg^{-1})/(counts min^{-1} $pl.^{-1}$ radioactive solution). This gave the uptake as pl. mg^{-1}, and when multiplied by the sodium concentration, which was usually 0·145 p-mole. $pl.^{-1}$, gave the labelled sodium which remained in the tissue as p-mole. mg^{-1}.

Measurement of total sodium and potassium in muscles. The method was that of Creese (1954).

Resting potentials. The arrangement for measurement of resting potentials with internal electrodes was similar to that used by Creese, Scholes & Whalen (1958). The muscle was vertical, with oxygenation from below, and the resting potentials of superficial fibres were recorded.

Fibre diameters. The muscles were mounted vertically in the bath used for microelectrode recordings. The microscope had an eyepiece and was calibrated by focusing on a reference graticule. Superficial fibres were observed at a magnification of × 32·5 and the diameters were measured.

Drugs. The drugs which were used are listed, with the molecular weight. Carbamylcholine chloride (carbachol: Koch-Light Laboratories, Ltd: 182·6); decamethonium bromide (Burroughs Wellcome Co.: 418·4); suberyldicholine iodide (gift from Dr R. S. Barlow and Dr A. Ungor, University of Edinburgh: 599·8); tetrodotoxin (Sankyo Co. Ltd: 319).

Statistical procedures. The variables which were measured usually had distributions with a positive skew (see below). Medians are given in the text, together with their non-parametric confidence limits (approximately 95 %), obtained from tables (Colquhoun, 1971; Geigy Scientific Tables, 1970). Procedures for the fitting of Gaussian curves and for weighted linear regression are described in an Appendix. Other standard methods are as Finney (1964), or Goldstein (1964).

RESULTS

Sodium entry at junctional region

Results obtained by exposure of diaphragms to labelled sodium for 15 sec followed by a wash for 6 min are shown in Fig. 1. The muscles were removed, frozen and sliced at intervals of 1 mm from tendon to rib, and the histograms give labelled sodium as p-mole.mg^{-1}. Fig. 1*A* is a control: in this and some of the muscles there is a higher count in the slice which is adjacent to the rib. Fig. 1*B* shows the uptake of ^{24}Na in the presence of 100 μM carbachol. Tetrodotoxin (0·1 μM) was also present to prevent

action potentials (Colquhoun, Rang & Ritchie, 1974). There is increased radioactivity in the muscle, with a peak in the slice of muscle which contained the band of end-plates, and the additional radioactivity, above the value assigned to the ends of the muscle was summed to give the uptake induced by the action of the depolarizing drugs. This sum was

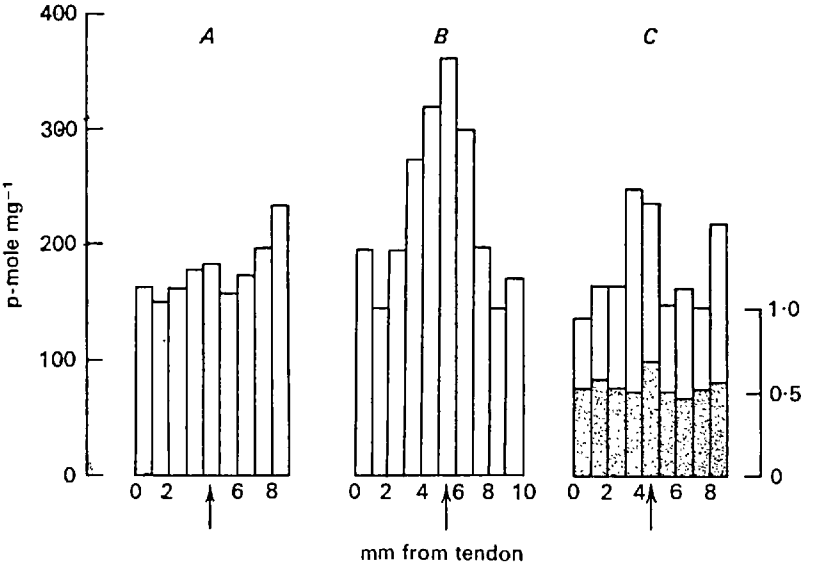


Fig. 1. Labelled sodium in rat diaphragm following exposure to ^{24}Na for 15 sec and wash for 6 min. Ordinate on the left is sodium as p-mole. mg^{-1}, abscissa is distance from the tendon in mm. Fig. 1*A* is a control, without depolarizing drug. Fig. 1*B* and *C* show the effects of carbachol 100 μM: in each case there is an increase in labelled sodium, with a peak in the slice which contained the band of end-plates which is marked by the arrows. In 1*A* and *C* there is increased radioactivity in the slice adjacent to the rib. All solutions contained tetrodotoxin 0·1 μM. The stippled histogram in 1*C* shows the uptake of [^{14}C]carbachol in a separate experiment, with exposure 15 sec and wash 6 min. The scale on the right is p-mole. mg^{-1} and there is a small peak in the end-plate region.

expressed as p-mole. mg^{-1}, and gives the *additional* sodium in the junctional region associated with 1 mg muscle or approximately 380 fibres (see below). Fig. 1*C* shows another muscle treated in a similar way in which the uptake in the junctional region is smaller and that at the rib end is comparatively large. Fig. 2*A* shows the uptake produced by suberyldicholine (100 μM) and by decamethonium (100 μM) with 30 sec exposure and wash 6 min.

The histograms in Fig. 1 and Fig. 2 show that depolarizing drugs induced in each case an additional uptake of labelled sodium, but the results are irregular with much

scatter. In practice the mean value of the ends of the muscle was obtained, with omission of the slice adjacent to the rib. The high value at the rib end was increased in some diaphragms with large amounts of rib, and may indicate re-entry of ^{24}Na from the rib or may possibly be due to receptors in tendon as found by Katz & Miledi (1964). The histograms become much more regular if results from several muscles are combined (see Figs. 6, 7 and 10 below).

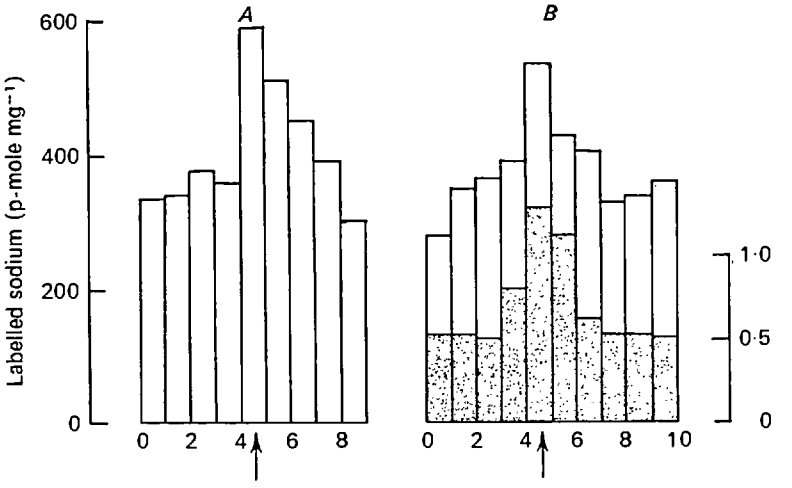


Fig. 2. As Fig. 1: labelled sodium in rat diaphragm following exposure to (*A*) suberyldicholine 100 μM and (*B*) decamethonium 100 μM. Exposure 30 sec in each case with wash 6 min. The left hand scale is sodium in p-mole. mg^{-1}. In the experiment shown in *B* the solution also contained [^{3}H]decamethonium, and the stippled histogram gives the uptake of the drug in p-mole. mg^{-1} (right hand scale).

In Fig. 2*B* the solution used for 30 sec exposure also contained [^{3}H]-decamethonium. The stippled histogram gives the uptake of labelled drug in p-mole. mg^{-1} (right hand side). The uptake is low because the concentration of decamethonium (100 μM) is much less than that of sodium (145 mM). The stippled histogram of Fig. 2*B* also shows a peak at the junctional region, and the counts appear to show less variation than those obtained with ^{24}Na. This may be due to the binding of decamethonium which takes place soon after entry (Case, Creese, Dixon, Massey & Taylor 1977).

The stippled histogram of Fig. 1*C* shows the result of exposure to [^{14}C]carbachol in a separate experiment (300 μM for 15 sec, with wash 6 min). The results are expressed as p-mole. mg^{-1} (right hand scale). There is a small additional uptake in the slice which contained the band of end-plates. This was found in each case, although the ratio of peak uptake to that at the ends of the muscle was low, with median 1·5 (twelve muscles, range 1·13–1·82).

Duration of exposure to labelled sodium

The open circles in Fig. 3 show the effect of exposure to carbachol 100 μM for times which varied from 5–60 sec, with a wash of 6 min. The additional uptake in the junctional region was estimated as in Fig. 1*B* and *C* as the value above that assigned to the ends of the muscle. The filled circles show sodium in the ends of the muscle. There is a progressive

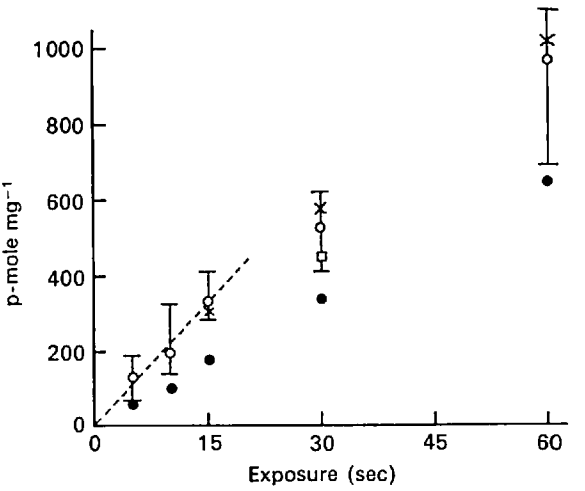


Fig. 3. Effect of varied exposure on the uptake of labelled sodium. The open circles show the additional or junctional sodium in the muscle following exposure to carbachol 100 μM. Each point is the median of twelve to forty-one muscles, and the limits give the confidence limits (approximately 95 %). The interrupted line is the initial regression. The crosses show the effect of decamethonium 100 μM. The square represents suberyldicholine 100 μM. The filled circles are the pooled values at the ends of the muscles.

increase in uptake with time: the filled circles show a linear increase while the open circles which represent junctional sodium show some curvature. For the filled circles the regression is 644 p-mole.mg^{-1} min^{-1} while for the open circles the initial rate (to 15 sec) is 22·5 p-mole.mg^{-1} sec^{-1} or 1350 p-mole.mg^{-1} min^{-1}, so the ratio (junctional sodium)/(sodium at the end) is 2·1.

Fig. 3 also shows some results obtained with decamethonium (100 μM) and with suberyldicholine (100 μM), and these were similar to those with carbachol (100 μM). When the distribution of these various measurements were examined it was found that they gave a positive skew, suggestive of a lognormal distribution. Fig. 4 (open circles) shows the results from forty-one estimates of the junctional sodium obtained as in Fig. 1*B* and *C*. The values were ranked and the probits (or deviates) have been plotted

against the uptake on a logarithmic scale. The linear plot is consistent with a lognormal distribution: the interrupted line is the weighted regression. Fig. 4 also shows other measurements, which were found to have similar distributions. For this reason the median (rather than the mean) has been used as a measure of central tendency in Fig. 3 and elsewhere.

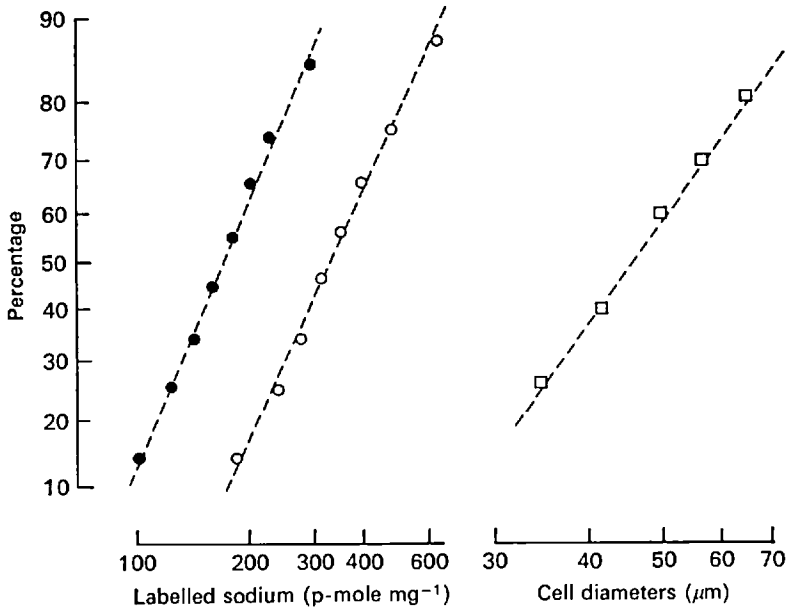


Fig. 4. Distributions of some variables. The cumulative percentages have been plotted on a probit scale against a logarithmic ordinate, so that a straight line indicates a lognormal distribution. The open circles show the junctional sodium induced by carbachol 100 μM for 15 sec with wash 6 min (forty-one muscles). The median is 335 p-mole.mg^{-1} and has been used in Fig. 3. Filled circles show the sodium in the ends of the forty-one muscles. The squares give cell diameters of 261 fibres, obtained as in the text.

Extrapolation and effects of washing: calculation of results

The labelled sodium which has accumulated at the junctional region can only be demonstrated when the extracellular spaces have been cleared by a wash, and it becomes necessary to extrapolate to obtain an estimate of the value at the end of the exposure. Fig. 5 shows the effect of the wash on labelled sodium. The muscles were exposed for 30 sec to solutions containing ^{24}Na and decamethonium 100 μM. Tetrodotoxin (0·1 μM) was present, and in some cases [^{3}H]decamethonium was also added. The results for individual muscles were similar to those shown in Fig. 2*B*, and the additional sodium at the junctional region which remained

after 3, 6, 9 and 12 min of wash is shown in Fig. 5 (open circles). The labelled sodium has been expressed for convenience as a clearance, being (counts mg^{-1})/(counts $pl.^{-1}$), and plotted on semilogarithmic paper. Each point gives the median of at least twelve diaphragms. The triangles show the labelled sodium in the ends of the muscles, and the values can be used for extrapolation to zero time.

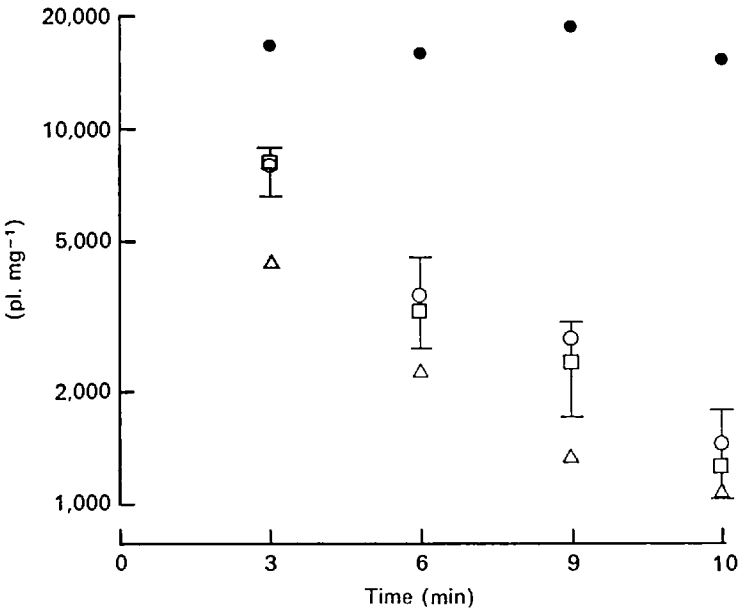


Fig. 5. Effects of washout following exposure to labelled sodium and decamethonium 100 μM for 30 sec. [^{3}H]decamethonium and tetrodotoxin 0·1 μM were also present in the test solution. Ordinate gives the uptake in the junctional region expressed as a clearance, being (counts min^{-1} mg^{-1})/(counts min^{-1} $pl.^{-1}$ test solution). Open circles show junctional sodium (median of at least twelve muscles), with 95 % confidence limits. The squares are values for labelled sodium obtained following pretreatment 30 min with decamethonium 100 μM. The triangles show labelled sodium in the ends of the muscle. Filled circles give labelled decamethonium in the same units.

The movements of labelled sodium can be interpreted in terms of the findings shown in Figs. 1, 2, 3 and 5 and previous measurements. From results similar to that of Fig. 1*A* the uptake in control muscles (sixteen with 15 sec exposure, twenty with 30 sec) was 10·6 p-mole.$mg.^{-1}$ sec^{-1} (median of 36, 95 % limits 7·6, 13·0). In Fig. 3 the filled circles give the labelled sodium which remains in the ends: the slope is 10·7 p-mole.$mg.^{-1}$ sec^{-1}, so that the *uptake at the ends* (uncorrected for wash) is similar to the controls.

Creese, El-Shafie & Vrbova (1968) found that the rate constant for outward movement following equilibration with labelled sodium was 0·137 min^{-1} (half-time 5·1 min), obtained from the slope of the washout after 6 min. In Fig. 5 *the rate*

constant for the washout from 6 min was 0·123 min^{-1} for the triangles (so that in 6 min the labelled sodium falls by a factor of 2·1), and 0·143 min^{-1} for the circles (junctional sodium).

In previous studies the fibre sodium was founded by extrapolation to zero time from points starting at 6 min (Creese, 1968, Fig. 2), with a correction factor of 1·5 to allow for the effects of diffusion lag (Huxley, 1960; Creese *et al.* 1968). The uptake of sodium at the ends of the muscle (exposure 15 sec with wash 6 min) is seen in Fig. 1 *B* and *C*. The median value is 147 p-mole. mg^{-1} (forty-one muscles, 95 % limits 125, 204), and the distribution is seen in Fig. 4 (filled circles). The uptake has to be corrected for the effects of the wash, and multiplication by 2·1 and division by 1·5 (for diffusion lag) gives a corrected uptake of 309 p-mole. mg^{-1}. If the fibre sodium is taken as 6220 p-mole. mg^{-1} (Creese *et al.* 1968) the fractional uptake f is 0·0357 in 15 sec. For simple exponential uptake f is $[1-\exp(-kt)]$ where k, *the rate constant for inward movement at the end of the muscle*, is 0·133 min^{-1} (half-time 5·1 min). In Fig. 3 the filled circles which represent the pooled values for the uptake at the ends of the muscles have a regression with a slope of 644 p-mole. mg^{-1} min^{-1}; calculations as above give a rate constant of 0·155 min^{-1} (half-time 4·5 min). In Fig. 5 the triangles represent sodium in the ends of the muscle following uptake for 30 sec. By extrapolation to zero time by simple regression from values at 6, 9 and 12 min the origin is 4600 pl. mg^{-1} or 667 p-mole. mg^{-1} which becomes 443 p-mole. mg^{-1} after correction for diffusion lag; the fractional uptake is then 0·0712 in 30 sec and the calculated rate constant is 0·148 min^{-1} (half-time 4·7 min).

The junctional sodium or additional sodium (given by the sum of values above the level assigned to the ends of the muscles) is shown by the clear circles in Fig. 3 (carbachol 100 μM 5–60 sec with wash 6 min) and from the regressions the ratio of (junctional sodium)/(sodium at ends) is 2·1, so that the *influx of labelled sodium at the end-plate* is increased by a factor of at least 3·0 as compared with that at the ends. In Fig. 5 the clear circles show the effect of the wash on the junctional sodium following exposure to decamethonium (100 μM for 30 sec). Extrapolation to zero time of values obtained at 6, 9 and 12 min gives 8270 pl. mg^{-1} or 1199 p-mole. mg^{-1}. The (uncorrected) value for sodium in the ends of the fibres is 667 p-mole. mg^{-1}, so that for decamethonium 100 μM the ratio junctional/end-plate sodium is 1·8. If in Fig. 5 the open circles are extrapolated from points at 3, 6, 9 and 12 min, the origin is 14,950 pl. mg^{-1}. The loss of labelled sodium from the junctional region occurs chiefly by exchange with the external solution, with an additional factor due to diffusion along the fibres which is considered below. Corrections for loss of junctional sodium due to longitudinal diffusion have not been made when interpreting the results shown in Fig. 5, and the extrapolated value for junctional sodium represents a lower limit.

From histograms similar to those of Figs. 1 and 2, the *ratio* of additional or junctional sodium to that at the end of the muscle is 2·28 for carbachol 100 μM (exposure 15 sec, median of forty-one muscles), so with carbachol the total sodium at the end-plate exchanges at least 3 times faster than at the ends of the muscle. For suberyldicholine 100 μM the ratio of extra sodium to that at the end is 2·0 (15 sec exposure, from results similar to that in Fig. 3). For decamethonium 100 μM the ratio is 1·9 (15 sec, median of eighteen muscles), and 1·74 for exposure of 30 sec (thirty-two muscles). These results are consistent with estimates obtained from the graphs shown in Figs. 3 and 5.

The exchange of sodium at the ends of the muscles exposed to depolarizing drugs is similar to control values and is comparable to previous results. At the end-plate the entry of labelled sodium is markedly

increased as estimated from histograms (Figs. 1 and 2), from uptake with time (Fig. 3) and by extrapolation from washouts (Fig. 5), and with carbachol (100 μM) the influx at the junctional region is increased by a factor of at least 3 when compared with that at the ends of the muscle.

In some cases [^{3}H]decamethonium was also present in addition to

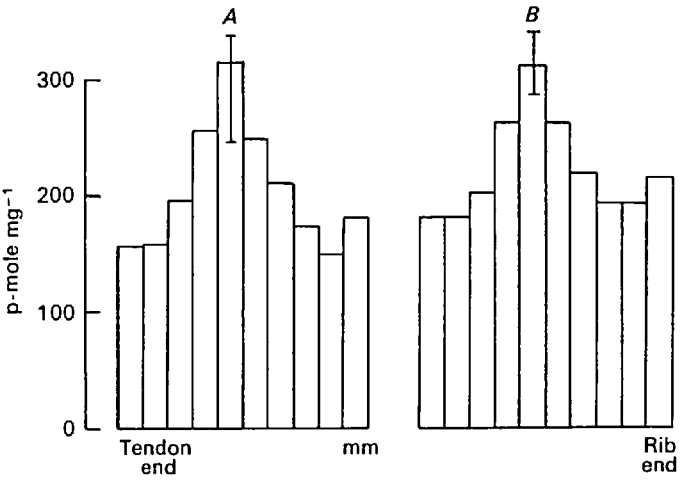


Fig. 6. Effect of prolonged application of carbachol 100 μM. 6*A* gives pooled values from fifteen diaphragms exposed for 15 sec to ^{24}Na and carbachol 100 μM and washed for 6 min. Ordinate is p·mole.mg^{-1}. 6*B* was obtained from fourteen diaphragms pretreated for 30 min in solution containing carbachol 100 μM and then treated as 6*A*.

^{24}Na. In Fig. 5 the filled circles show the labelled decamethonium which remained after known periods of wash. The values were estimated in the same way as for sodium (see Fig. 2*B*), and are given as the additional label in the junctional region above the level assigned to the ends of the muscle. The uptake has been expressed as a clearance in pl. mg^{-1} as before. It can be seen from Fig. 5 that the radioactivity shows little if any decline during the short wash, and this behaviour is consistent with previous results (Case *et al.* 1977). When extrapolated to zero the clearance for decamethonium is 16,670 pl. mg^{-1}, which is twice as great as that for sodium. If the higher estimate is used in the extrapolation for sodium (Fig. 5) then the discrepancy would be much smaller. In any case it is clear that the permability to labelled decamethonium at this concentration, when expressed in this manner, is comparable to that of labelled sodium, as expected if the cations enter through the same channels.

Sodium entry following continued exposure to carbachol and to decamethonium. It is known that continued application of depolarizing drugs

produces desensitization (reviewed by Ginsborg & Jenkinson, 1976), and it is therefore important to determine whether continued exposure is accompanied by a change in the entry of sodium. Fig. 6*B* shows results from fourteen muscles which were pretreated for 30 min in solution containing carbachol 100 μM and then exposed to ^{24}Na and carbachol 100 μM for 15 sec with wash of 6 min. The histogram in Fig. 6*A* was obtained from fifteen controls without pre-treatment. Tetrodotoxin 0·1 μM was present in all cases.

The radioactivity was expressed as p-mole.mg^{-1} for each slice of the muscles, and the histograms in Fig. 6*A* and *B* were obtained by pooling the results in such a way that the peaks were aligned. Each rectangle in the histogram represents the median from all the muscles in the series.

In these experiments particular care was taken to have as small a rib as possible, and this may account for the comparative symmetry of the histogram, with only a small increase in radioactivity in the slice at the rib end of the muscles. The limits for the peaks in Fig. 6*A* and *B* are the confidence limits (approximately 95 %) for the median.

The results in Fig. 6*A* and *B* are similar and pre-treatment has not produced any drastic effect on the entry of labelled sodium. The additional sodium above the level assigned to the ends was 398 p-mole.mg^{-1} for the controls (median of 15, limits 240, 636), and for pre-treated muscles 353 p-mole.mg^{-1} (median of 14, limits 273, 515). These are not significantly different.

The effect of prolonged exposure to decamethonium (100 μM) is seen in Fig. 5 in which the squares give results following pre-treatment for 30 min with subsequent exposure to labelled sodium for 30 sec and wash for 3, 6, 9 and 12 min. Each point is the median of at least 12, and the points in Fig. 5 differ little from those obtained without pre-treatment. Comparison by a *t* test at each time showed no significant difference ($P > 0{\cdot}05$ in each case). It was concluded that the effects of decamethonium at a concentration of 100 μM did not appreciably alter with prolonged application for 30 min.

For muscles pretreated with decamethonium the values for washes of 3, 6, 9 and 12 min are, respectively 102, 91, 87 or 88 % of the values obtained without pretreatment. If the washout for pretreated muscles (squares) is initially faster than the controls (open circles), which could arise if the effect of the decamethonium persists during the early wash for presoaked muscles, this would give somewhat lower levels of labelled sodium (squares) during the final stages of the wash in Fig. 5.

Prolonged application of carbachol (100 μM for 1 hr) did not appreciably affect the total potassium or sodium of diaphragm muscle. The values for *treated* muscles were 90·8 μmole.mg^{-1} for potassium (median of 10, 95 % limits 87, 96) and 44·0 μmole.mg^{-1} for sodium (limits 38, 53): values for *control* muscles were 89·8 μmole.mg^{-1} for potassium (median of 40, limits 88, 96) and 45·8 μmole.mg^{-1} for sodium (limits 43, 49).

Longitudinal diffusion of labelled sodium

Fig. 7 shows the effects of prolonged washout, from which the diffusion coefficient of labelled sodium can be estimated. The muscles were exposed to ^{24}Na plus decamethonium 100 μM for 30 sec. The upper histogram shows labelled sodium which remained after a wash of 6 min (pooled results from

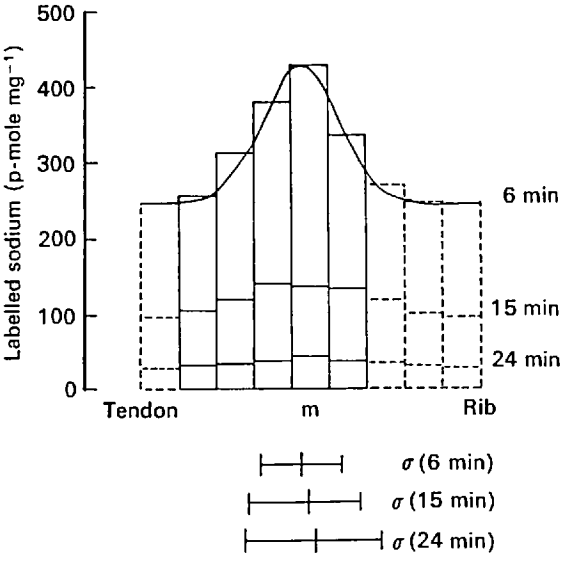


Fig. 7. Effect of wash on the spread of labelled sodium along the fibre. Exposure 30 sec to ^{24}Na with decamethonium 100 μM. Upper histogram shows pooled results from twelve muscles with wash of 6 min. Ordinate gives labelled sodium in p-mole. mg^{-1}. A Gaussian curve has been fitted to the junctional sodium (above the level assigned to the ends of the muscle). In practice the Gaussian curve was obtained from five of the slices (continuous lines), so that only the left or tendon end of the histogram is complete. Results are also shown for washes of 15 min (fourteen muscles), and for 24 min (twenty-four muscles). The mid-points of the curves and the calculated standard deviations are shown in the lower part of the diagram. There is a progressive widening of the fitted curves with time.

twelve muscles); other histograms show the effects produced by a wash of 15 min (fourteen muscles) and by 24 min (twenty-four muscles). The lower part of Fig. 7 gives the S.D. obtained from Gaussian curves fitted to these results. As the wash is increased there is a fall in the labelled sodium and also a widening of the Gaussian curves which is consistent with longitudinal diffusion of labelled sodium along the fibres.

In each muscle the labelled sodium was expressed as p-mole.mg^{-1}, as in Fig. 2 *B*. In Fig. 7 the slice which contained the band of end-plates is labelled *m*: other slices,

each of 1 mm width, may be termed $(m-1)$, $(m-2)$, $(m-3)$ on the tendon side, and $(m+1)$ etc. on the rib side. Counts from twelve muscles with wash of 6 min were pooled by aligning the figures such that the rectangle marked m in Fig. 7 gives the median from twelve values, and the results from the other slices were treated similarly. In practice, the values from the rib end $(m+2, m+3)$ gave high values in some of the muscles, possibly because of the proximity of the rib with its large reservoir of labelled sodium, and for this reason the results in slices $(m-3)$, $(m-2)$, $(m-1)$, m and $(m+1)$ were used for estimation of the extra sodium which remained in the junctional region. This is indicated by the continuous lines in the histograms in Fig. 7; other parts of the histogram are shown by dotted lines. Gaussian curves were fitted to the truncated histograms as described in the Appendix, and the standard deviations from results obtained at 6, 15, and 24 min are displayed in the lower part of Fig. 7.

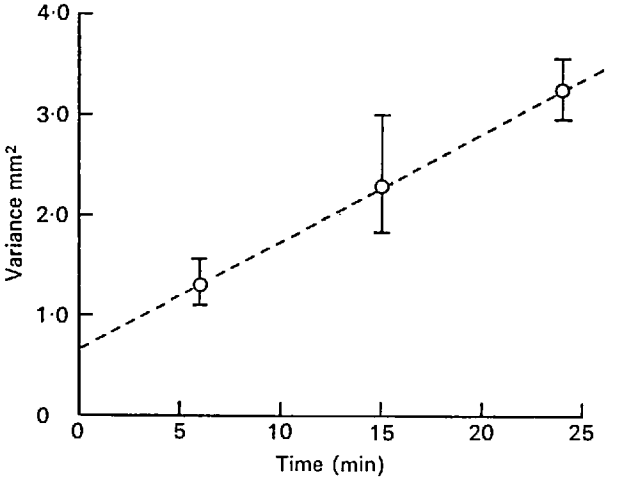


Fig. 8. The variance σ^2 obtained from the standard deviations in Fig. 7 is plotted against time (see text). Confidence limits (95 %) are shown. The slope is proportioned to the longitudinal diffusion coefficient.

The standard deviation σ_t at time t may be expected to increase by the relation

$$\sigma_t^2 = \sigma_0^2 + 2D't, \tag{1}$$

where D' is the apparent diffusion coefficient and σ_0^2 is a constant. Fig. 8 shows the variance plotted against time, and the interrupted line is the regression, obtained as in the Appendix. The slope is 0·108 $mm^2\ min^{-1}$ which gives D' as 9·0 $cm^2\ sec^{-1} \times 10^{-6}$ (95 % limits 6·6, 13·0 $\times 10^{-6}$). If the diffusion coefficient of sodium at 38 °C is taken as 17·3 $\times 10^{-6}$ $cm^2\ sec^{-1}$ (Creese, 1968), then D' is close to 50 % of the value to be expected in free solution. The value of σ_0 is 0·80 mm, and this is a measure of the initial distribution of the labelled sodium before spread by lateral diffusion. The value obtained with labelled sodium is close to the figure of 0·79 found with labelled decamethonium in a different series (Case *et al.* 1977).

Dose–response effect of carbachol obtained with labelled sodium

The entry of sodium induced by carbachol varied with concentration, and Fig. 9 shows the relation. The additional uptake of labelled sodium in the junctional region following exposure for 15 sec and a wash of 6 min was obtained as before and expressed as pl. mg^{-1}. The circles in Fig. 9 give the median, with limits (approximately 95 %) for twelve or more muscles in each case. The uptake of sodium increases with concentration to a peak at 1 mM, and by probit analysis the median effective dose was 72 μM (95 % limits 53, 106 μM: the statistic g is 0·55).

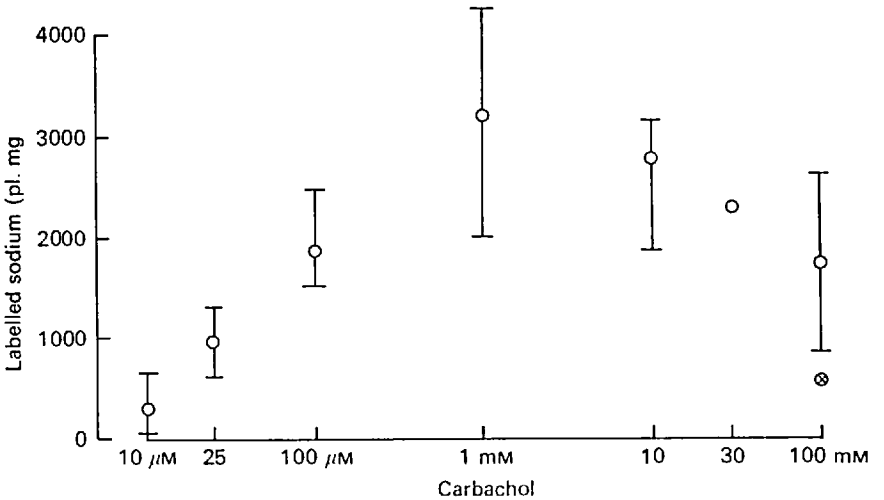


Fig. 9. Dose–response effect with carbachol. Ordinate gives the summed junctional uptake of labelled sodium (p·mole. mg^{-1}) following exposure of 15 sec to solution containing ^{24}Na, carbachol, and tetrodotoxin 0·1 μM, with wash 6 min. The results are given as a clearance, (counts min^{-1} mg^{-1})/ (counts min^{-1} $\mu l.^{-1}$ radioactive solution). Each circle gives the median of at least twelve muscles (except for the value at 30 mM) with the confidence limits obtained as in the text. The circle with cross at 100 mM gives values obtained following pretreatment for 15–60 min at 100 mM (median of 16).

Fig. 9 also shows the effect of higher concentrations, obtained by equivalent reduction of sodium chloride. There is some diminution in the uptake, expressed as pl. mg^{-1}. This effect is accentuated with pre-treatment at high concentrations. The circle with the cross (Fig. 9) gives the results of sixteen muscles pretreated in solution containing carbachol (100 mM) for 15 min (five muscles), 30 min (five muscles) or 60 min (six muscles). These were similar and the values have been pooled in Fig. 9. At this concentration the pretreated muscles had a lower uptake than those without presoaking ($P < 0·01$ by a two sample t-test). The significance of the effect of high concentration is not clear; a similar effect is found with concentrations of decamethonium in the mM range (Creese & England, 1970).

Effect of potassium on resting potential and entry of ^{24}Na

Fig. 10 shows effects on the entry of junctional sodium produced by depolarization with potassium. In Fig. 10*A* the muscles were in solution with potassium 5·0 mM; sodium was decreased to 88 mM, osmotic balance being maintained by sucrose. The muscles were exposed to labelled sodium

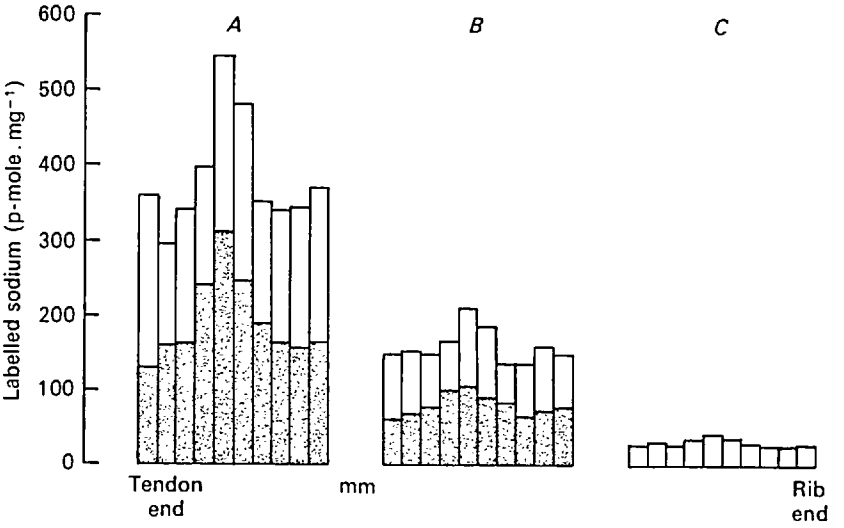


Fig. 10. Effect of potassium on uptake of sodium produced by carbachol 100 μM. Potassium concentrations were 5 mM for *A* and 60 mM for *B:* potassium methyl sulphate was added for *B*, and sucrose for *A:* sodium was 88 mM in each case. Ordinate gives labelled sodium (p-mole. mg^{-1}): each histogram in *A* and *B* gives pooled results from six diaphragms. Exposure was 15 sec with 3 min wash (open histograms), or 6 min wash (stippled). The histogram in (*C*) gives pooled results from eight muscles in solution containing 141 mM-potassium and 6 mM-sodium: exposure 30 sec and wash 3 min.

and carbachol 100 μM for 15 sec, with a wash of 3 min, or 6 min (stippled). Each rectangle of the histogram gives the median of slices from six muscles. Fig. 10*B* gives the uptake of muscles in solution with raised potassium (60 mM); the uptake of ^{24}Na is considerably diminished. The resting potential with 5 mM-potassium was -75 mV (median of 100 fibres from four diaphragms, 95 % limits -71, 77 mV). With 60 mM-potassium the potential was -20 mV (median of seventy-five fibres from three diaphragms, 95 % limits -18, 22 mV). In Fig. 10*C* the muscles were depolarized in solution with 141 mM-potassium and sodium 6 mM. The histogram was obtained from eight muscles, and the entry of sodium, expressed as p-mole. mg^{-1} is low, but increased uptake in the junctional region is still

found. In parallel experiments the resting potentials of muscles in solution with 141 mM-potassium was −4 mV (median of seventy-five fibres from three muscles, 95% limits −3, −5 mV).

Fig. 11 shows a dose–response curve obtained in the presence of 141 mM-potassium methyl sulphate (muscles exposed 30 sec with 3 min wash). The extra entry, obtained from histograms similar to those of Fig. 10*C*

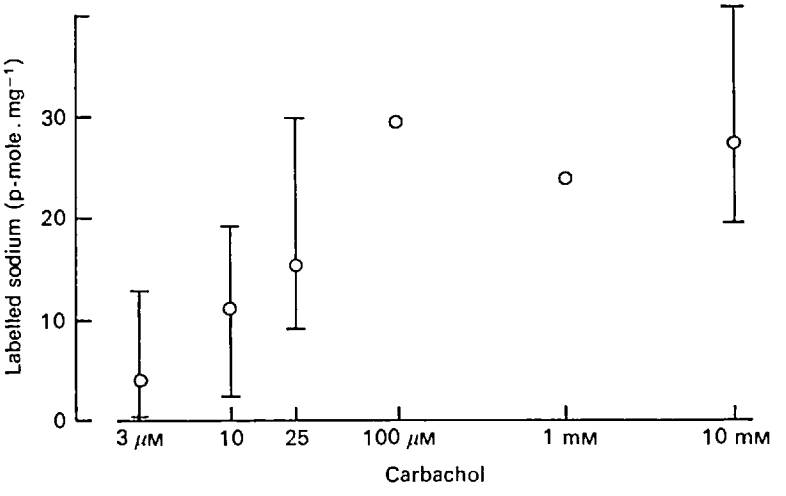


Fig. 11. Dose–response effect produced by carbachol in the presence of 141 mM-potassium methyl sulphate and 6 mM-sodium. Ordinate gives the junctional sodium as p-mole.mg^{-1}. Each point is the median of seven to eleven muscles. Exposure 30 sec, with wash 6 min. The confidence limits shown at 10 mM are limits for all the values at the plateau, and were obtained by pooling results at 100 μM, 1 mM and 10 mM (twenty-four muscles).

has been plotted against carbachol on a logarithmic scale. Each point is the median of seven to eleven muscles. The uptakes were small and variable, but the median values appear to rise with concentration to produce a plateau, and there was little change over the range 0·1–10 mM. The half-maximal concentration in these diaphragms was 18 μM carbachol, which is appreciably lower than that shown in Fig. 9.

Fibre diameters and calculation of sodium entry

The diameters of superficial fibres from muscles suspended in solution were measured with a microscope and the results were pooled. The distribution was lognormal as shown in Fig. 4 with median value 46 μM (261 fibres, 95% limits 45, 47 μM).

The fibres in the diaphragm muscle of the rat extend from tendon to rib: with an extracellular space of 0·33 by volume (Creese, 1968) and specific gravity of 1·07 (Klaus, Lüllmann & Muscholl, 1960) the volume of myoplasm is 0·626 μl. mg^{-1}.

If the fibres are treated as cylinders there would be 377 per mg muscle. From the results shown in Fig. 9, exposure to 1 mM carbachol for 15 sec with wash of 6 min produced an additional uptake of labelled sodium of 3212 pl. mg^{-1} (median of seventeen muscles), which corresponds to 466 p-mole.mg^{-1}. This has to be corrected for the effects of the wash of 6 min: multiplication by a factor of 2·1 and division by 1·5 (correction for diffusion lag) gives a value of 652 p-mole.mg^{-1} in 15 sec or 44 p-mole.mg^{-1} sec^{-1}. This is the lower estimate: extrapolation based solely on the open circles in Fig. 5 (3, 6, 9 and 12 min) would give a half-time for wash of 3·3 min so that the corrected uptake would be 1640 p-mole.mg^{-1} or 109 p-mole.mg^{-1} sec^{-1}.

From the peak uptake in Fig. 9 the rate of influx of sodium when corrected for the effects of the wash is 652 p-mole in 15 sec for 1 mg muscle or 377 fibres, so the influx is 0·115 p-mole.sec^{-1} $fibre^{-1}$ or $6{\cdot}9 \times 10^{10}$ ions sec^{-1} $end\text{-}plate^{-1}$. If the number of receptors (and of channels) is $4{\cdot}7 \times 10^{7}$ per end-plate (Miledi & Potter, 1971), then the influx of sodium is $1{\cdot}5 \times 10^{3}$ ions sec^{-1} $channel^{-1}$. If the upper estimate is used the influx is $3{\cdot}7 \times 10^{3}$ ions sec^{-1} $channel^{-1}$. This is smaller by a factor of 10^{4} then the rate of influx of ions estimated by analysis of end-plate noise (Katz & Miledi, 1972; Anderson & Stevens, 1973), and is considered below.

DISCUSSION

The change in permeability induced in the end-plate by drugs and transmitters has usually been studied by electrical measurements. For the direct or isotopic method the exposure has to be brief, and it requires detection of the small extra uptake in the end-plate region in the presence of a large excess of extracellular sodium. A long exposure would allow sodium which has accumulated at the end-plate to diffuse along the fibres, and extra radioactivity at the end-plate was not detected in mouse muscle following exposure for 20 min (Evans, 1974). A similar explanation may be given for the failure to detect an extra uptake at the end-plate following prolonged exposure to labelled carbachol (Taylor, Creese, Nedergaard & Case, 1965). The simple procedure adopted in the present study is only feasible in muscles in which the end-plates form a regular band (e.g. England, 1970). The method involves a wash to clear the extracellular spaces, and corrections are needed to allow for the loss of radioactivity during the wash. The half-time of sodium exchange in rat diaphragm is normally 5·1 min (Creese, 1968), and in the presence of depolarizing drugs the influx is at least three times that at the ends. The exchange at the end-plate region can be inferred by comparison with that of the ends of the muscle which are largely unaffected by the drug, and this has enabled the rapid influx at the end-plate region to be estimated from measurements in whole muscles.

Dose–response curves can be obtained by direct measurement of the

increased flux of labelled sodium. Half-maximal values for carbachol are 72 μM for the end-plate region of rat diaphragm at 38 °C, 70 μM in microsacs from the electric eel at room temperature (Hess, Andrews, Struve & Coombs, 1975), 32–54 μM for *Torpedo* microsacs (Popot, Sugiyama & Changeux, 1976) and 300 μM for cultured muscle cells at 2 °C (Catterall 1974). The method can be compared with those used to obtain dose–response curves in muscle end-plates by electrical measurements (Dreyer & Peper, 1975; Adams, 1975; Rang, 1975; Peper, Dreyer & Müller, 1976).

The influx of sodium can be demonstrated in depolarized muscle, so the effect is not dependent on the change of membrane potential which is induced by the drug. The method can also be used to infer the state of the sodium channels. In rat diaphragm exposed to depolarizing drugs there is a spontaneous recovery of the membrane potential in the presence of the drug (Thesleff, 1955; Creese, Franklin & Mitchell, 1976). It has been found that the entry of sodium following prolonged exposure to carbachol (100 μM) or to decamethonium is similar to that obtained with the initial application of the drug, so pre-treatment did not prevent the channels from opening in rat diaphragm at body temperature. This is in contrast to cultured chick fibres (Catterall, 1974), and to membrane fragments of *Torpedo* (Sugiyama, Popot & Changeux, 1976) which showed desensitization to carbachol when sodium movements were measured.

Labelled sodium was found to spread along the fibres with an internal rate of diffusion approximately half of that expected in the external saline. A similar conclusion was reached by Kushmerick & Podolsky (1969) who worked with single fibres of the frog at room temperature. The present results have been obtained on whole tissues and the values from several muscles have been pooled at each time interval. The method of analysing the results is given below, and included the use of weighting factors which enabled Gaussian curves to be fitted in spite of the scatter and low count of the individual muscles. The rapid mobility of sodium ions is in contrast to the slow drift of labelled decamethonium which enters and becomes bound (Case *et al.* 1977). The permeability of the end-plate region as affected by depolarizing drugs appears to be similar for sodium and decamethonium, as expected if they enter through the same channels (Cookson & Paton, 1969).

The influx of labelled sodium is very much smaller than that estimated from fluctuation analysis of end-plate noise in amphibian end-plates where, expressed as the number of ions traversing a channel, the rates are 5×10^7 sec^{-1} (Katz & Miledi, 1972), and 2×10^7 sec^{-1} (Anderson & Stevens, 1973). The estimate for the action of carbachol on rat diaphragm is $1{\cdot}5 \times 10^3$ sec^{-1}, which is 1000 times smaller than that obtained by measurement of electrical noise. Catterall (1974) worked on cultured chick fibres and

his figure is $3{\cdot}2 \times 10^6$ min^{-1} or $5{\cdot}3 \times 10^4$ sec^{-1} at 2 °C: if various other corrections are applied the figure comes to 2×10^7 min^{-1} or $3{\cdot}3 \times 10^5$ sec^{-1}. The value obtained by Kasai & Changeux (1971) who studied microsacs from the electric eel was only 5×10^3 min^{-1} or 80 sec^{-1}, though the figure has been revised upwards by more recent measurements (Hess *et al.* 1975). The marked discrepancy between the steady-state fluxes and the electrical measurements is explicable if the channels rapidly close so that only a small fraction of the total are open at any one time, and this fraction may be 0·01–0·1 % of the total (Anderson & Stevens, 1973), or possibly as high as 2–8 % as calculated by Catterall (1975).

APPENDIX

Fitting of Gaussian curves

Fig. 7 shows histograms obtained by pooling results from five slices from each of twelve to twenty-four muscles. It is necessary to fit Gaussian curves to these truncated histograms in which the left or tendon side is complete but part of the right or rib side is missing. The results are in p-mole.mg^{-1} and in these muscles the values at the tendon end were used to obtain a base line for the extra or junctional sodium. In practice the pooled results from five slices were available for curve fitting, as shown in the filled-in histogram of Fig. 7.

The peak of the Gaussian curve is in the slice which contains the band of the end-plates labelled *m* in Fig. 7, but the exact location in the strip of 1 mm is not known. If a value is assigned to this midpoint, then the uptake in the slice marked *m* in Fig. 7 can be allocated so that the area in the left hand side of the curve is known, and hence the total area. This enables the uptake in the rectangles to be obtained as fractions, and so the cumulant values can now be found and expressed as probits and plotted against the distance in mm. With five slices there will be five points in the probit plot. In practice by trial and error the location of the midpoint was found which gave the minimum variance about regression of the weighted probit: from the slope of the probit the standard deviation of the Gauss curve is obtained. The locations of the midpoints and the standard deviations are shown in Fig. 7 for the curves at three different times of wash.

With few points on the probit line, the weighting is important. Weighting values for probit analysis as in Finney (1964) are inappropriate as the values are not quantal. Weighting values for homoscedastic quantitative probit analysis are given by Aitcheson & Brown (1969), and are suitable if the errors about the cumulative sigmoid curve are normally distributed. The use of these weighting factors has the effect of constraining the curve about the values near the midpoint of the curve.

Weighted regression analysis

A desk calculator was programmed to give the regression and also s^2, the squared deviations about regression, from values of x, y and w. The term s^2 was used in the

form $(1-r^2)S_{yy}$ where r is the correlation coefficient and S_{yy} is defined by Finney (1964). Then the variance about regression is $s^2/(n-2)$ where n is taken as the number of points. Initial values were used for the weights and the regression was obtained by iteration (Finney, 1964).

Dose-response curves (Fig. 9, Fig. 11) were obtained by weighted probit analysis, with the weighting coefficients of Aitcheson & Brown (1969). Weighted regressions were also used for the plots shown in Fig. 4.

Fig. 7 shows the relation between variance σ^2 and time for longitudinal diffusion. This is unsuitable for the regression analysis as the distribution of σ^2 is certainly not normal. If the interrupted line in Fig. 8 is prolonged it cuts the abscissa at a point which can be termed $(-d)$ min where d is close to 6 min. If time is measured from this point then eqn. (1) becomes

$$\sigma^2 = 2D'(t+d) \quad (2)$$

or

$$\sigma^{-1} = (2D')^{-\frac{1}{2}}(t+d)^{-\frac{1}{2}}. \quad (3)$$

Now σ^{-1} is given by the slope of the probit line for the appropriate value of t, and the regressions have already been calculated as for a normal distribution of errors. Hence from eqn. (3) the slope of the probit should give a linear relation when plotted against $(t+d)^{-\frac{1}{2}}$, and the weighting factors can be obtained from the variance about regression of the probit and the numbers of muscles at each time t. In practice the value of d was found which gave the minimum deviations about regression in the plot of σ^{-1} in eqn. (3). The term d was 5·9 min, and the slope was used to give D' and its confidence limits. The method is a variation of that used by Case *et al.* (1977).

This research was supported by the Medical Research Council. The authors also wish to thank Dr R. B. Barlow and Dr A. Ungar, University of Edinburgh, for a gift of suberyldicholine; Dr J. M. England, St Mary's Hospital Medical School, who supplied a programme for the desk calculator, and a table giving interpolation values for the weighting factors of Aitcheson & Brown (1969); and Mr G. Stolarczyk for skilled assistance.

REFERENCES

ADAMS, R. P. (1975). An analysis of the dose–response curve at voltage-clamped frog end-plates. *Pflügers Arch. ges. Physiol.* **360**, 145–153.

ANDERSON, C. R. & STEVENS, C. F. (1973). Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.* **235**, 655–691.

AITCHESON, J. & BROWN, J. A. C. (1969). Factors for a homoscedastic quantitative probit analysis. In *The Lognormal Distribution*, p. 164. Cambridge: Cambridge University Press.

CASE, R., CREESE, R., DIXON, W. J., MASSEY, F. J. & TAYLOR, D. B. (1977). Movement of labelled decamethonium in muscle fibres of the rat. *J. Physiol.* **272**, 283–294.

CATTERALL, W. A. (1974). Sodium transport by the acetylcholine receptor of cultured muscle cells. *J. biol. Chem.* **250**, 1776–1781.

COOKSON, J. C. & PATON, W. D. M. (1969). Mechanisms of neuromuscular block. *Anaesthesia* **24**, 395-416.

COLQUHOUN, D. (1971). Nonparametric confidence limits for the median. In *Lectures on Biostatistics*, pp. 397–398. Oxford: Clarendon.

COLQUHOUN, D., RANG, H. P. & RITCHIE, J. M. (1974). The binding of tetrodotoxin and α-bungarotoxin to normal and denervated mammalian muscle. *J. Physiol.* **240**, 199–226.

CREESE, R. (1954). Measurement of cation fluxes in rat diaphragm. *Proc. R. Soc.* B, **142**, 497–513.

CREESE, R. (1968). Sodium fluxes in diaphragm muscle and the effects of insulin and serum proteins. *J. Physiol.* **197**, 255–278.

CREESE, R., EL-SHAFIE, A. L. & VRBOVA, G. (1968). Sodium movements in denervated muscle and the effects of antimycin A. *J. Physiol.* **197**, 279–294.

CREESE, R. & ENGLAND, J. M. (1970). Decamethonium in depolarized muscle and the effects of tubocurarine. *J. Physiol.* **210**, 345–361.

CREESE, R., FRANKLIN, G. I., HUMPHREY, P. P. A. & MITCHELL, L. D. (1976). Dose–response curves with labelled sodium and labelled decamethonium in rat muscle. *J. Physiol.* **254**, 43–44*P*.

CREESE, R., FRANKLIN, G. I. & MITCHELL, L. D. (1976). Two mechanisms for spontaneous recovery from depolarizing drugs in rat muscle. *Nature, Lond.* **261**, 416–417.

CREESE, R. & MACLAGAN, J. (1970). Entry of decamethonium in rat muscle studied by autoradiography. *J. Physiol.* **210**, 363–386.

CREESE, R. & MITCHELL, L. (1975). Sodium entry in junctional region of rat muscle. *J. Physiol.* **246**, 44–45*P*.

CREESE, R. & NORTHOVER, J. (1961). Maintenance of isolated diaphragm with normal sodium content. *J. Physiol.* **155**, 343–357.

CREESE, R., SCHOLES, N. W. & WHALEN, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

DREYER, F. & PEPER, K. (1975). Density and dose–response curve of acetylcholine receptors in frog neuromuscular junction. *Nature, Lond.* **253**, 641–643.

ENGLAND, J. M. (1970). The localization of end-plates in unstained muscle. *J. Anat.* **106**, 311–321.

EVANS, R. H. (1974). The entry of labelled calcium into the innervated region of the mouse diaphragm muscle. *J. Physiol.* **240**, 517–533.

FINNEY, D. J. (1964). *Statistical Method in Biological Assay*, 2nd edn. London: Charles Griffin & Co.

GEIGY. *Scientific Tables* (1970). 7th ed., pp. 105, 189, ed. DIEM, K. & LENTNER, C. Basle: Ciba-Geigy Ltd.

GINSBORG, B. L. & JENKINSON, D. H. (1976). Transmission of impulses from nerve to muscle. In *Handbook of Experimental Pharmacology*, ed. BURN, G. V. R., EICHLER, O., FARAR, A., HERKEN, H. & WELCH, A. D., vol. 42, ed. ZAIMIS, E., ch. 3, pp. 229–364. Berlin: Springer Verlag.

GOLDSTEIN, A. (1964). *Biostatistics*. New York: Macmillan.

HESS, G. P., ANDREWS, J. P., STRUVE, G. E. & COOMBS, S. E. (1975). Acetylcholine-receptor-mediated ion flux in electropax membrane preparations. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4371–4375.

HUXLEY, A. F. (1960). In *Mineral Metabolism*, ed. COMAR, C. L. & BRONNER, F., vol. 1, part A, pp. 163–166. New York: Academic.

KASAI, M. & CHANGEUX, J.-P. (1971). *In vitro* excitation of purified membrane fragments by cholinergic agonists. *J. Membrane Biol.* **6**, 58–80.

KATZ, B. & MILEDI, R. (1964). Further observations on the distribution of acetylcholine-reactive sites in skeletal muscle. *J. Physiol.* **170**, 379–388.

KATZ, B., & MILEDI, R. (1972). The statistical nature of the acetyl and choline potential and its molecular components. *J. Physiol.* **224**, 665–699.

KLAUS, W., LÜLLMAN, H. & MUSCHOLL, E. (1960). Der Kalium-Flux des normalen und denervierten Rattenzwerchfells. *Pflügers Arch. ges. Physiol.* **271**, 761–775.

KUSHMERICK, M. J. & PODOLSKY, R. J. (1969). Ionic mobility in muscle cells. *Science, N.Y.* **166**, 1297–1298.

MILEDI, R. & POTTER, L. T. (1971). Acetylcholine receptors in muscle fibres. *Nature, Lond.* **233**, 599–603.

PEPER, K., DREYER, F. & MÜLLER, K.-D. (1976). Analysis of cooperativity of drug-receptor interaction by quantitative iontophoresis at frog motor end-plates. *Cold Spring Harb. Symp. quant. Biol.* **40**, 187–192.

POPOT, J.-L., SUGIYAMA, H. & CHANGEUX, J.-P. (1976). The permeability response of the receptor-rich membrane fragments to cholinergic agonists *in vitro*. *J. molec. Biol.* **106**, 469–483.

RANG, H. P. (1975). Acetylcholine receptors. *Q. Rev. Biophys.* **7**, 283–399.

SUGIYAMA, H., POPOT, J.-L. & CHANGEUX, J.-P. (1976). Pharmacological desensitization *in vitro* of the receptor-rich fragments by cholinergic agonists. *J. molec. Biol.* **106**, 485–496.

TAYLOR, D. B., CREESE, R., NEDERGAARD, O. A. & CASE, R. (1965). Labelled depolarizing drugs in normal and denervated muscle. *Nature, Lond.* **208**, 901–902.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of end-plate membranes during the action of transmitter. *J. Physiol.* **154**, 52–67.

THESLEFF, S. (1955). The effects of acetylcholine, decamethonium and succinylcholine on neuromuscular transmission in the rat. *Acta physiol. scand.* **34**, 386–392.

(Reprinted from Nature, Vol. 261, No. 5559, pp. 416–417, June 3, 1976)

Two mechanisms for spontaneous recovery from depolarising drugs in rat muscle

THE action of depolarising drugs on the endplate of skeletal muscle is difficult to interpret because the depolarisation is often transient and followed by spontaneous recovery during the continued presence of the drug, and because of differences between species. In frog muscle, depolarising drugs produce an initial increase in conductance at the endplate, followed by a decline in the conductance as 'desensitisation' develops[1–3]. In cat muscle, depolarisation is maintained while the drug is applied[4]. Rat muscle has long been considered anomalous in that the depolarisation is transient and limited[5]. We now report an unexpected finding for rat diaphragm, in which the recovery is attributed to the action of an electrogenic sodium pump[6], stimulated by the entry of sodium.

Figure 1 shows the action of carbachol (100 μM) recorded with an internal electrode with tetrodotoxin (0.1 μM) in the outside bath to prevent action potentials. The recovery of the membrane potential in the presence of the drug might be due to closure of the ion channels after the initial action of the drug, or to another process. There are methods by which the entry of labelled sodium in the junctional region can be studied directly in diaphragm muscle[7], and dose-response curves have been obtained[8].

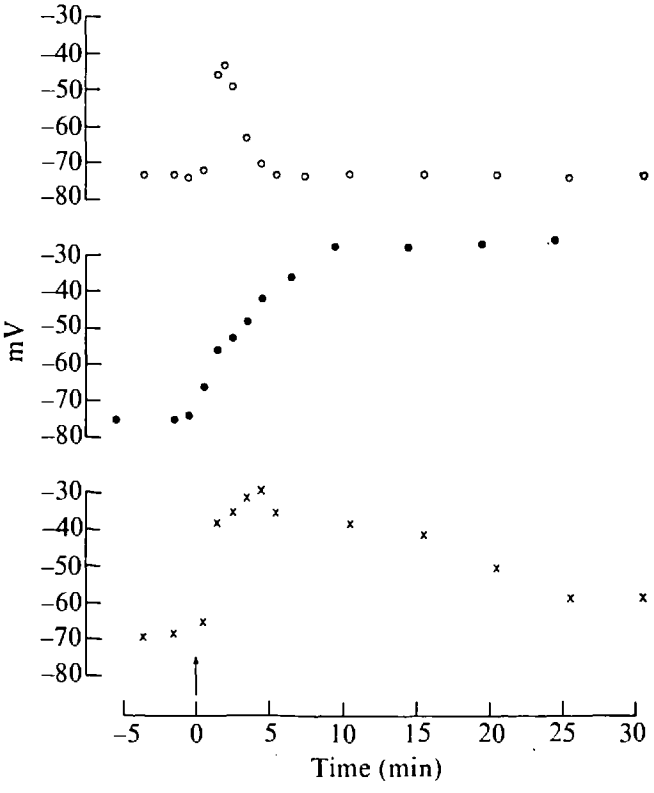


Fig. 1 ○, Effect of 100 μM carbachol at endplate, with internal recording[19]: muscle vertical, arranged for continuous register and rapid changeover, at 38 °C. The solution contained 5 mM potassium and 0.1 μM tetrodotoxin. The ordinate gives membrane potential, with depolarisation upwards. The abscissa gives time after the solution was changed. Miniature endplate potentials disappeared when carbachol was applied. ●, Effects of 100 μM carbachol plus 100 μM ouabain. The reading after 25–60 min with ouabain plus carbachol 100 μM was −31 mV (median of six estimations). ×, Effect of 3 mM decamethonium plus 100 μM ouabain: final reading, compared with initial resting potential, showed depolarisation of 9 mV (median of nine estimations).

Figure 2*a* shows the uptake of ^{24}Na in a diaphragm exposed for 15 s to a solution containing ^{24}Na and carbachol (100 μM) followed by washing for 6 min. Compared with the control (Fig. 2*b*), there was an extra uptake of ^{24}Na in the junctional region in the presence of the depolarising drug. The effect was similar (Fig. 2*c*) when the diaphragm was pretreated with carbachol (100 μM) for 30 min. These results (and others to be reported elsewhere) are consistent with the view that the ion channels continue to open while the drug is in contact with the tissue, and this differs markedly from the situation in frog muscle.

The recovery of the resting potential could be attributable to an electrogenic sodium pump, and in this case the process would be affected by certain enzyme inhibitors. When ouabain[9] and carbachol (100 μM) were added concomitantly (Fig. 1) there was a profound depolarisation and little evidence of recovery. In rat diaphragm, ouabain alone (100 μM at 38 °C) produced a depolarisation of 9 mV (median of 10 estimations, range 6–17 mV) by sampling methods used before[10]. This is a greater effect than that found in frog muscle at room temperature with strophanthidin[11].

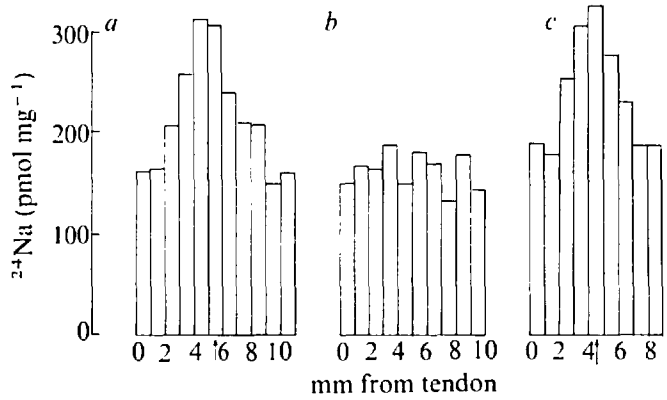


Fig. 2 *a*, Effect of carbachol on influx of labelled sodium in rat diaphragm in the presence of 0.1 μM tetrodotoxin. The diaphragm was exposed for 15 s to solution containing carbachol (100 μM) plus ^{24}Na, (1 mCi in 10 ml), then washed for 6 min in inactive saline, frozen, sliced and counted[7,20]. Ordinate gives ^{24}Na (pmol mg^{-1}); abscissa is distance from tendon (mm). The arrow indicates the strip of muscle which contained the band of endplates[20]. *b*, Control without carbachol. The uptake in *a*, above the level assigned to the ends of the muscle, gives the extra sodium which entered in the presence of carbachol and which remained after washing for 6 min: extrapolation to zero time is needed to give the uptake at the end of the exposure. *c*, Uptake in a diaphragm which had been incubated for 30 min with 100 μM carbachol and then treated as in *a*.

Figure 1 shows that the membrane potential returned, in the presence of carbachol, to a level close to the initial reading, and in nine experiments the difference ranged from −9 mV to +4 mV. It is not clear how the voltage could be regulated to give recovery to the previous value, and in other situations, with a higher initial resting potential, the recovery from depolarising drugs was incomplete in rat muscle[5].

Decamethonium (100 μM) in the presence of ouabain had an effect similar to that shown in Fig. 1 (closed circles). But with large concentrations of decamethonium the situation was different. Figure 3*a* shows the uptake of ^{24}Na in a muscle exposed for 15 s to decamethonium (3 mM) and washed for 6 min. There was an extra uptake in the junctional region compared with the value at the ends of the fibres, and this initial entry was comparable with the effect shown in Fig. 2*a*. Pretreatment with decamethonium (3 mM) for 30 min (Fig. 3*b*) gave little or no indication of an extra

uptake of sodium in the junctional region, and we infer that after 30 min at this concentration the channels for sodium had mostly been closed. When a solution containing ouabain (100 μM) and decamethonium (3 mM) was applied, there was an initial depolarisation (Fig. 1, crosses) followed by marked spontaneous recovery.

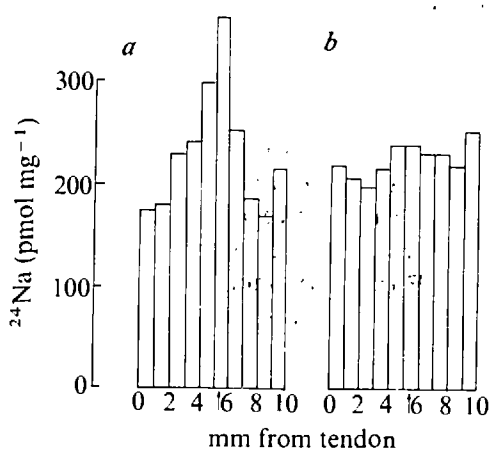


Fig. 3 Effect of 3 mM decamethonium on entry of ^{24}Na: exposure for 15 s, followed by washing for 6 min and treatment as in Fig. 2. The arrow shows the slice of muscle which contained the band of endplates. In *b* the muscle was incubated for 30 min with 3 mM decamethonium and then treated as in *a*.

In rat muscle there seems to be at least two mechanisms for the recovery of the resting potential. The first is an ouabain-sensitive process[12] in which sodium channels continue to open under the influence of the drug. This may explain why labelled decamethonium, which enters at the endplate region[13], accumulates steadily for many hours[14] in spite of desensitisation. The second process is seen with high concentrations of decamethonium and is largely unaffected by ouabain, apparently involving closure of sodium channels. The permeability to decamethonium falls in the mM range in certain conditions[15], and this could be due to channel block as postulated for frog muscle[3]. The alternative explanation in terms of receptor mechanisms is that the pharmacological receptors can be transformed to a desensitised state[16–18] in which ion channels become secondarily closed.

This research was supported by the MRC.

R. Creese
G. I. Franklin
L. D. Mitchell

Department of Physiology,
St Mary's Hospital Medical School,
London W2 1PG, UK

Received February 26; accepted April 13, 1976.

1 Manthey, A. A., *J. gen. Physiol.*, **49**, 963–976 (1966).
2 Jenkinson, D. H., and Terrar, D. A., *Br. J. Pharmac.*, **47**, 363–376 (1973).
3 Adams, P. R., *Pflugers Arch. ges. Physiol.*, **360**, 135–144 (1975).
4 Head, S. D., *J. Physiol., Lond.*, **241**, 102–103P (1974).
5 Thesleff, S., *Acta physiol. scand.*, **34**, 386–392 (1955).
6 Thomas, R. C., *Physiol. Rev.*, **52**, 563–594 (1972).
7 Creese, R., and Mitchell, L., *J. Physiol., Lond.*, **246**, 44–45P (1975).
8 Creese, R., Franklin, G. I., Humphrey, P. P. A., and Mitchell, L. D., *J. Physiol., Lond.*, **254**, 43–44P (1976).
9 Scou, J. C., *Biochim. biophys. Acta*, **42**, 6–23 (1960).
10 Creese, R., Scholes, N. W., and Taylor, D. B., *J. Pharmac. exp. Ther.*, **124**, 47–52 (1958).
11 Terrar, D. A., *Br. J. Pharmac.*, **51**, 259–268 (1974).
12 Mooji, J. J. A., Evers, C. D. J., and Ras, D., *Eur. J. Pharmac.*, (in the press).
13 Creese, R., and Maclagan, J., *J. Physiol., Lond.*, **210**, 363–386 (1970).
14 Nedegaard, O. A., and Taylor, D. B., *Experienta*, **22**, 521–522 (1966).
15 Creese, R., and England, J. M., *J. Physiol., Lond.*, **210**, 345–361 (1970).
16 Katz, B., and Thesleff, S., *J. Physiol., Lond.*, **138**, 63–80 (1957).
17 Rang, H. P., and Ritter, J. M., *Molec. Pharmac.*, **6**, 357–382 (1970).
18 Sugiyama, H., and Changeux, J. P., *Eur. J. Biochem.*, **55**, 505–515 (1975).
19 Creese, R., Scholes, N. W., and Whalen, W. J., *J. Physiol., Lond.*, **140**, 310–317 (1958).
20 England, J. M., *J. Anat.*, **106**, 311–321 (1970).

Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

Circuitry

The circuit (Fig. 1) is designed to give audiovisual signals via loudspeakers and light emitting diodes. One such circuit is required for each 'receptor'. The frequency of both sound and light signals follows the time course of the transistor, Q_1, output waveform, modified by the resistance of the strain gauge. The audiovisual signals thus simulate the passage of action potentials along the '1a' or '1b' afferent nerves. Both static and dynamic properties of the receptors can be demonstrated by appropriate adjustment of variable resistance VR_2, whilst sensitivity is adjusted by VR_3 and threshold by VR_1. The gain of the operational amplifier OPA_1 and the value of resistance R_3 are chosen to suit the characteristics of the strain gauge. The duration of the LED flashes, to be clearly seen, is controlled by a pre-set variable resistor.

The model has been used in a tutorial-demonstration to small groups for several years, as well as for practical illustration in lectures. Student response in a recent questionnaire was very favourable, and indicated that the model was successful in creating interest and expediting understanding of the working of the muscle spindle.

D. 7

Method for internal recording from diaphragm in a vertical position

By L. D. Mitchell (introduced by R. Creese). *Department of Physiology, St Mary's Hospital Medical School, London, W2 1PG*

For internal recording the rat diaphragm is usually mounted horizontally in a bath with flowing saline (e.g. Liley, 1956). The muscle can also be mounted vertically without flow in experiments designed to conserve drugs, and in situations when the oxygenation of the whole tissue is necessary. The rib was secured to one leg of a vertical holder, and the micro-electrode approached at an angle. The present method has been used for end-plate recording (Creese, Franklin & Mitchell, 1976), and is modified from Creese, Scholes & Whalen (1958).

This research was supported by a grant from the Medical Research Council.

REFERENCES

Creese, R., Franklin, G. I. & Mitchell, L. D. (1976). *Nature, Lond.* **261**, 416–417.
Creese, R., Scholes, N. W. & Whalen, W. J. (1958). *J. Physiol.* **140**, 301–317.
Liley, A. W. (1956). *J. Physiol.* **132**, 650–666.