SPONTANEOUS RECOVERY

FROM

DEPOLARIZING DRUGS

IN

GUINEA-PIG DIAPHRAGM

by

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ABSTRACT

Carbachol (80 μM), decamethonium (10 μM) and other depolarizing drugs produced an initial neuromuscular block in isolated guinea-pig diaphragm at 37°C, which was followed by spontaneous recovery of contractions elicited by nerve stimulation, with the later development of a slow secondary neuromuscular block.

With this preparation it was found that continuous microelectrode recording could be made at the end-plate for over 2 hr. Carbachol and decamethonium produced initial depolarization at the end-plate followed by spontaneous recovery of membrane potential which then remained steady indefinitely.

The involvement of an electrogenic sodium-potassium pump was implicated by the reversal or halting of the recovery process if external potassium was removed during spontaneous repolarization in the presence of carbachol or of decamethonium; by the small and incomplete repolarization in the absence of potassium; by the absence of repolarization in the presence of ouabain (10 μM); and by the consistent hyperpolarization which followed removal of the drug when spontaneous recovery of membrane potential had occurred in the presence of carbachol or of decamethonium. The results indicated that the sodium pump was switched on

at an early stage and that the effect was maintained while the muscle was exposed to the depolarizing drug.

With decamethonium and carbachol the input resistance, measured with 2 electrodes at the end-plate when the membrane potential had recovered in the presence of the drug, was diminished as compared with control muscles in the absence of drug. This finding is interpreted as indicating that channels continue to open in the presence of the stimulating drug. In other experiments it was found that decamethonium continued to accumulate for at least 2 hr in muscle exposed to tritium-labelled drug. Estimates of the end-plate current at this stage were 12 nA by electrical and 10 nA by isotopic measurements.

The space constant was determined to be 0.9 mm. The reversal potential in control muscle was -3.6 mV, measured by interpolation of miniature end-plate potentials. With certain assumptions, these measurements allowed the pump current and the conductance changes associated with the action of carbachol to be estimated.

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GLOSSARY OF ABBREVIATIONS

ACh	:	Acetylcholine
AP	:	Action potential
ATP	:	Adenosine triphosphate
Ca^{2+}	:	Calcium ion
Epp	:	End-plate potential
K^{+}	:	Potassium ion
Mepp	:	Miniature end-plate potential
Na^{+}	:	Sodium ion
V	:	Resting potential: also represents a voltage signal as recorded by internal electrode.
V_i	:	The battery formed by combining the 'ionic batteries" (Hubbard et al, 1968, p48).
V_{tr}	:	Transmitter equilibrium potential. The potential difference across the cell membrane that is unaffected by the action of transmitter (Ginsborg, 1967; see also Introduction p42).
V_{rev}	:	Potential at which no net current flowsthrough open end-plate channels.
R	:	Apparent or effective input resistance, measured as V/I.
R'	:	Apparent input resistance in the presence of agonist drug, being V'/I'.
R_{in}	:	Value of V/I when extrapolated to zero interelectrode distance, as in Fig. 6.7. Equal to $\frac{1}{2}(r_m r_i)^{\frac{1}{2}}$.
R_m	:	Transverse resistance x unit area of surface membrane, in Ωcm^2.
R_i	:	Specific internal resistance of the myoplasm, in Ωcm.
r_m	:	Resistance x unit length of the surface membrane in Ωcm. $r_m = R_m/2\pi a$, and also $2R_{in}\lambda$.
r_m^{-1}	:	Membrane conductance per unit length, in $\mu S\ cm^{-1}$.
r_i	:	Resistance per unit length of the myoplasm, in Ωcm^{-1} $(=R_i/\pi a^2)$
I	:	Current.
x	:	Interelectrode distance.
a	:	Radius of fibre.
λ	:	Space constant. Equal to $(r_m/r_i)^{\frac{1}{2}}$.
Ω	:	Ohm. Unit of resistance
S	:	Siemen (reciprocal ohm). Unit of conductance.

References for electrical nomenclature, other than those given above: Hodgkin and Rushton, 1946; Fatt and Katz, 1951.

C H A P T E R 1

INTRODUCTION

INTRODUCTION

Depolarizing drugs are used as chemical analogues of the transmitter acetylcholine and also as muscle relaxants in anaesthesia and in some neurological disorders. Their action as muscle relaxants was studied in cats by Paton and Zaimis (1948) and Burns and Paton (1951) who showed that the neuromuscular block was accompanied by depolarization in the region of the end-plate. The concept of 'Depolarization Block' was and is widely accepted as the basis of the neuromuscular block produced by such compounds as decamethonium and suxamethonium.

It soon became apparent however, that there was a wide variety of response in different species and in different members of the same species; to different drugs and to the same drug at different stages following prolonged use, and some of these effects are briefly described below. It has become clear that a number of different mechanisms need to be invoked to account for the prolonged actions of depolarizing drugs.

Neuromuscular blocking agents may be studied in preparations *in vitro*, in the whole animal *in vivo* and in anaesthesia and in human experimentation. Information regarding neuromuscular block can be obtained by stimulation of the motor nerve followed by recording of the electrical or mechanical response, and information obtained by other observations will not usually be reviewed in the rest of this Chapter.

It became evident from laboratory studies that on prolonged exposure of the end plate to depolarizing relaxants the characteristics of the block appeared to change. It was found by Jenden and his associates (1951; 1954) that depolarizing relaxants produced a two-phase block of neuromuscular transmission in vitro. They observed a phase I block of short duration, the intensity of which decreased spontaneously. Subsequently, the intensity of the neuromuscular block again increased, but this time very slowly. The characteristics of this block were quite different from those of the phase I block and it was not followed by spontaneous recovery.

The short term effects of the depolarizing neuromuscular blocking agents are now well documented, but the process of spontaneous recovery in their continued presence remains to be satisfactorily resolved. The investigations to be described here were designed to measure the neuromuscular blocking activity of various depolarizing drugs during prolonged application. A preparation has been found which displays complete recovery and a technique developed to continuously measure electrical events at the end-plate for long periods.

This introduction contains a review of the responses of selected species to depolarizing drugs . The section on the responses of human muscle is largely comprised of clinical accounts. Contenders for theories to explain recovery are introduced and current views briefly discussed. A plan of investigation completes the introduction.

Some terms used throughout the introduction and indeed, in the rest of the text, are briefly defined first.

Nomenclature

A brief resume of the origins of some terms relevant to this area of work should be beneficial before a more detailed review is made.

As already mentioned, the first reference to different phases of neuromuscular block by a depolarizing drug was made by Jenden, Kamijo and Taylor (1951). They reported two phases of action produced by decamethonium, as measured from the depression of indirectly-elicited contractions of rabbit lumbrical muscle *in vitro*, and assigned to them the terms 'phase I' and'phase II'. The characteristics of these blocks differed, as determined by the effect of d-tubocurarine, potassium and anticholinesterases.

Zaimis (1953) coined the term 'Dual Block' after examining the mechanical response of muscles to depolarizing drugs *in vivo*. Stressing the variation in response between species, Zaimis suggested that in some types of muscle these compounds exhibited a dual mode of action, beginning as depolarization but changing to competition with acetylcholine.

Thesleff (1955b) described the effects produced by large concentrations of depolarizing drugs in rat muscle, recorded by intracellular electrodes. Initial depolarization was

followed by partial recovery and the development of neuromuscular block without depolarization, and this was termed 'desensitization'. The word can be used as a description of the diminished response which occurs following continuous application of stimulant drugs.

Tachyphylaxis is a term commonly used to describe the waning effects of depolarizing drugs. Jewell and Zaimis (1954) detected changes in the depth of block produced by repeated intravenous injections of suxamethonium and decamethonium, at regular intervals, into cat tibialis muscle. No change in the mode of action of these drugs was reported and tachyphylaxis was used to describe the reduction of the degree and duration of block with subsequent doses.

The term 'fade' was used by Paton (1961) as a descriptive term for the waning effect produced by continuous application of drugs. It has the advantage that it implies no assumptions as to mechanism.

Species Variations in the Action of Depolarizing Drugs

There is a marked variation in the response to depolarizing drugs between different species and also between individuals of the same species. In addition, various muscles from the same animal are known to exhibit differing reactions. In mammals the sensitivity of different species to depolarizing drugs seems to be highest in the cat, and lowest in the rat.

The location of man in this scale of sensitivity remains to be satisfactorily resolved; Zaimis and Head (1976) have maintained that the responses in man and cat are similar. Reports of the responses of cat, rat and man to depolarizing drugs will thus be reviewed, with particular reference to accounts of spontaneous recovery and to 'changing modes of action'.

(i) Cat

Burns and Paton (1951), examining the action of decamethonium on a preparation of the cat's gracilis muscle in vivo, reported that a single intravenous administration led to a peak depolarization 3 - 5 min after injection recorded by wick electrodes, followed by a steady repolarization to the normal values, taking 30 - 40 min. They found that "decamethonium causes a depolarization centred around that part of the muscle which contains motor end-plates" but which subsequently extended beyond this area, the extent of the distribution increasing with time. It was suggested that the characteristic features of block by decamethonium and indeed, acetylcholine at the neuromuscular junction were simply those of any persistent cathode;as shown by such features as initial excitation, latency of onset while initial superexcitability passes to inexcitability, the spatial spread of inexcitability with time, the reversal of the block by an anode, and the lessening of the block by procedures which also lessen the associated excitation.

Prolonged or repeated application however, may produce different features. Paton and Waud (1962) demonstrated partial

repolarization in the cat in vivo by intra-arterial injections of suxamethonium at repeated intervals. Moreover, Creese and Maclagan (1976) have shown that prolonged infusion of ^{3}H-decamethonium in the cat produces initial paralysis in both fast and slow muscles, followed by recovery of the twitch response despite a plasma concentration of the drug which was maintained approximately constant. Extracellular recording of depolarization showed that this recovery of the twitch was accompanied by a waning of the end-plate depolarization produced by the drug. There was, however, no sign of the secondary block which was found in vitro and termed phase II block by Jenden (1954; 1955).

Zaimis has maintained that neuromuscular block is accompanied by prolonged depolarization of the end-plate region when either decamethonium or suxamethonium are used in the cat (Zaimis and Head, 1976). Jewell and Zaimis (1954) showed that in the cat a single depolarizing drug may produce different modes of neuromuscular block depending on the skeletal muscle being examined. Tibialis proved to be very sensitive to decamethonium, while soleus was particularly resistant.

It was also demonstrated that while interruption of neuromuscular transmission in tibialis showed all the characteristics of a typical depolarization block, there seemed to be a 'dual' mode of action in soleus during which a depolarizing phase was followed by a competitive one.

Although prolonged depolarization seems not to have been demonstrated in cat muscle in vivo, Wray (1981), working in Zaimis' laboratory, has shown that prolonged depolarization may occur when isolated cat tenuissimus muscle is treated with a low concentration of acetylcholine in the presence of an anti-esterase drug.

(ii) Rat

From experiments on isolated nerve-diaphragm preparations of rats, Thesleff (1955b) concluded that "neuromuscular block by acetylcholine, decamethonium and succinylcholine is not due to depolarization of the muscle membrane, and that the block is presumably caused by a decrease in the sensitivity of the end-plate to the transmitter substance". While using a Tyrode solution containing 2.7 mM potassium, Thesleff measured a depolarization from about -95 mV to about -65 mV with these drugs. The threshold depolarization for causing a neuromuscular block in the majority of the fibres was, as demonstrated by the use of high concentrations of potassium, -50 to - 55 mV. Thesleff attributed this limited depolarization in rat skeletal muscle to the layer of endothelium (pleura and peritoneum) covering the diaphragm, preventing instantaneous contact with end-plate 'receptors'. In addition, he observed that in the rat the whole muscle membrane showed a reduced membrane potential, which was in contrast to the frog where only the end-plate regions were depolarized. That the non-synaptic membrane influences the observed depolarization in response to transmitter, or an analogue, was proposed by

Fatt and Katz (1951) and has more recently been reviewed by Ginsborg and Jenkinson (1976). Extra-junctional conductance is thus implicated in explaining the limited depolarization in rat muscle.

The insensitivity of rat muscle to depolarizing neuromuscular blocking drugs has been reported by other workers (Ireson, Ford and Loveday, 1969; Derkx, Bonta and Lagendijk, 1971) the latter of these showing that neostigmine produced instant reversal of the block by suxamethonium. Galindo (1971; 1974) proposed that end-plate depolarization plays a minor role in the mode of action of succinylcholine or decamethonium during the initial neuromuscular block in rat, suggesting instead prejunctional failure of transmission. Galindo also described spontaneous recovery, followed by a 'phase II' block from which he inferred reductions in transmitter release and mobilization, increase in threshold for initiation by epps of muscle action potentials, and depression of cholinergic receptors. Gibberd (1966) suggested that the type of response to decamethonium depends on the method by which the drug reaches the muscle and the state of the muscle at the time. In a rat diaphragm preparation maintained by perfusion via the inferior vena cava, it was claimed that decamethonium produced a steady paralysis, whereas, in immersed muscles a biphasic neuromuscular block to decamethonium occurred.

Humphrey (1975) perfused rat diaphragm via the inferior vena cava and found that a steady level of neuro-

muscular block was not obtained by a constant concentration of decamethonium.

Humphrey (1973) also performed parallel experiments, in one series recording isometric contractions of the rat diaphragm *in vitro* and in another series recording changes in membrane potential via external electrodes. Using decamethonium he found that the time course and the concentration required for depolarization were quite distinct from those required for neuromuscular block, and hence concluded that neuromuscular block in rat muscle *in vitro* is not the result of a prolonged post-synaptic depolarization. Head (1975) demonstrated, in the isolated extensor digitorum longus muscle of the rat, that at no concentration of decamethonium did depolarization exceed 25 mV, that is, never depolarizing beyond a membrane potential of -60 mV.

Whole or partial recovery from neuromuscular block despite the continued application of a depolarizing drug has been demonstrated in rat muscle *in vivo* (Creese and Maclagan, 1970). Thesleff's observations (1955b) indicated partial recovery of potential in the presence of various depolarizing agents in a preparation of the rat *in vitro*, and this phenomenon has recently been investigated (Creese and Mitchell, 1981) using repeated sampling by internal microelectrode. These workers described a spontaneous recovery of membrane potential in the presence of carbachol and of suberyldicholine which is sensitive to potassium and to ouabain.

For rat muscle then, sensitivity to depolarizing drugs is generally believed to be low. Indeed, Zaimis and Head (1976) proposed that, of the species tested, rat muscle forms an extreme of insensitivity to decamethonium, while at the same time requiring the smallest dose of tubocurarine to block competitively.

(iii) Man

That certain bisquaternary salts might have a clinical usefulness was reported in 1948 by Paton and Zaimis. From their preliminary trials on cats they proposed decamethonium (C10) as a possible substitute for tubocurarine and emphasised that, in addition, it was potent and could be antagonised by a lower member of the same salt series, pentamethonium (C5). It was noted that eserine and prostigmine did not antagonise the C10 compound.

The first trials of decamethonium on man were performed by Organe, Paton and Zaimis (1949) at the Westminster Hospital using the authors as subjects. The similarity in the sensitivity to decamethonium of man and cat prompted Organe et al to point out that there appeared to be a relative sparing of respiration in man when compared with the action of tubocurarine.

These first reports formed the basis for the concept of 'respiratory sparing' whereby it was believed that abdominal relaxation could be obtained without interfering with spontaneous respiration. In search of this elusive goal

anaesthetists were initially loath to administer positive pressure ventilation to patients showing signs of prolonged apnoea, prefering the use of pentamethonium or hexamethonium as antagonists. Armstrong Davison (1950) however, stressed the need for caution when using these drugs because they were known to lead to cardiovascular collapse.

Questioning the concept of respiratory sparing, Dripps (1953) made an analysis of 1135 patients to whom Syncurine (decamethonium), had been administered. In comparing this group with that of 1335 individuals who had received d-tubocurarine during the course of surgical anaesthesia, he found no statistical difference in the incidence of prolonged apnoea or respiratory depression. Dripps thus dispelled the myth of respiratory sparing, indeed concluding that in some particular patients respiratory depression by muscle relaxants could occur without even a block of nerve-muscle conduction. He implicated a depressant action on the respiratory centre in association with other peripheral actions (via potassium metabolism, body temperature, elimination of drugs).

The nature of the neuromuscular blockade in man induced by decamethonium has induced much speculation. The first clinical report noted that the antidote for decamethonium was not neostigmine and later experience proved that instead of counteracting the relaxant's effect it could potentiate its action (Paton and Zaimis, 1949). This observation, when considered with the random spontaneous fasciculations which often precede the block either by decamethonium or suxamethonium

in man, made it quite clear for Zaimis (1959) that these drugs interrupt neuromuscular transmission by long-lasting depolarization. Zaimis also studied the effect of tetanic stimulation applied during a partial block and used this as a further indication of depolarizing block.

Bearing Zaimis' contention in mind, it is ironic that it was her report in 1953 suggesting a dual mode of action for suxamethonium and decamethonium in monkeys, rabbits, hares and dogs which provided substance for the claims of a changing nature of the block in man made by other workers (Payne & Holmdahl,1959;Katz, Wolf and Papper, 1963). Brennan (1956) described an analysis of 62 patients who had received suxamethonium by intravenous drip throughout operations and were given either 2 mg neostigmine or 10 mg gallamine. He reported that "neostigmine usually had an antidotal effect, gallamine always caused potentiation" and speculated that breakdown products of suxamethonium during the course of an operation might become "antidepolarizing agents"; he supported the use of neostigmine "in the treatment of prolonged apnoea or reduced pulmonary ventilation due to suxamethonium".

Churchill-Davidson and Christie (1959) presented evidence from patients undergoing routine surgery at St Thomas's Hospital, London. Neuromuscular transmission was measured by the application of supramaximal stimuli to the ulnar nerve at the elbow and the detection of electrical activity over the muscle mass of the abductor digiti minimi. They reported that following a single dose of either decamethonium or suxamethonium

the pattern of block was initially of the depolarization type: that is, well maintained action potentials with fast rates of stimulation and no post-tetanic facilitation. Edrophonium potentiated the block. Continuous infusion of either of the agents, however, led to a change in the pattern of the block as the total dose increased; fade in a series of action potentials, which was reversed by the injection of neostigmine. The authors thus concluded that block following the infusion of both suxamethonium and decamethonium gradually underwent a change and likened this observation to those made by Zaimis (1953) in animal studies.

Churchill-Davidson, Christie and Wise (1960) presented further clinical evidence for this change in block, and called upon the work of Creese, Dillon, Marshall, Sabawala, Schneider Taylor and Zinn (1957) to lend support to their suggestion. These workers examined the action of decamethonium on an isolated preparation of human intercostal muscle and reported an initial rapid block, followed by recovery and then the development of a slow secondary block, which resembled the results in guinea-pig (Jenden, 1955).

An extreme view point was taken by de Jong and Freund (1967) who recorded EMG's evoked by nerve stimulation and muscle tension in the thumb simultaneously. In their studies a 'phase I' block was never seen and they attributed neuromuscular block solely to a 'phase II' action, reporting fade of tetanic EMG, fatigue of tetanic tension and development of post-tetanic facilitation from the onset of relaxation.

The opposing attitude was maintained by Zaimis (1969) whose observations still suggested that no such alteration in response occurred at all. Cannard and Zaimis (1959) studied 16 subjects under anaesthesia and reported that the nature of the blockade, measured as the effect on isometric contractions of the leg muscles, produced by either suxamethonium or decamethonium in both warm and cold conditions appeared constant, however long the paralysis lasted.

Assessment of such findings is hampered by the multiplicity of techniques used by these workers when investigating the actions of neuromuscular blocking agents in man. As Zaimis (1969) pointed out, the fact that anticholinesterases reverse the block induced by suxamethonium or decamethonium cannot be taken as incontroversible evidence for the nature of the block. The increase in muscle contraction, seen by some workers, could have been the result of a direct effect of the anticholinesterase drug on a proportion of the muscle fibres which had already recovered from the effects of the drug. In addition, the prolonged administration of anaesthetics, under which these observations were made, is known to lead to prejunctional inhibition of acetylcholine release and postjunctional inhibition of end-plate depolarization (Karis, Gissen and Nastuk, 1967). Such actions depend on the nature and partial pressure of anaesthetic used but could produce some degree of blockade of a competitive type.

Interpretation is rendered all the more difficult because of the alteration of prejunctional function which has been

suggested with both decamethonium and suxamethonium (eg Standaert and Adams, 1965; Blaber, 1970). Although such evidence was obtained in the cat, impairment of neuromuscular action in man by such a process must be considered when a change in the nature of a block is being proposed.

Respiratory centre effects of the muscle relaxants, as proposed by Dripps (1953), formed the basis of Zaimis' criticism (1959) of studies in which the decreased activity of respiratory muscles was used as an indication of neuromuscular blockade. Concomittant central effects due to the presence of other drugs or to other factors during anaesthesia complicated such measurements.

Tachyphylaxis, the development of decreased sensitivity after the repeated administration of both decamethonium and suxamethonium, has been variously described in man. Foldes, Wnuck, Hamer Hodges, Thesleff and de Beer (1957) made an analysis of the prolonged application of these drugs in both anaesthetised patients and in isolated nerve-muscle preparations of frogs and reported a decrease in the sensitivity of the end-plate to these agents.

Reports differ, however, in the frequency of occurrence of tachyphylaxis. Payne and Holmdahl (1959) used an intermittent dose of 10 mg suxamethonium and reported that 73% of the patients developed tachyphylaxis under light anaesthesia. Freund (1969) gave repeated doses (0.05 mg/kg) when adduction of the thumb had recovered ¾ of its control twitch tension and reported tachyphylaxis in all (21) subjects. He also

showed a simultaneous change in the type of blockade as demonstrated by the reversal of the block by edrophonium. By comparison, Crul, Long, Brunner and Coolen (1966) used an intermittent dose of suxamethonium (0.125 mg/kg - 10 mg/kg) during anaesthesia and found 28.4% of the patients developed tachyphylaxis.

Such discrepancies probably result from differing experimental conditions. In a study by Naosuke Sagai, Hughes and Payne (1975) tetanic and single twitch contractions of the adductor pollicis muscle in man were recorded during repeated injections of suxamethonium (0.2 mg/kg or 0.1 mg/kg) every 15 min, while the patient was under the lightest possible halothane anaesthesia. Tachyphylaxis developed in every subject when single contractions were observed, but recovery was found later with tetanic contractions. To these workers, this result suggested a changing mode of action of suxamethonium, which they investigated by the administration of edrophonium during block. Blockade was first potentiated by this action, but when tachyphylaxis had fully developed on the single twitch, blockade of tetanic contraction was reversed. They concluded that "tachyphylaxis and the change in nature of the block caused by suxamethonium take place simultaneously in man and might be part of the same phenomenon". Another study using the same technique (Calder, Hughes and Payne, 1979) demonstrated tachyphylaxis after repeated dosage of decamethonium in anaesthetised man and also a change in the properties of the block to resemble more those of competitive

neuromuscular blocking agents.

Tachyphylaxis and prolonged apnoea are thus clinically associated with the use of both decamethonium and suxamethonium. The natural hydrolysis of suxamethonium by pseudocholinesterase in the circulation ensures that its action is the more brief and its use is preferred to that of decamethonium. Indeed despite re-appraisals of the use of low doses of decamethonium (Enderby, 1959) and the absence of muscle pains which usually follow the use of suxamethonium, decamethonium has fallen from grace with the anaesthetist.

Suxamethonium continues to be used at the end of an operation where a non-depolarizing relaxant has been employed. Neuromuscular function is assessed using indirectly elicited train-of-four muscle responses, a technique developed by Ali, Utting and Gray (1971) in an attempt to quantitatively assess residual block after the administration of a muscle relaxant. Rouse and Bevan (1979) used this procedure and determined that the effect of suxamethonium varied depending on the degree of recovery from the initial block produced by pancuronium bromide. Early in recovery suxamethonium reversed the block without paralysis whereas when administered later, initial recovery was followed by paralysis. In addition, injection of neostigmine after suxamethonium enhanced recovery from blockade, while its prior administration prolonged the paralysis. These workers concluded that suxamethonium produced a "mixed block" with "depolarizing" and "non-depolarizing" features.

Small doses of non-depolarizing relaxants are often administered 2-3 min before suxamethonium to limit some of the drug's adverse side-effects. 'Precurarization' seems to reduce the incidence of muscle fasciculations (Ferguson and Bevan, 1981) and post-operative muscle pains (Brodsky, Brock-Utne and Samuels, 1979). However, reports indicate variable success and the risk of prolonged paralysis limits the usefulness of pretreatment for short procedures.

Depolarizing neuromuscular blocking agents, by their very nature, increase the permeability of the end-plate membrane, which is accompanied by the release of an excessive amount of potassium ions into the extracellular space. This alteration in ion distribution may have an undesirable effect on the activity of various organs; in patients with neurological injuries or with severe burns, suxamethonium has been known to induce cardiac arrest due to its hyperkalaemic action. It is quite natural therefore, that the main trend in the search for a new neuromuscular blocking agent has aimed at creating short acting compounds with a non-depolarizing mode of action. Several compounds have been introduced: clinical evaluation of another has recently appeared (Fahey, Morris, Miller, Sohn, Cronnelly and Gencarelli, 1981) in which the pharmacology of ORG NC45, a pancuronium analogue, is reviewed. This relaxant appears to be more potent and to have a shorter duration of action with both initial and repeated doses than does pancuronium, is more easily antagonised with neostigmine and does not block at the vagal ganglion (Lee Son, Waud and Waud, 1981).

Desensitization

(The term has been introduced earlier). Desensitization of the post junctional membrane has been implicated as a likely mechanism to explain spontaneous recovery from depolarizing drugs. The gradual decrease of the end-plate response to various quaternary ammonium compounds was described by Thesleff (1955a) who reported that cells became repolarized in the continued presence of the drug almost to the normal level in 10 to 20 min. The desensitization of receptive sites was later studied by iontophoretic means by Katz and Thesleff (1957) who used a double-barreled micropipette labelled with acetylcholine or other choline derivatives in high concentration. They concluded that desensitization and recovery occur with a half time amounting to only a few seconds. Katz and Thesleff commented that the rate of desensitization shown in these experiments was much greater than that seen in Thesleff's earlier work. It has been demonstrated since that agonist concentration, extracellular ions, membrane potential, the nature of the preparation and temperature influence this process. The first three of these have been widely investigated and will be considered here.

(i) Agonist Concentration

Nastuk and Gissen (1965) studied the effects of increasing concentrations of carbachol in frog muscle. At 27 μM, the tension output curve fell and later recovered (cf Jenden, Kamijo & Taylor, 1951; 1954); the 'early' block appeared to be

associated with a reduction of the potential below the -55 mV level. With time, as the membrane potential recovered, electrical excitability returned and at this stage stimulation of the motor nerve produced end-plate potentials (epps) which were reduced in amplitude. In some junctions, no epps were detected.

When the carbachol concentration was increased still further to 55 μM, the tension output rapidly fell to zero and it remained at or near that level thereafter. Under these conditions the membrane potential remained low, but there was a partial return towards the control value. This repolarization was associated with a loss of response to carbachol.

During continuous application of carbachol, the rate and extent of repolarization of the post junctional membrane was found to increase as the drug concentration was elevated. Nastuk and Gissen concluded that the extent of desensitization increases as concentration increases.

The rates of repolarization in these experiments were less than those observed by Thesleff (1955). However, with local application by means of a pipette filled with 100 mM carbachol, Manthey (1966), obtained half-times for 'receptor-inactivation' of 8 - 10 seconds. These values approach the range of half-times for desensitization (1 - 7 seconds) reported by Katz and Thesleff (1957) in their iontophoresis experiments.

The rapid times found using iontophoresis probably result from the relatively high concentration of drug which is

effectively applied at close range to the postjunctional membrane. The resultant small depolarization indicates that only a fraction of the total number of ionic channels are activated by the iontophoretic application. However, these few sites which open channels are saturated and inactivated by the high concentration of agonist which is rapidly achieved in a relatively small region near the tip of the pipette. In an effort to achieve the most uniform concentration possible over the end-plate region consistant with rapid application, Scubon-Mulieri and Parsons (1977) used large perfusion pipettes to demonstrate the influence of carbachol concentration on the rate of desensitization. Their time constant for onset (7 seconds with 10 mM) were comparable to those obtained using iontophoretic agonist application and proved to be dependent on agonist concentration.

Scubon-Mulieri and Parsons used a potassium-depolarized preparation in these studies and employed input conductance changes as a measure of receptor desensitization. This they considered appropriate because it eliminated the change in membrane potential associated with receptor activation in polarized fibres. Rate of desensitization onset is known to be influenced by membrane potential (see later section). In addition, large ion shifts during the prolonged application of agonists prevent quantitative analysis of this rate from depolarization alone (Jenkinson and Terrar, 1973).

Voltage clamping of the membrane at the resting level avoids such drawbacks and has proved a useful quantitative tool for studies on desensitization (Adams, 1975; Kuba and

Koketsu, 1976; Lambert, Spannbauer and Parsons, 1977; Miledi, 1980). Studying the action of carbachol and other agonists on voltage-clamped frog end-plates, Adams (1975) showed that desensitization has an exponential onset with a rate constant which is proportional to the agonist concentration, and that this constant varies with the agonist used. The current rises and then falls to a low value, and the rate of fall is faster as the concentration is increased until it reaches a maximum rate.

Changes in the frequency of channel opening at the post-synaptic membrane induced by agonist action may be used as a measure of desensitization. Wray (1981) has made an analysis of membrane noise fluctuation during prolonged exposure of the cat tenuissimus muscle to bath-applied acetylcholine. At higher concentrations (10-50 μM), he demonstrated that desensitization occurred with an exponential fall to a plateau, while at low concentrations (1-2 μM) only slight desensitization occurred at a much lower rate.

(ii) Extracellular Ions

The concentration of external calcium ions is another important factor which influences the rate of receptor desensitization as Manthey has shown (1966; 1970; 1972). Manthey simultaneously measured the effective membrane resistance and membrane potential of frog muscle fibres during the continued application of carbachol by a pipette. Similarities in the change of both parameters showed that the desensitization

process affects both ionic permeability of the post-synaptic membrane and the potential. From measurements of input resistance, Manthey showed that the half time for desensitization is decreased when the external calcium is raised from 1.8 to 10 mM. The half-time is increased if calcium is removed.

Magazanik and Vyskocil (1970) used double-barrelled micropipettes to evoke test responses following a conditioning application of acetylcholine in frog muscle fibres. They too observed that calcium enhanced the rate of desensitization and proposed that, during the prolonged action of acetylcholine free calcium ions accumulate in the membrane and can be bound to the phosphate groups of phospholipids, forming complexes which control the pathways for ion permeability. Under conditions when increased binding of multivalent cations in the membrane can be expected (with a raised calcium concentration), the rate of desensitization is higher. From their own observations, Nastuk and Parsons (1970) suggested that such a reaction occurred between free intracellular calcium and anionic sites on the inner surface of the postjunctional membrane. Other evidence has supported this proposal (Cochrane and Parsons, 1972; de Bassio, Parsons and Schnitzler, 1976).

Scubon-Mulieri and Parsons (1977) made the interesting observation that desensitization onset was accelerated by a prior activation-desensitization sequence, and put forward a scheme to explain it. Internal calcium may accelerate the transition from the activated agonist/receptor complex to

the inactive state. Normally the concentration of intracellular ionized calcium is maintained at very low levels but rises in the vicinity of the end-plate during the action of an agonist, which causes not only an increase in sodium and potassium permeability but also in calcium permeability. A second application of agonist during the period when the internal level of ionized calcium is still elevated would further increase the rate of desensitization.

Usingiontophoretic application of acetylcholine to voltage-clamped end-plates in frog muscle, Miledi (1980) investigated this role of calcium. Miledi obtained desensitization when the concentration of calcium ions in the external medium was reduced to very low levels by chelating agents. He concluded that external calcium ions alter desensitization not by a direct interaction with the channels as they pass through them, but more probably because they raise the level of ionized calcium on the inside of the membrane. This calcium may exert its action directly on the receptor itself or may act indirectly through a combination with other macromolecules and thus regulate the time during which the receptor channels complex remains in an inactive form.

Other ions can influence the rate of desensitization. The effect of calcium is inhibited by both sodium and potassium (Manthey, 1966) and it is postulated that the monovalent ions competitively antagonise the action of calcium. Magazanik and Vyskocil (1970) investigated the ability of various multivalent

cations to increase the rate of desensitization and discovered that magnesium cannot be a substitute for calcium, whereas lanthanum led to an increased rate comparable with the effect of 18 mM calcium (see also Lambert and Parsons, 1970). It has been proposed that other ions which effect the rate of desensitization could act at the same specific site at the inner surface of the membrane as calcium does.

(iii) Membrane Potential

Magazanik and Vyskocil (1970) have shown in frog muscle that desensitization to ionophoretically applied acetylcholine depends on the membrane potential. Changes in the potential produced either by altering the external potassium concentration or by passing current revealed that the depolarization resulted in a decrease, hyperpolarization in an increase of the rate of desensitization. It was tentatively proposed that desensitization is not only restricted to the receptor level but that it also occurs, at least partly, at the terminal stages of the activation system.

The voltage dependance of desensitization onset has been confirmed by Scubon-Mulieri and Parsons (1978), who also showed a voltage dependance of the time-course of recovery from desensitization. Recovery proceeded more rapidly at -40 mV that at +40 mV with potassium-depolarized muscle fibres which were voltage clamped. The mechanism is unknown but it was suggested that because onset and recovery are influenced in a similar direction by membrane potential, the recovery is linked to onset.

Desensitization develops exponentially at all voltages and the time constant varies as a logarithmic function of membrane voltage in frog muscle (Fiekers, Spannbauer, Scubon-Mulieri and Parsons, 1980). Somewhat surprisingly, the voltage dependance remained in calcium-deficient solutions and was not altered by elevating either the level of extracellular or intracellular calcium. From the analysis of results obtained with bath-applied agonist, these workers postulated that the voltage sensitivity of desensitization primarily resides in the transition of the receptor/channel complex from a conducting to a non-conducting state. Voltage sensitive macromolecules in the membrane were postulated which become inactivated in "all or none" fashion, the decay of current reflecting a progressive decrease in the number of activated complexes.

(iv) The Reversal Potential During Desensitization

The transmitter reversal potential (null potential) is the membrane potential at which there is no net flow of current actross the subsynaptic membrane, and hence no change in membrane potential, when receptors are activated by transmitter. This does not necessarily mean that the net flux of any one ion is zero.

The reversal potential represents the maximum potential change that a transmitter can produce and has been found to be in the region of 0 to -15 mV (del Castillo and Katz, 1954b;Axelsson and Thesleff, 1959; Takeuchi and Takeuchi, 1960). This estimate represents an equivalent increase in conductance

of the end-plate membrane to sodium and potassium ions and hence lies between the Nernst potentials for the two ions.

Kuba and Koketsu (1976) estimated that the reversal potential for acetylcholine is shifted to more negative values from approximately -10 to -35 mV at desensitized end-plates. However, Katz and Miledi (1977) have queried the values obtained by Kuba and Koketsu on the grounds that they have been obtained by linear extrapolation of the current-voltage relation during Ach ionophoresis. The excitability of the muscle cells in the experiments of these latter workers prevented the clamping of the membrane potential at values less negative than -50 mV and hence they were obliged to make this extrapolation. Katz and Miledi avoided this source of error by either glycerol pre-treatment or the use of a hypertonic bathing medium to prevent contraction artefacts. The value of the reversal potential was then found by displacing the membrane potential until the response reversed sign, and then by 'bracketing' the null-point as closely as possible so that only a minimum interpolation was needed. Using this procedure, Katz and Miledi obtained a mean value for the change in reversal potential, due to either ionophoretically-applied acetylcholine or induced epps, of only -0.35 mV. They concluded that there is no significant alteration of the reversal potential attributable to desensitization.

A similar conclusion was reached by Lambert, Spannbauer and Parsons (1977) using neurally-induced end-plate currents in voltage-clamped frog muscle. These workers induced desensitization by bath-applied or pipetted carbachol, the solution being made 2.3 times hypertonic to minimise contraction. The reversal potential obtained from suitably curarized junctions (-2.4 mV) was similar to that obtained from carbachol-desensitized junctions (-4.4 mV). The determination of reversal potentials is beset by complications, but it is possible to avoid some of the problems by the use of interpolation rather than extrapolation. Methods used to prevent contractions of the muscle fibres to obtain such a goal may introduce their own artefacts. Efforts are worthwhile however, because knowledge of transmitter reversal potentials provides insight into the induced permeability changes.

(v) Models for Desensitization

Molecular models which have been considered for the mechanism underlying desensitization have been summarized as follows (Magleby and Pallotta, 1981):

(a) a sequential model in which some of the receptor-channel complexes with open channels proceed to the desensitized state at a characteristic rate (Katz and Thesleff, 1957; Rang and Ritter, 1970).

(b) a model where desensitization occurs as a result of agonist block of the open channel state of the receptor channel complex (Adams, 1975, 1976; Adams and Sakmann, 1978b).

(c) a model where receptors can interconvert directly between normal and desensitized states in a cyclic (reversible) fashion. Agonists bind to the desensitized state and shift the equilibrium towards a larger percentage of desensitized receptors (Katz and Thesleff, 1957; Rang and Ritter, 1970).

Realizing that the method of agonist application affects the desensitization process, Magleby and Pallotta (1981) have recently made a study using nerve-released acetylcholine in the frog. Their results proved to be inconsistent with the possibility that desensitization was a consequence of receptor activation (category (a)), resulted from the binding of acetylcholine to an open-channel state (category (b)) or developed from the binding of transmitter to receptors already in the desensitized state with a shift in the receptor distribution (category (c)).

The model for desensitization to nerve released transmitter proposed by Magleby and Pallotta is formulated from three non-conducting states of the receptor, where in the first step a "desensitizable state" is obtained and in the second, this state is desensitized. Their results suggest that the proposed desensitizable state of the receptor can have a lifetime of up to 25 msec and thus that desensitization does not occur if the interval between nerve impulses is 30 msec or greater (and the esterase is active).

The other main category of theories regarding desensitization includes models in which the process does not involve any

molecular change in receptors. This disagreement is based on experimental observations which are not predicted by the models above. As already mentioned, the rate of desensitization can be increased by raising extracellular calcium concentration (Manthey, 1966; Lambert and Parsons, 1970), by raising the tonicity of the bathing medium, or by substituting sulphate for chloride ions (Nastuk and Parsons, 1970). Furthermore, the rate of desensitization is increased by membrane hyperpolarization (Magazanik and Vyskocil, 1970). Finally, the rate of desensitization can be influenced by injecting pharmacological agents inside muscle cells (Magazanik and Vyskocil, 1972), though acetylcholine receptors are not affected by intracellular injection of acetylcholine (del Castillo and Katz, 1955). The alternative mechanism is that local accumulation of ions interfere in some way with end-plate currents, possibly by inducing a change in the properties of the ionic channel ('ionophore') itself; the most plausible candidate could be calcium ions which probably accumulate in the junctional region.

Recent advances in the resolution of membrane currents may serve to clarify the mechanism of desensitization. Neher and Sakmann (1976) first succeeded in observing directly, the current through individual channels by the 'patch clamp' technique, and analyses of the actions of various drugs have been made using this technique. It seems that whereas local anaesthetics block currents through single channels (Neher and Steinbach, 1978), acetylcholine at high concentrations in denervated frog muscle produces initial opening of channels

with pulses of current which lead to long periods of electrical silence with bursts of pulses at intervals (Sakmann, Patlak and Neher, 1980). It appears that the groups of pulses, seen between these gaps, all originate from the same channel, thus ruling out a channel-blocking mechanism. These findings have been confirmed for suberyldicholine (Colquhoun and Sakmann, 1981), and give some explanation for the fall in conductance associated with desensitization, but at present there seems to be no model which is entirely satisfactory.

The Sodium Pump

It is possible that a rise in intracellular sodium, caused by the prolonged application of a depolarizing drug, could initiate electrogenic transport. Such an exchange system might then contribute to the spontaneous recovery of the membrane potential in the presence of an agonist.

(i) Contribution to Membrane Potential

The electrogenicity of the sodium pump in other situations has been widely reported. Firm evidence (Straub, 1961; den Hertog and Ritchie, 1969) shows that at least part of the post-tetanic hyperpolarization in mammalian non-myelinated nerve fibres results from the activity of an electrogenic sodium pump that is normally dependent on the presence of external potassium. It also appears that the sodium pump in snail neurones is always electrogenic, with a sodium: potassium coupling ratio in the range 4.3 to 3.2 (Thomas, 1972).

Most investigations of the sodium pump in skeletal muscle have been performed on frog striated muscle. The most common method of study has been to load the muscle cells with sodium by soaking in ice-cold potassium free Ringer, thus inhibiting the pump, and then transferring the loaded muscle to room temperature with a recovery solution containing normal or elevated potassium. Using such conditions, a large number of studies have confirmed the electrogenicity of the sodium pump when it is extruding sodium ions from loaded cells (Desmedt, 1953; Frumento, 1965; Martirosov and Mikayelyan, 1970).

Determination of the steady state contribution, however, of the pump to the resting potential is not a straight forward affair. Inhibition of the pump is usually accomplished by decreasing extracellular potassium or by adding a specific inhibitor, such as ouabain, to the bathing medium. There are several problems in estimating electrogenic activity by pump inhibition, and these will be considered in the Discussion. However, it is generally accepted that depolarizations following within a few minutes of pump inhibition before marked changes in ion distribution can occur, indicate that there is an electrogenic contribution to the resting potential (Thomas, 1972). Locke and Solomon (1967) found that intravenous or topical ouabain caused significant depolarizations of the resting potential measured in vivo; gastrocnemius muscles were depolarized from -82 mV to about -66 mV and soleus from -69 mV to about -59 mV. Bray, Hawken, Hubbard, Pockett and Wilson (1976), in addition to showing that denervation

produces a marked fall in membrane potential of rat diaphragm, determined that ouabain (1 mM) produces a rapid (within 15 min) depolarization of 20 mV or more in innervated muscle. Their interpretation was that an electrogenic sodium pump makes a considerable contribution to the resting potential in normal muscle, and that this factor is removed by denervation and by application of ouabain; in denervated rat diaphragm ouabain produced no rapid effect but only a slow fall in membrane potential. However, an estimation of the maximum possible contribution of the sodium pump to a steady state resting potential by Thomas (1972) produced a figure of only about 10 mV.

(ii) Biochemical Mechanism

The biochemical mechanism of the sodium pump is well known (see reviews by Jørgensen, 1975; Wallick, Lane and Schwartz, 1979; Levitt, 1981). Elegant and quantitative characterisation of active transport of sodium and potassium has been accomplished using resealed red-cell ghosts (Glynn, 1968) and reconstitution of the ATPase from sarcoplasmic reticulum (Racker, 1972). It appears that each functional pump unit consists of two large subunits that span the membrane and two smaller glycoprotein subunits that are exposed on the outside of the cell. Each functional unit has one binding site for cardiac glycosides (available only on the exterior of the cell), one phosphorylation site (only on the interior), three binding sites for sodium and two binding sites for potassium (Wallick

et al, 1979). Energy from ATP hydrolysis drives active transport by causing conformational changes in the pump, with ions crossing the membrane by hydrophilic interactions with charged residues at the interfaces of two or more protein subunits in the membrane.

(iii) Coupling Ratio

The crucial question about the sodium pump with respect to its electrogenic nature is obviously the coupling ratio of the number of sodium ions extruded to the number of potassium ions taken up. The higher this ratio, the more electrogenic the pump, assuming that sodium and potassium are the only ions involved. Certainly for active transport across the erythrocyte membrane, the coupling ratio has been clearly defined as 3 sodium: 1 potassium (Post, Albright and Dayaru, 1967). Much evidence on the value of the coupling ratio in nerve and skeletal muscle cells is consistent with a figure of 3 sodium: 2 potassium, but it is possible that the exchange ratio may, in fact, be variable, depending on salient environmental factors. The pump's activity is a function of intracellular concentration of sodium and the extracellular concentration of potassium (Thomas, 1972; Akera and Brody, 1977), so it is a reasonable assumption that the coupling ratio may also be dependent on these factors (Brinley and Mullins, 1974). Indeed, Wang, Lindenmayer and Schwartz, (1977) have derived a kinetic model showing that the hydrolysis of ATP within the pump does not require the binding of a fixed ratio of sodium to potassium ions. These authors

have proposed that there may be various enzymes complexes, containing one and two potassiums and two or three sodiums, depending on the concentration of potassium and sodium. The data are consistent with the transport model of simultaneous interactions of inner and outer ligands with the pump binding sites, and not with a consecutive system.

Considerable evidence indicates that the sodium pump is often electrogenic, most probably because of an unequal coupling between ionic fluxes. The contribution of such a mechanism to the membrane potential may vary according to the tissue being investigated and also to the concentration of certain ions in the internal and external media.

Plan of Investigation

An investigation of spontaneous recovery from depolarizing drugs requires a tissue preparation with several attributes. Ideally, it should be relatively easy to isolate and to maintain its physiological status. The cat is a long established animal in neurophysiology and is sensitive to depolarizing drugs (Zaimis, 1953). It is, however, an expensive animal to use and a smaller mammal was sought. The limited response to agonists in the rat (Thesleff, 1955b) made this preparation impracticable. The guinea-pig hemi-diaphragm and phrenic nerve preparation appeared suitable in many respects. The muscle is sensitive to the blocking action of depolarizing drugs (Hall and Parkes, 1953) and, in addition, the

comparatively large fibre size (see Chapter 7) facilitated microelectrode studies.

A series of experiments involving the muscle contractions were intended to demonstrate spontaneous recovery of tension in the presence of various depolarizing compounds and also to determine suitable drug concentrations for subsequent experiments. Single microelectrode experiments were used to investigate electrical events at the end-plate which accompanied neuromuscular blockade. It was planned to study the recovery process by removal of potassium from the bathing medium or by the addition of ouabain, thus assessing any possible electrogenic component.

The permeability of the end-plate membrane during the prolonged application of a depolarizing drug was of interest, and so this was examined in two ways. Firstly, two-electrode experiments were performed to determine the input resistance of the muscle fibre. Secondly, radiolabelled drug was used as an indication of the duration of the permeability change. Pilot studies showed that the preparation could be used for prolonged experiments with microelectrodes and it proved possible to exploit this property.

C H A P T E R 2

METHODS

METHODS

Animals

All the guinea-pigs used in these experiments were random-bred albino males of the Dunkin-Hartley strain. The animals were delivered at the age of 2 to 3 weeks and were used within 7 days of this time. Delivery weight was specified as 150 to 200 g and upon experimentation was of the order of 225 g (median weight of a batch of 84 animals).

The techniques for dissection of the hemi-diaphragm varied slightly depending upon the nature of the experiment to be performed and are described in relevant sections of this Chapter.

Solutions

Table 2.1 shows the constituents of the physiological salines used in these experiments and gives the concentration of ionic species in mM.

The solution used to bathe the contracting preparations resembled that which flowed over the preparation in the microelectrode studies. Both were modifications of the mammalian saline developed by Krebs and Henseleit (1932). The potassium concentration of the normal saline for use in the electrode experiments was 4 mM, while it was 5 mM in the contraction

Table 2.1 Physiological Salines

	MW	Krebs (1932)	For Contractions	For Micro-electrode
NaCl	58.5	0.9 g 100 ml	6.923 g	6.923 g
$NaHCO_3$	84.0	1.3 g 21 ml	2.1 g	2.1 g
KCl	74.5	1.15 g 4 ml	0.376 g	0.3 g
$MgSO_4.7H_2O$	246.4	3.82 g 1 ml	0.294 g	0.294 g
KH_2PO_4	136.0	2.11 g 1 ml	-	-
$NaH_2PO_4.2H_2O$	156.0	-	0.185 g	0.185 g
Glucose	180.0	0.26 g	2.0 g	2.0 g
$CaCl_2$	111.0	0.11 M 3 ml		
$CaCl_2$ (1M)			1.9 ml	2.5 ml
		130 ml	to 1000 ml	to 1000ml
[Concentrations]		mM	mM	mM
NaCl		118.3	118.3	118.3
$NaHCO_3$		25.0	25.0	25.0
KCl		4.7	5.0	4.0
$MgSO_4 7H_2O$		1.2	1.2	1.2
KH_2PO_4		1.2	-	-
$NaH_2PO_4.2H_2O$		-	1.2	1.2
$CaCl_2$		2.5	1.9	2.5
Na^+		143.3	144.5	144.5
K^+		5.9	5.0	4.0
Mg^{2+}		1.2	1.2	1.2
Ca^{2+}		2.5	1.9	2.5

Solutions modified from Krebs and Henseleit (1932). Analar grade salts (BDH) in deionized water. Saline bubbled with 5% CO_2 and 95% O_2 before addition of $CaCl_2$ (BDH 1 molar solution).

experiments. The diminished potassium concentration which was used for electrical recordings increased the polarity of the cell membrane such that the actions of depolarizing drugs were made more apparent.

The calcium concentration in the microelectrode saline was 2.5 mM, as used by Krebs. It is probable that the ionized calcium concentration of this solution was higher than that of serum but it served to aid the establishment of a stable resting potential following insertion of the electrode tip.

The potassium free saline used in the microelectrode studies was made by omitting potassium chloride from the solution. In such cases the potassium chloride was replaced by sodium chloride, the substitution was such that the solution was of the same ionic strength as the normal saline.

All the solutions were gassed with 5% carbon dioxide and 95% oxygen for 1 hr previous to use. At such a time and at 37^{o}C, measurement of the pH yielded a figure between 7.3 and 7.4.

One l of saline was usually concocted for experiments involving the contracting preparation while the flowing nature of the bath used in the microelectrode studies necessitated 5 l. Such volumes were suitably subdivided for the addition of relevant compounds.

Drugs

The drugs used in these investigations were obtained in powder form and periodically made up to a stock solution in 0.9% saline. This solution was usually 5 mM in concentration and was stored in a refrigerator until required. Exceptions to this were acetylcholine and physostigmine which were dissolved shortly before the experiment.

Drugs used were:

Acetylcholine chloride (BDH Chemicals, MW 181.7)

Carbachol (Koch-Light, MW 182.7)

Decamethonium bromide (Burroughs Wellcome, MW 418.3)

Ouabain (strophanthin-G) (BDH Chemicals, MW 728.8)

Physostigmine sulphate (BDH Chemicals, MW 648.8)

Suberyldicholine iodide (Dr A. Ungar, University of Edinburgh, MW 600.0)

For experiments with uptake of radiolabelled drugs the following were used:

Decamethonium-(methyl-H3) chloride (Radiochemical Centre, MW 329.0)

Decamethonium iodide (Koch-Light, MW 512.3)

Methods for Experiments Involving the Contracting Preparation

(i) Dissection Technique

Guinea-pigs were stunned by a blow to the back of the neck and laid prone such that the ventral aspect was uppermost. Skin and connective tissue were rapidly cleared and the thorax

opened by incisions parallel with the caudal ribs which were then extended laterally to meet at the sternum.

A fine thread was attached to the most rostral portion of the left phrenic nerve which was accessible in the thorax. The nerve was severed distally to this knot and was then carefully cleared of the connective tissue which intimately bound it to the pericardium. Ensuring that the thread was not jerked, a 2 - 3 cm length of nerve could be laid out of the way over the ribs which remained attached to the diaphragm.

A rectangular slip of diaphragm was isolated by 2 incisions parallel with the muscle fibres, 1 either side of the point of attachment of the left phrenic nerve. These cuts transected the caudal ribs and extended to almost meet at the tendinous end of the diaphram fibres. Just before totally severing this remaining connection, the hook of a platinum wire was inserted through the tendon. After the final cut, the entire preparation could be removed from the animal and placed in warm aerated saline. The total time from stunning to immersion was kept to a minimum and was usually less than 2 min.

(ii) Mounting, Stimulation and Recording

The preparation was attached to a suitable holder while immersed in a shallow bowl of saline. The holder was fabricated from Perspex and was L-shaped, with an additional cross-piece about 1/3 up from the base which contained 2 rings of platinum wire for stimulation purposes.

Two platinum hooks about 1 cm apart at the base of the holder served as the attachment points for the arc of the transected ribs. The cord which was tied to the phrenic nerve was passed through the pair of stimulating rings and the nerve placed into contact with each of these. Care was taken not to put any strain on the nerve.

The holder, with muscle, was removed from the shallow bath and mounted in a jacketed glass flask with parallel sides, which just contained the entire assembly. The slip of diaphragm was immersed in the 50 ml of saline which filled the flask. The solution was aerated with the aforementioned gas mixture via a sintered glass bubbler at the base of the flask. The jacketing formed part of a closed loop system through which water at 37^{o}C was circulated by means of a Circon Unit.

The tendon of the diaphragm was connected to a force-displacement transducer (Dynamometer U.F.I: Bioscience) by means of the platinum wire. Tension developed by the diaphragm was displayed as a curvilinear displacement of the ink trace on the moving paper of a Washington 400 MD 2C Oscillograph via a direct input coupler (FC100) and an amplifier (CD 10).

Suitable connections were made between a Grass SD 9 stimulator and the holder such that it was possible to indirectly stimulate the diaphragm via the phrenic nerve. It was arranged that the electrode closest to the muscle carried the cathodal current. The stimulator was set to deliver a square pulse of 0.05 msec duration every 5.6 sec. Before the experiment

was commenced the voltage/response relationship of the preparation was determined, the voltage at which the response was just maximal being doubled to ensure supra-maximal stimulation throughout the proceedings. The resting tension of the muscle was also optimally set at this stage to yield the maximum force that could be developed upon each stimulation. This was accomplished by adjustment of the transducer which was mounted on a Palmer rack and pinion.

The solutions were heated to 37^{o}C and gassed via sintered bubblers. A storage flask (100 ml) was clamped above the muscle bath. By means of glass taps and lengths of polythene tubing the saline could be gravity fed to the bath containing the preparation which, in turn, could be voided of its contents via a tap at its base. Careful manipulation of the taps ensured that at no time during the change of solutions was the muscle exposed to the atmosphere. This technique whereby a larger volume of fluid replaced the original volume, enabled the rapid application of drug to the tissue. Total time for transference of solution did not exceed a few seconds.

Tension developed by the muscle following each stimulation was monitored for at least a quarter of an hour prior to the administration of any drug. During this time observations were made to ensure that the magnitude of the contractions was constant and that the resting tension (as depicted by the baseline) did not fluctuate.

Following the cessation of contractions in the presence of a drug, the preparation was stimulated directly by attaching a lead to the platinum wire which perforated the tendon, the

other electrode being provided by one of the platinum hooks which secured the rib. It usually proved necessary to increase the stimulating voltage to a figure approaching 100 V to obtain a strength of contraction akin to that recorded at the beginning of the experiment.

Methods for Experiments with Microelectrodes

(i) Dissection Technique

Guinea-pigs were stunned by a blow to the back of the neck and the thorax opened as previously described. An incision in the abdomen made it possible to grasp the caudal ribs, which remained attached to the diaphragm, with a pair of forceps. So doing, 2 parallel cuts were made about 0.5 cm apart, transecting the ribs and extending about 1.5 cm to reach the tendinous region of the diaphragm. A transverse cut of the tendon was made to join these and the rectangle of muscle removed and pinned to the floor of the experimental bath. At such a time the solution would be already flowing. Total time from stunning to immersion was less than 90 sec. Experience dictated the optimal points for cutting, such that the resultant section would not be too heavily sheathed in connective tissue and would contain a number of terminating nerve fibres and associated end-plates.

One series of microelectrode experiments involved the analysis of neurally-evoked end-plate potentials (epps) and it was thus necessary to dissect out an area of diaphragm which

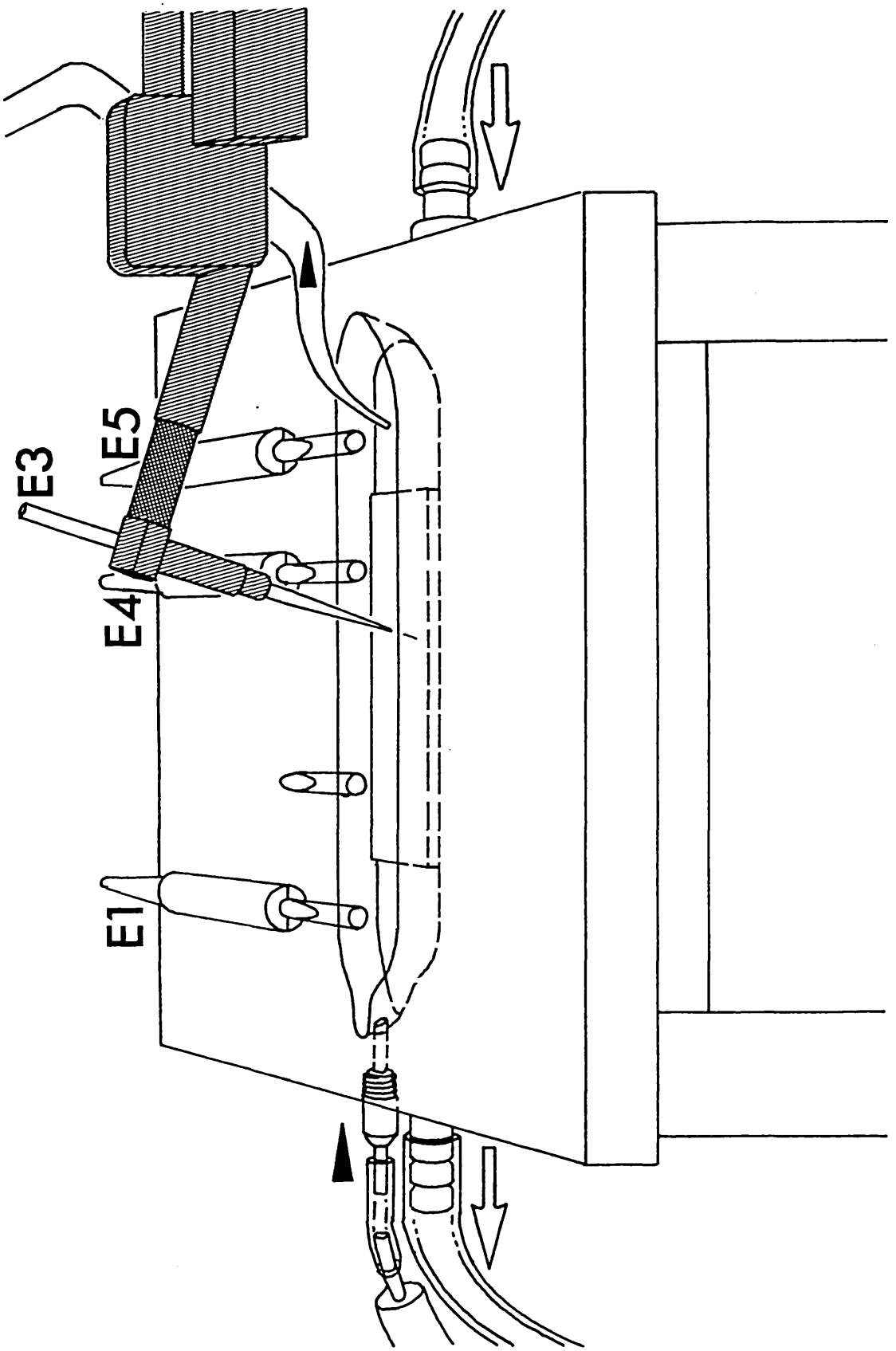
would be suitable for electrode insertions but would also retain its connection with the phrenic nerve. Such a preparation involved the sectioning-out of a rectangular area of muscle of the dimensions described above and which was still connected to the rib. Furthermore, a 2 - 3 cm length of nerve remained in situ with the preparation, this being accomplished by the technique described earlier.

(ii) Mounting of the Preparation

After removal from the animal, the diaphragm preparation was transferred directly to the chamber of the bath. Fig. 2.1 is a diagrammatic representation of this bath and shows the 3 mm thick layer of Sylgard resin which lay at its base. By means of fine stainless steel pins, the muscle was carefully spread over the resin, pleural face uppermost, with the rib section overhanging. Excessive connective tissue was removed from the surface of the muscle by means of micro-dissection scissors while observation was made via the microscope.

The bath was machined from a solid block of Perspex. Only the base plate was removable, this being secured by nylon screws and sealed with a reinforced gasket. The open arrows in Fig. 2.1 indicate the direction of flow of the heating water. A large proportion of the bath, both beneath and to the sides of the muscle chamber, was hollow and was connected to a heating circuit (described later). The temperature of saline in the muscle chamber could be maintained at $37^{o}C \pm 0.5^{o}C$ and was periodically checked by a thermistor

Figure 2.1. Diagrammatic representation of the tissue bath used in the microelectrode experiments. E1, E4 and E5 are reference electrodes and E3 is the glass microelectrode. Solid black arrows indicate the flow of physiological saline. Open arrows show the flow of the water which maintained the bath and its contents at 37°C. The shaded area represents the micromanipulator and electrode holder, while the dark (cross-hatched) area indicates the insulating nylon section.


E3
E5
E4
E1

inserted into the bathing medium. A sheet of fine wire mesh mounted in the hollow base of the bath was connected to the earth circuit and provided a shield against radio interference.

Three glass flasks each containing 1 l of saline, were mounted such that their bases were about 70 cm above the level of the bath. These are depicted in Fig. 2.2 and may be seen in the left hand photograph of Fig. 2.3. Relevant flasks held drugs in solution while sintered glass bubblers aerated their contents with a gas mixture of 5% carbon dioxide and 95% oxygen. Each flask tapered to a glass tap which led to a short length of narrow-bore silicon tubing. By means of small clamps, these tubes could be constricted to regulate the flow rate of the solution. The flow rate was usually set at about 25 ml/min.

Three glass tubes conducted the solutions from their respective reservoirs through a 45 cm-long heat exchanger. The jacket of this was maintained at a constant 55^{o}C by means of a Circon Unit and a remote water bath. The flow of heating water passed in a direction counter to that of the saline. Upon exit from the exchanger, the solutions had attained a temperature of 35 - 37^{o}C.

A nylon multiway tap mechanism located just within the Faraday cage made it possible to switch rapidly between the solutions draining from the 3 flasks. The design of the tap allowed saline to run to waste before it was admitted to the

Figure 2.2. Diagrammatic representation of the apparatus used for heating, bathing and viewing the preparation in the microelectrode experiments.

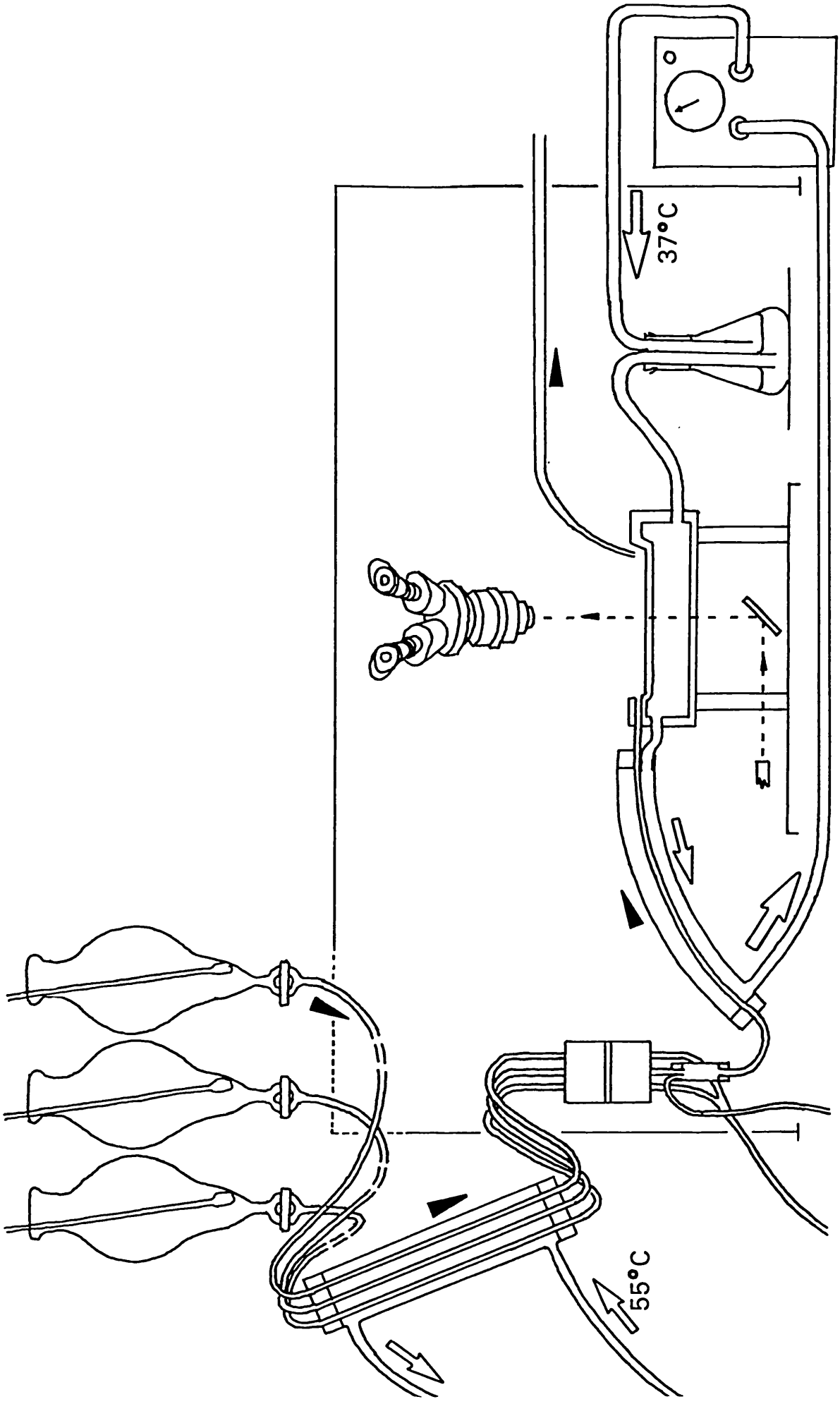
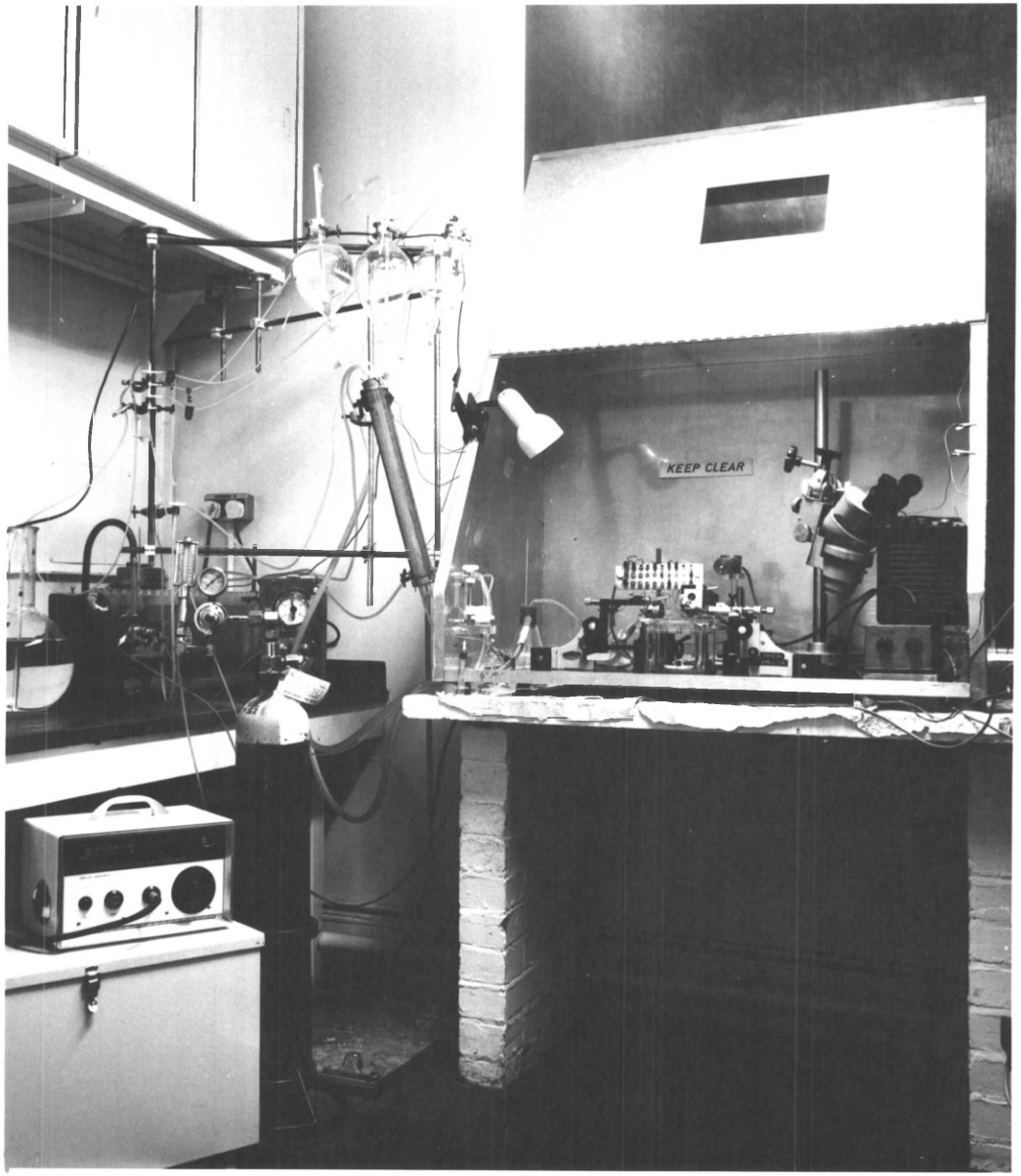

37°C
55°C

Figure 2.3. Views of the microelectrode equipment taken from 2 angles. The left-hand plate shows the Faraday cage which contained the tissue bath and first stage of the recording equipment. To the left of the cage are the flasks for the solutions, the water heaters, the gas cylinder and the fibre-optic light source. The anti-vibration system comprised 1) felt pads and rubber blocks between the brick pillars and the concrete table, and 2) rubber bushes (Dunlop) beneath the steel plate on which the bath was mounted.
The right-hand plate shows the rack system flanking the cage which bore the digital multimeter, oscilloscope, stimulator, calibrator and pen recorder. The length of the co-axial screened cable which linked this apparatus to that in the cage was kept to a minimum to avoid interference.


KEEP CLEAR


KEEP CLEAR

muscle chamber. A small glass bubble trap was located just beneath the tap in the line that fed the bath and enabled any gas which came out of solution to be removed from the system.

A second heating circuit was used for fine control of the temperature of the solution entering the muscle chamber and this is illustrated in Fig. 2.2 (open arrows show direction of flow). Water was maintained at 37^{o}C and circulated through the system by a remotely-positioned Churchill fluid pump. An anti-surge tank formed by a half litre flask was interposed between the pump and the bath to minimise vibration due to turbulance. Having passed through the cavity in the bath, the water flowed along a 60 cm length of 1 cm diameter silicon tubing before returning to the Churchill unit. This tube contained a concentric nylon tube of smaller diameter which bore the saline flowing out of the bubble trap in a countercurrent direction (black arrows). The temperature of the solution which ultimately bathed the preparation was thus identical to that set on the Churchill.

It was sometimes necessary to insert a small piece of silicon rubber as a baffle across the muscle chamber to minimise vibration caused by turbulence on the surface of the solution. This would be lodged across the bath in the path of the incoming fluid at the position of the electrode El (Fig. 2.1).

Saline was removed from the muscle chamber of the bath via a glass suck-out tube. This is depicted at the right hand end

of the chamber in Fig. 2.1 and consisted of a low-profile curved length of tube which tapered to a fine nozzle. The rate of suck-out just exceeded that of the incoming flow and thus the depth of fluid in the bath could be altered by vertical adustments of the nozzle, which was mounted on a Palmer rack and pinion for this purpose. Suction was provided by a Watson-Marlow Flow Inducer which was mounted outside the cage and drained the solution to waste via a sealed flask which ensured electrical discontinuity.

Transillumination of the muscle preparation was accomplished using a fibre optic cable and a Barr and Stroud Light Source, the intensity of the light being variable up to a maximum of 150 W. Remote positioning of the Light Source, which contained a fan for cooling purposes, ensured no vibration was transmitted to the bath and provided cold light. The termination of the cable beneath the bath is illustrated in Fig. 2.2, as is the concave mirror used for focusing and alignment of the light.

Polarizing filters were interposed between the mirror and the Nikon SMZ-6 Stereo Zoom Microscope, one being mounted directly beneath the Perspex bath and the other attached to the rotatable nosepiece of the microscope. Adjustment of these filters provided a visual contrast when viewed through the microscope such that the localisation of end-plates was enhanced (Creese and Mitchell, 1981). By replacement of the eyepieces the microscope provided a range of magnification from X9 to X80 and was thus suitable for all of the required observations, from pinning out of the preparation to positioning of micro-

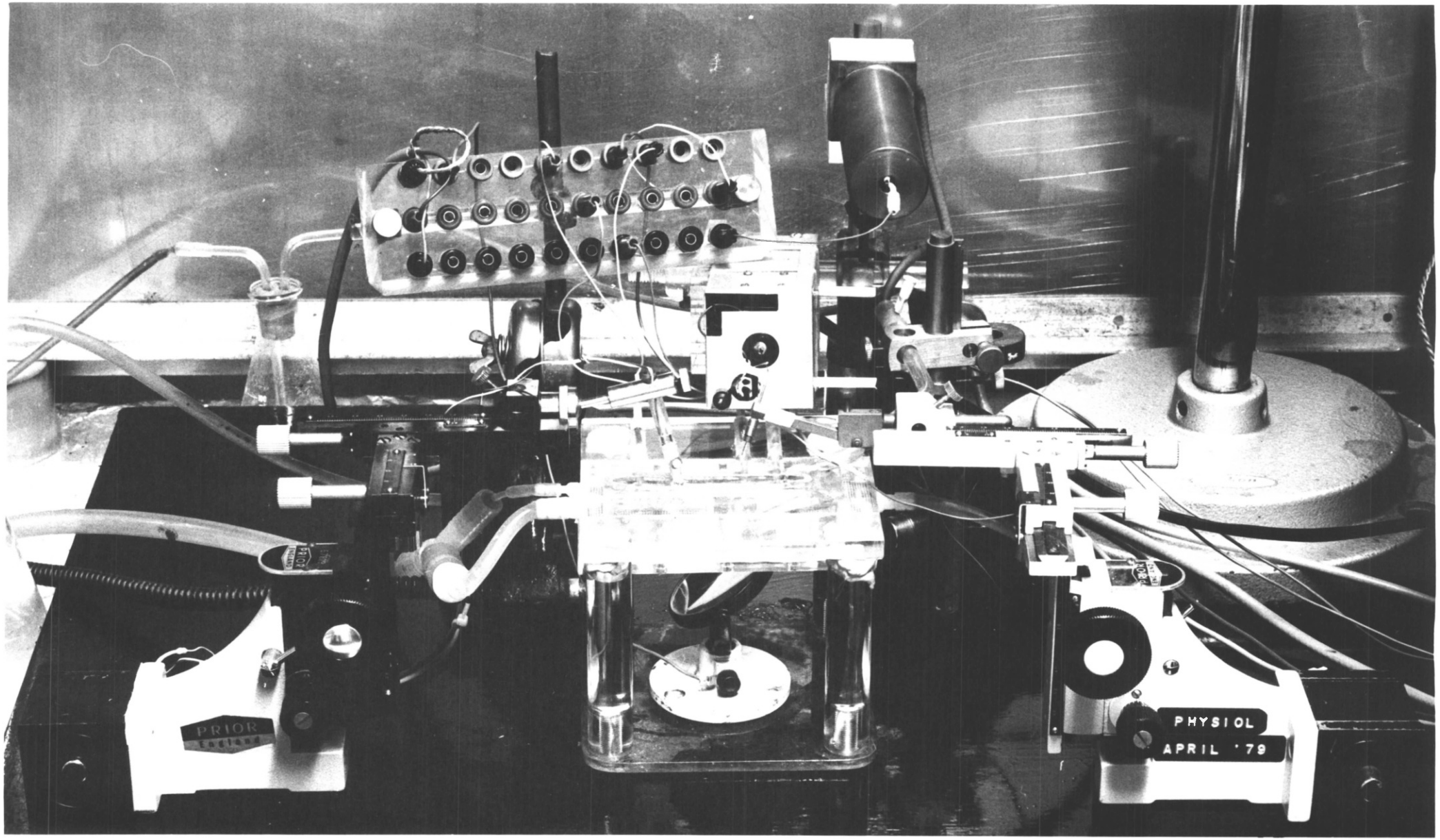
electrodes. Earthing of the microscope and its stand provided an additional screen against interference.

Isolation of the muscle bath and closely associated microelectrode equipment from external sources of vibration was an important consideration during the design of this apparatus. Relative motion between the electrode and its inserted position in the cell membrane was known to be a major difficulty in the establishment of a steady resting potential and so efforts were made to minimise this. The photographs comprising Fig. 2.3 show the brick pillars which were cemented directly to the concrete floor and which thus provided a rigid base for the suspension system. A solid concrete table bridged the pillars being supported via strips of impregnated felt (Adhaesium Machine Mounting) and rubber blocks. The mounting compound was claimed to absorb high-frequency vibrations.

A heavy steel base plate, 1 cm thick, stood on the concrete table suspended on 6 Dunlop Instrumounts, low-frequency-absorbing rubber bushes. Situated on this plate (Fig. 2.4) was the bath, the micromanipulators, the microscope, the cathode-follower valves and associated electrical connection board. All possible sources of vibration were kept off this plate.

The aforementioned equipment was contained within the aluminium Faraday cage which was mounted on thick rubber supports. The shield also encompassed the multiway tap, anti-surge tank, the batteries and the cathode follower control unit, other apparatus being positioned outside it (Fig. 2.3).

Figure 2.4. Apparatus mounted on the base plate within the Faraday cage. In the centre is the Perspex tissue bath, flanked on either side by the micro-manipulators. The right hand manipulator bears the microelectrode while the left hand one holds the stimulating electrode. The two valves of the cathode follower are above the bath, the lower being connected to the microelectrode via a screened length of chlorided wire.


PRIOR
England
PHYSIOL
APRIL '79

(iii) Electrodes

(a) Microelectrodes

15 cm long capillary tubes (nominal diameters; 2mm external 1 - 1.2 internal) were sorted according to their internal/external diameter ratios. Tubes were first selected with external diameters between 0.072 - 0.076 inch by means of a brass plate with holes drilled in it; drill-bits of known size were used to select internal diameters between 0.046 and 0.051 inch. The ratio internal diameter/thickness (where thickness is external diameter minus internal diameter) was found empirically to be 3.9; ratios between 3.2 and 4.3 were acceptable.

After sorting each tube was divided into 2 and washed in alcohol, then in hot water with detergent, rinsed, dried, and stored in a sealed container to prevent contamination by dust. The electrodes were pulled by a two-stage instrument (Narishige). in which the tubes were clamped vertically and heated until the glass began to draw and separate, at which time an elecro-magnet cut in and pulled the 2 halves apart. Two useable microelectrodes were thus obtained from each tube. The settings of the heating coil and of the electromagnet were empirically adjusted to give an acceptable appearance of the tip and a suitable electrode resistance.

The microelectrodes were filled with 3M KCl by boiling them in an evacuated flask, their shanks being secured to a rigidly fixed Perspex rod by an elastic band. The KCl was passed through a 0.22 μm Millipore filter before each filling.

Excessive agitation of the electrodes during the boiling process was avoided, as this tended to fracture the tips.

About 20 electrodes would be made by this method and used within 2 days. Before any experiment was commenced, samples from the batch of new electrodes were tested by measuring their tip potential, resistance (see below) and noise. Ideal figures for freshly filled microelectrodes were respectively 4 - 8 mV (Adrian, 1956), 20 - 30 MΩ and 0.2 - 0.3 mV noise (peak to peak). Usage of the electrodes after a prolonged period was not advisable because of increased electrical noise.

Precise movements of the microelectrode in relation to the surface of the muscle preparation were made possible by the use of Prior Micromanipulators. These are seen flanking the bath in Fig. 2.4 and the terminal holder of one is depicted by the shaded area in Fig. 2.1. The shank of the microelectrode (E3) was an interference fit in the brass holder which was cathode screened. The cross-hatched area in Fig. 2.1 indicates the nylon section which insulated the holder from the remainder of the micromanipulator.

(b) Reference electrodes

The reference electrodes are depicted in Fig. 2.1 as E1, E4 and E5. E1 and E5 served to earth the solution: E4 was connected to the input cathode follower. Each one was fabricated from machined Perspex tubing and provided electrical contact between the solution in the muscle chamber and relevant apparatus. This was accomplished via a small coil of

electrolytically-chlorided silver wire which was set in a potassium chloride based Agar medium. The hollow nozzles of the reference electrodes were exposed to the saline.

Reference electrodes typically had a resistance less than 5 K Ω. Reference electrodes and microelectrodes contained chlorided silver wire prepared by passing a current of 0.1 mA for 24 - 48 hr.

(iv) Electrical Apparatus

(a) General

A high-impedence differential cathode follower comprised the input stage to the recording equipment (shown in electrical flow-chart, Fig. 2.5). The electrical circuit of the cathode follower is shown in Fig. 2.6. The cathode follower allowed the voltage signal to be passed to the oscilloscope without amplification but also without distortion. The two ME 1400 (Mullard) thermionic valves were positioned close to the bath (see Fig. 2.4), being mounted on insulating stands and shielded by cathode screens. A link between the 'active' (lower) valve and the microelectrode E3 was provided by a length of chlorided wire inserted into the latter and soldered to a short cathode-screened cable.

Reference electrode E4 provided continuity between the bath solution and the appropriate valve via a millivolt calibrator. Electrodes E1 and E5 earthed the solution in the muscle chamber to a common point through the insulated peg-board visible in the photograph. Hence the cathode follower

Figure 2.5. Flow-chart of electrical equipment. The tissue bath is represented at the bottom, the numbers indicating electrodes referred to in the text as E1 to E5. Electrode E3 is shown cathode-screened and is the microelectrode used for voltage recording. E2 is a microelectrode used for current injection in some experiments. E1 and E5 serve to earth the solution. E4 is the reference electrode and passes to the grid of the second valve.

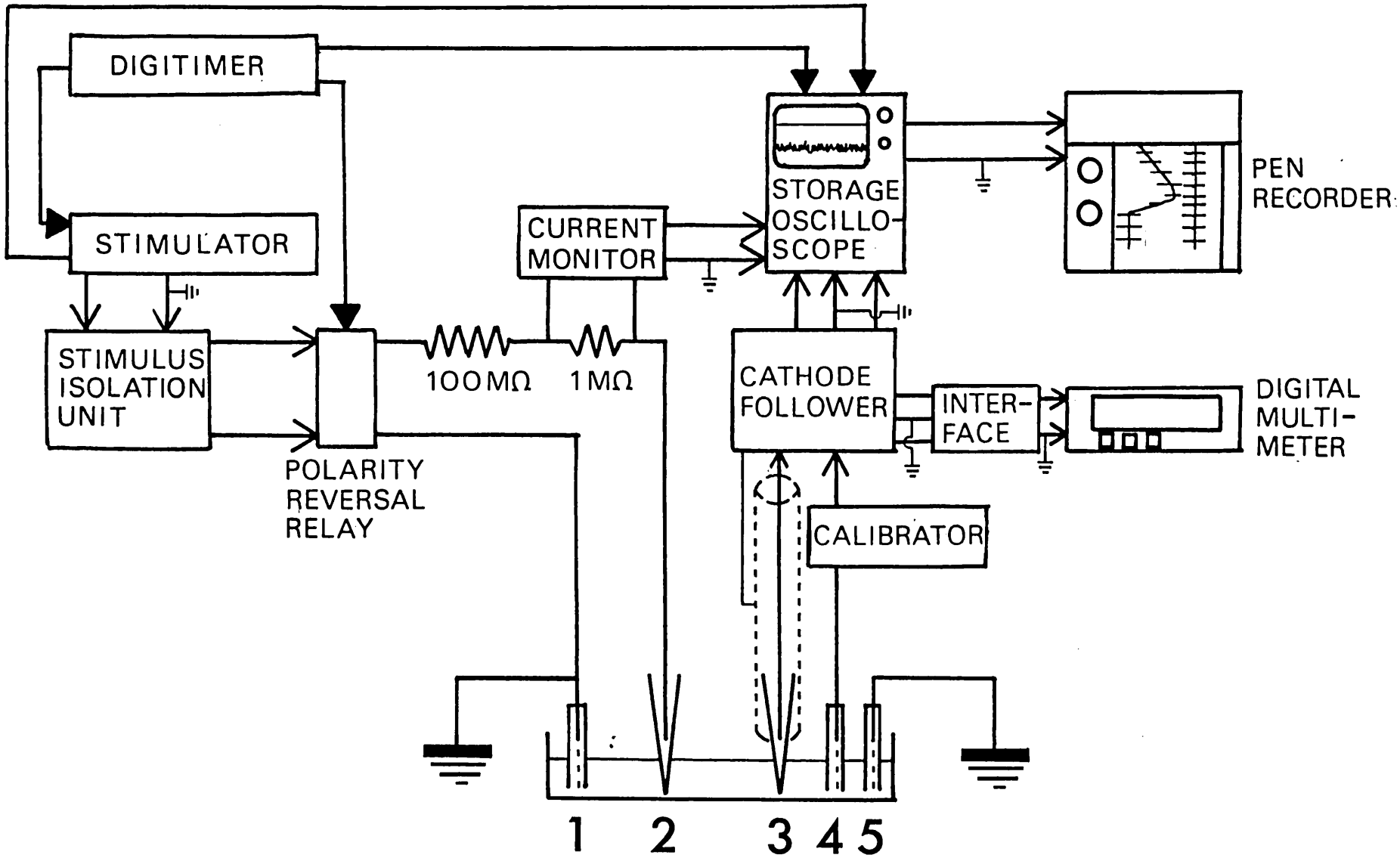
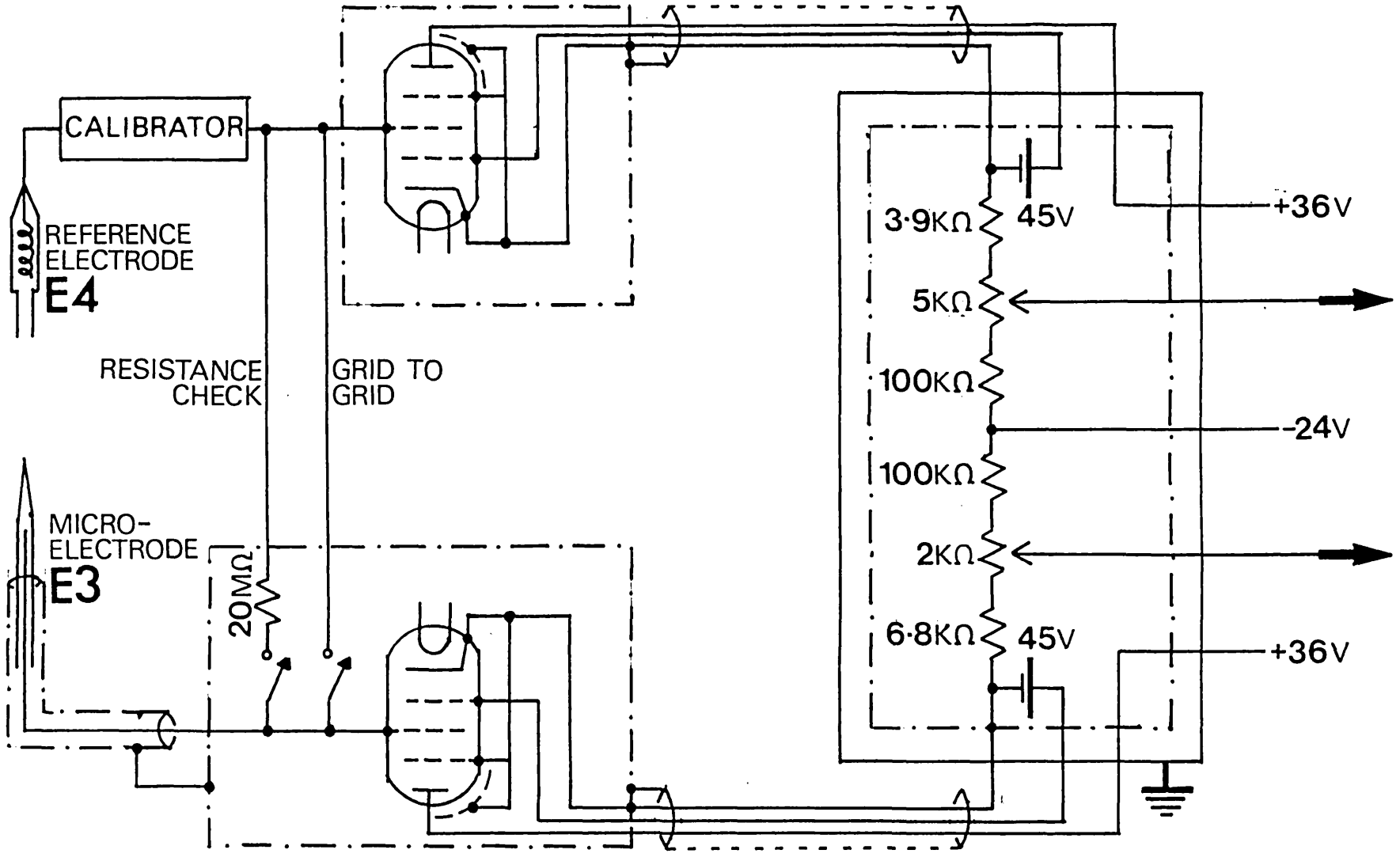

DIGITIMER
STIMULATOR
STIMULUS ISOLATION UNIT
POLARITY REVERSAL RELAY
100MΩ
1MΩ
CURRENT MONITOR
STORAGE OSCILLO-SCOPE
PEN RECORDER
CATHODE FOLLOWER
INTER-FACE
DIGITAL MULTI-METER
CALIBRATOR
1 2 3 4 5

Figure 2.6. Circuit diagram for cathode follower (after K. Copeland). Two ME 1400 valves were respectively connected to a reference electrode and a microelectrode. Each valve was cathode-screened, as were the main unit and the leads supplying it. The main unit was encased in an earthed box. Connections between the valves enabled either the grids to be linked, or the interposition of a 20 M Ω resistance in series with the microelectrode such that the resistance of the latter could be checked. Outputs from the cathode follower (black arrows at right of figure) led to differential amplifier of oscilloscope.


CALIBRATOR
REFERENCE ELECTRODE
E4
RESISTANCE CHECK
GRID TO GRID
MICRO-ELECTRODE
E3
20MΩ
3·9KΩ
45V
+36V
5KΩ
100KΩ
-24V
100KΩ
2KΩ
6·8KΩ
45V
+36V

was electrically semi-'floating', its closest position to earth being the bathing medium. The calibrator provided a voltage source with low constant resistance.

The valves were connected to the control unit of the cathode follower (visible at the right of the cage in Fig. 2.3) by cables which bore current to the heating coils and the signals from the cathodes. Potentiometers on this unit enabled the cathode follower system to be balanced such that when there was a zero differential input there was a zero differential output. Shielded co-axial leads carried the output to a Fluke 8000A Digital Multi-Meter via a high-impedence interface, and also to the differential amplifiers of a Tektronix 5115 Storage Oscilloscope, all of which were mounted in a rack system. The interface to the multi-meter was necessary to transform a differential input into a single-ended output, and also served to prevent radio frequency feedback to the recording equipment. The gain of the interface was set such that a direct reading of the potential, as detected by the microelectrode, was displayed on the meter. The facilities of the oscilloscope made it possible to simultaneously display gross resting potential as a direct-coupled (DC) trace and the same signal at a much higher magnification but as an AC coupled trace. Both channels had an upper band-width limit of 10 KHz.

Photographs of stored images could be taken from the oscilloscope screen using a Polaroid camera, loaded with high speed 'instant' film. Outputs from the oscilloscope fed the

signal to a Tekman flatbed pen recorder, calibration of which was accomplished using the millivolt calibrator.

Shielding from interference was accomplished by a variety of precautions. The cathode follower apparatus and muscle bath were contained within an aluminium Faraday cage while aluminium foil was used to form a shield beneath the steel plate. Both of these screens were earthed to a common point, as were all metallic objects within the cage. Water in both heating circuits and the physiological saline were also earthed to the same point by suitable connections. The 12 V battery which powered the thermionic heaters was negative ground. The cathode shield of the microelectrode has been described above: the control unit of the cathode follower was cathode-screened and also earth-screened (see Fig. 2.6).

Measurement of the electrical resistance of the micro-electrode was made with the electrode tip in the solution, by comparison of a signal obtained from a known voltage (calibrator) with that obtained when the microelectrode was placed in series with a resistance of 20 MΩ (which was incorporated into the input stage of the cathode follower).

(b) Specific for two electrode techniques

Three series of measurements required the use of a technique which involved two electrodes; space constant, input resistance and reversal potential. Positioning of the electrode for voltage measurement (E3 in Fig. 2.5) was accomplished as described in section (v) below . A second microelectrode (E2) for current injection was mounted on a left-hand Prior micro-manipulator.

The space constant, λ, was determined by square-pulse analysis (Fatt and Katz, 1951; Boyd and Martin, 1959). Current was passed through electrode E2, and the resulting change in transmembrane potential was detected by electrode E3. Current intensity was measured by recording the voltage drop across a 1 MΩ resistor in the current-passing circuit (see Fig. 2.5). Only hyperpolarizing pulses were used. Electrotonic potentials produced by a constant current pulse were recorded at different inter-electrode distances, as measured by micrometer. The order of insertion of electrode E3 at different distances along a muscle fibre was varied from fibre to fibre, and a run was only accepted if the resting potential did not fall below -75 mV. Transmembrane potential change and current were displayed on the dual beam oscilloscope and photographed using the Polaroid camera. The term V/I was plotted against inter-electrode distance on a semilogarithmic scale (see Fig. 6.6 and Fig. 6.7).

For experiments involving measurement of input resistance, R (= V/I), the second electrode E2 was positioned within 80 μm of the first microelectrode E3. This distance, x, was less than 0.1 of the space constant λ, (see Discussion).

Once both electrodes were in position, a preprogrammed timing device (Digitimer D4030) was employed to send commands to salient pieces of equipment (see Fig. 2.5). Using this technique, in sequence: (a) the oscilloscope screen was cleared and primed for external triggering, (b) the Grass S44 Stimulator passed one 500 msec pulse to the electrode E2 via the SIU5 Stimulus Isolation Unit, (c) a relay reversed the

polarity of the stimulus and (d) a second pulse was passed. Electrical pulses of alternating polarity were thus injected into the muscle fibre via the stimulating electrode at a rate which could be varied to suit that of the events occurring at the cell membrane.

The strength of the current pulses was present before the experiment at 10 nA by monitoring the potential difference across the 1 MΩ resistance. Use of the current monitor made it possible to measure the pulse strength during the course of the experiment. The pen recorder displayed membrane potential with electrotonic potentials, and also the output from the current monitor (eg. Fig. 6.4).

To measure the reversal potential, V_{rev}, electrode E3 was inserted in the end-plate and the other E2 within 80 μm of the first. Electrode E2 was used for passing direct current from the Grass S44 stimulator for 15 sec periods to displace the membrane potential. In the control experiments, the reversal of the sign of mepps was used to 'bracket' the null-point so that V_{rev} could be obtained by interpolation (see Katz and Miledi, 1977). Mepps at different holding potentials were recorded on an FM Store 4 Racal thermionic tape-recorder, and their mean amplitude determined on a PDP 11/10 Digital computer according to a program described by Head (1983). Computed values were occasionally compared with a manual estimate, and found to be in good agreement.

After prolonged exposure to carbachol, the membrane potential recovered in the presence of the drug, but mepps did not reappear. The reversal of the sign of neurally-evoked epps was therefore used in this second series to determine V_{rev}. The epps were small in these desensitized muscles (see Fig. 8.3) and usually did not evoke an action potential. No special precautions were necessary to prevent contractions in this preparation.

(c) Specific for end-plate potentials (epps)

The investigation of the action of depolarizing drugs on epps required a special preparation which has already been described. Stimulation of the phrenic nerve was accomplished using the electrode shown mounted to the left-hand manipulator in Fig. 2.4. The nerve was laid on the platinum wires of the electrode and was submerged in the bathing saline. The cathodal wire was closest to the muscle. Earthing of the solution made it unnecessary to isolate the stimulating electrode from the recording electrode, the stimulus artefact normally appearing only a few mV in height on the oscilloscope trace.

A Grass SD9 Stimulator was remotely positioned in the rack system (right hand photograph in Fig. 2.3) and its pre-pulse was used to trigger the oscilloscope. Stimulus duration was 0.05 msec and the voltage was set to obtain a maximal contraction of the muscle, as determined visually.

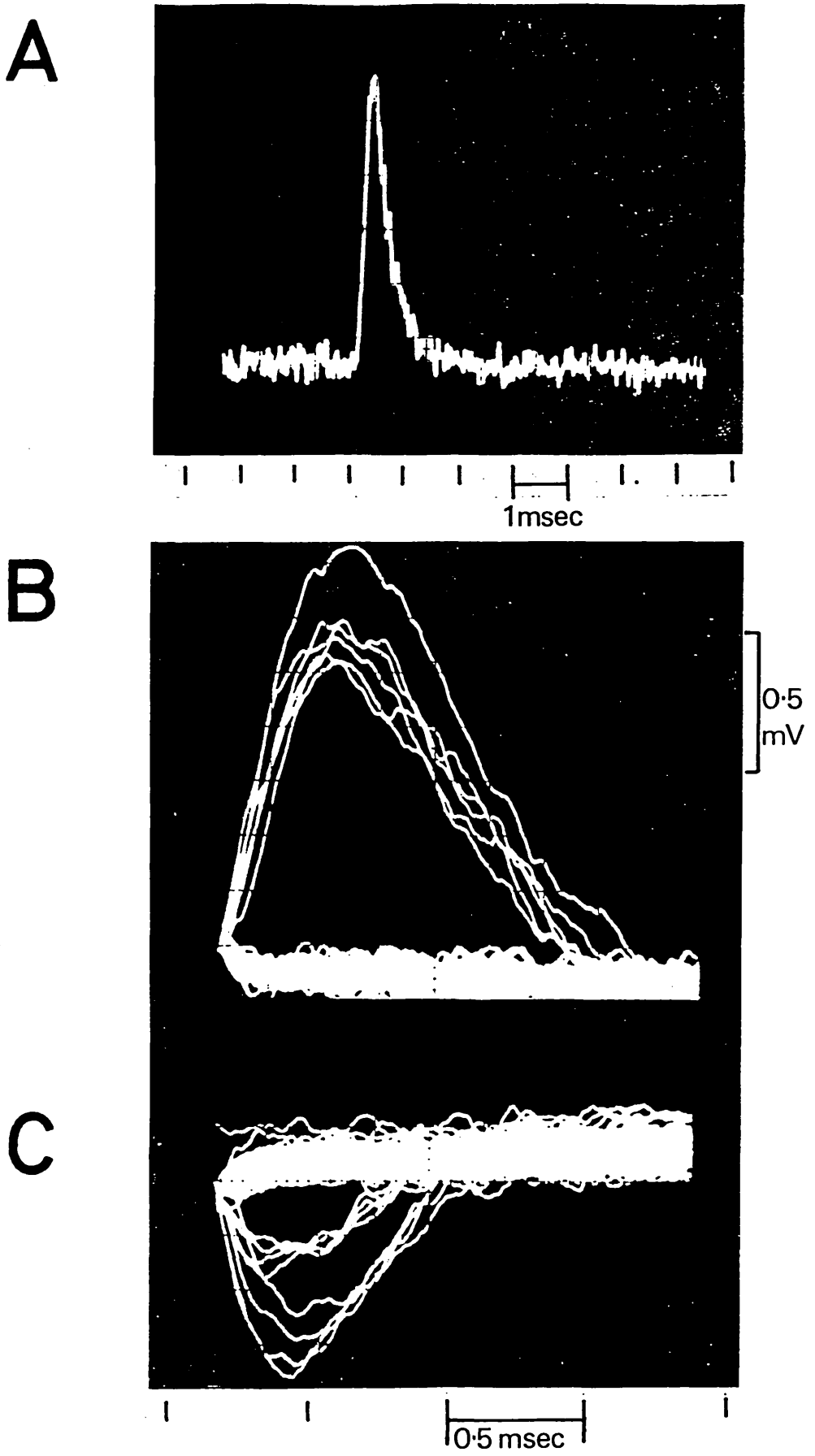
No precautions were taken to diminish the excitability of the muscle-fibres, and hence action potentials were usually accompanied by the expulsion of the electrode. If a further recording was required, the electrode was subsequently reinserted at the same point. Neuromuscular blockade induced by the depolarizing compounds enabled observations of epps to be repetitively made without the microelectrode being dislodged.

(v) Location of End-plates

All microelectrode recordings were made at the chemosensitive area of the muscle fibre, the end-plate. Location of such regions was enhanced by the use of polarizing filters in conjunction with the microscope (as already described). It was possible to follow the course of nerves branching off the trunk to their terminals, where groups of small dark spots were usually visible.

Plate A in Fig. 2.7 shows a miniature end-plate potential (mepp) recorded via a micro-electrode following its insertion at one of these spots. The horizontal calibration bars represent 1.0 msec intervals. In plate B the time base has been expanded (0.5 msec/division) and mepps with a rise time of 0.4 msec are shown. If an insertion yielded mepps with rise times in excess of this figure, the electrode was withdrawn and repositioned. Plate C shows inverted mepps recorded extracellularly following the withdrawal of the electrode from the membrane (same cell as Plate B). The detection of

Figure 2.7. Miniature end-plate potentials recorded via micro-electrode. Vertical calibration of 0.5 mV applies to all plates, horizontal calibration for 'A' shows 1 msec, and for 'B' and 'C' 0.5 msec. Plate B shows a sequence of superimposed mepps. Plate C illustrates inverted mepps recorded following withdrawal of the microelectrode to an extracellular position. Note that the extracellular records have a shorter duration.


A
1msec
B
0·5
mV
C
0·5 msec

these 'inverts' was a rare event but served to confirm the focality of the recording (del Castillo and Katz, 1956). The time course of the miniature potentials was briefer when recorded extracellulary. This has been attributed to the capacitive effect of the membrane which tends to distort intracellular recordings (Liley, 1956).

Measurement of Fibre Diameter

For the measurement of muscle fibre diameters the preparation was mounted vertically. A strip of diaphragm plus rib was removed and the rib and tendon were sutured to the vertical arms of a Perspex holder so that the fibres ran horizontally. The holder and tissue was then immersed in physiological saline contained within a Perspex jacketed bath which had vertical faces (Creese, Scholes and Whalen, 1958). Sintered bubblers gassed the solution, while a Circon unit maintained its temperature at 37^{o}C via water pumped through a jacket which surrounded 2 of the sides and the base of the bath. The muscle was viewed through the remaining 2 sides of the bath.

The muscle was transilluminated from the back of the bath using the Barr and Stroud Fibre Optic Light Source. The facilities of the Nikon Stereo Microscope made it possible to view the preparation in this position via the front of the bath.

Two graticules were used, one in an eyepiece of the microscope while another bearing a grid was mounted on the front of the bath. At a set magnification the eyepiece graticule could thus be calibrated and was arranged such that 1 eyepiece unit corresponded to 6.06 μm.

Radiolabelled Drug Experiments

Guinea-pigs were stunned and the thorax opened as previously described. In these experiments it was possible to obtain 2 muscle preparations from each animal by using left and right hemi-diaphragms. Each section was attached to a holder (Creese and Northover, 1961) which exerted a resting tension on the muscle (3 g) and was then immersed in a tube containing 10 ml of saline.

Muscles were transferred to tubes bearing a solution to which radioactive decamethonium had been added. After a suitable exposure period the holders were consecutively immersed in 10 tubes of saline at minute intervals. This process served to wash radioactivity from the extracellular fluid. (Creese, Taylor and Tilton, 1963).

Following the wash-out, muscles were frozen on brass plates while being stretched. A band of end-plates became apparent in each muscle as a white line which appeared upon freezing (England, 1970). A sequence of slices perpendicular to the muscle fibres and parallel to this line were made to divide the preparation into a series of strips 1 mm wide. Each

frozen strip was then rapidly weighed and trasferred to a plastic vial containing 0.5 ml N-KOH in methanol.

The tissues were dissolved by heating these vials in a water bath at 70°C for 30 min; the tray which contained the vials was connected to a shaking device which ensured that the tissues disintegrated. After cooling 10 ml scintillator was added (Creese, Taylor & Case, 1971). Background was measured in vials containing the KOH in methanol, plus scintillator, and was usually less that 10 counts per min. The radioactivity of the muscles was determined using a Packard automatic refrigerated counter with efficiency 25% (Creese and England, 1970).

The labelled compound was methyl-^{3}H Decamethonium chloride of specific activity 1 curie/m.mol, obtained from the Radiochemical Centre, Amersham. It was stored as a solution of 344 μM in a refrigerator. Then 0.1 ml in 10 ml solution gave 0.34 μM labelled decamethonium. In practice solutions of 1 μM, 3 μM, 10 μM and 100 μM (see Chapter 7) were obtained by adding 0.1 ml of the stock solution of labelled compound to 10 ml of solution which contained the appropriate concentration of unlabelled decamethonium iodide.

The ratio (counts per mg muscle)/(counts per μl external solution) is the <u>clearance</u> (μl mg^{-1}), and <u>uptake</u> in p-mole mg^{-1} is clearance x concentration in μM.

Statistical Procedures

Medians are given in the Tables together with the limits which are approximately 95% (Colquhoun, 1971). Values were compared by two-sample rank tests (Goldstein, 1964). The dose-effect curve in Fig. 3.2 was obtained by (unweighted) logit analysis (Finney, 1978), while the curve in Fig. 4.2 was obtained by a computer program (Waud, 1976) which gave estimates of the maximum as well as the median effective concentration and the Hill slope.

C H A P T E R 3

MUSCLE CONTRACTIONS

MUSCLE CONTRACTIONS

The neuromuscular actions of depolarizing drugs were studied as a preliminary investigation before microelectrode recordings were made. Contractions of isolated guinea-pig diaphragm were elicited by a series of maximal single stimuli to the phrenic nerve and recorded via a strain gauge Experimental results were obtained from guinea-pigs which were 2 to 3 weeks old, with a median weight of 225 g (84 animals, 95% confidence limits 215 - 230 g). Problems were encountered in some initial experiments with animals at the lower limit of the weight range, spontaneous contractions of these preparations giving rise to an unstable baseline for resting tension.

Actions of Decamethonium

Fig. 3.1 shows a sequence of events following the changing of bathing medium to one containing 10 μM decamethonium. An initial contracture was apparent as a rise in resting tension and this was accompanied by a small temporary increase in twitch tension. Neuromuscular block then developed which showed 2 distinct phases. The first phase consisted of a block of rapid onset which reduced the twitch height to 17% of its original size within 6 min. Despite the continued presence of the drug, the twitch height subsequently recovered in 18 min. Later, a slow secondary block developed, and

Figure 3.1. Action of decamethonium (10 μM) on indirectly elicited contractions. The bathing medium was switched to one bearing the drug at the solid arrow. Time in min. At 71 min the muscle was stimulated directly: this is indicated by period between open arrows. Vertical calibration shows 5 g.wt. The initial fall in amplitude is followed by recovery and later development of a slow secondary block.

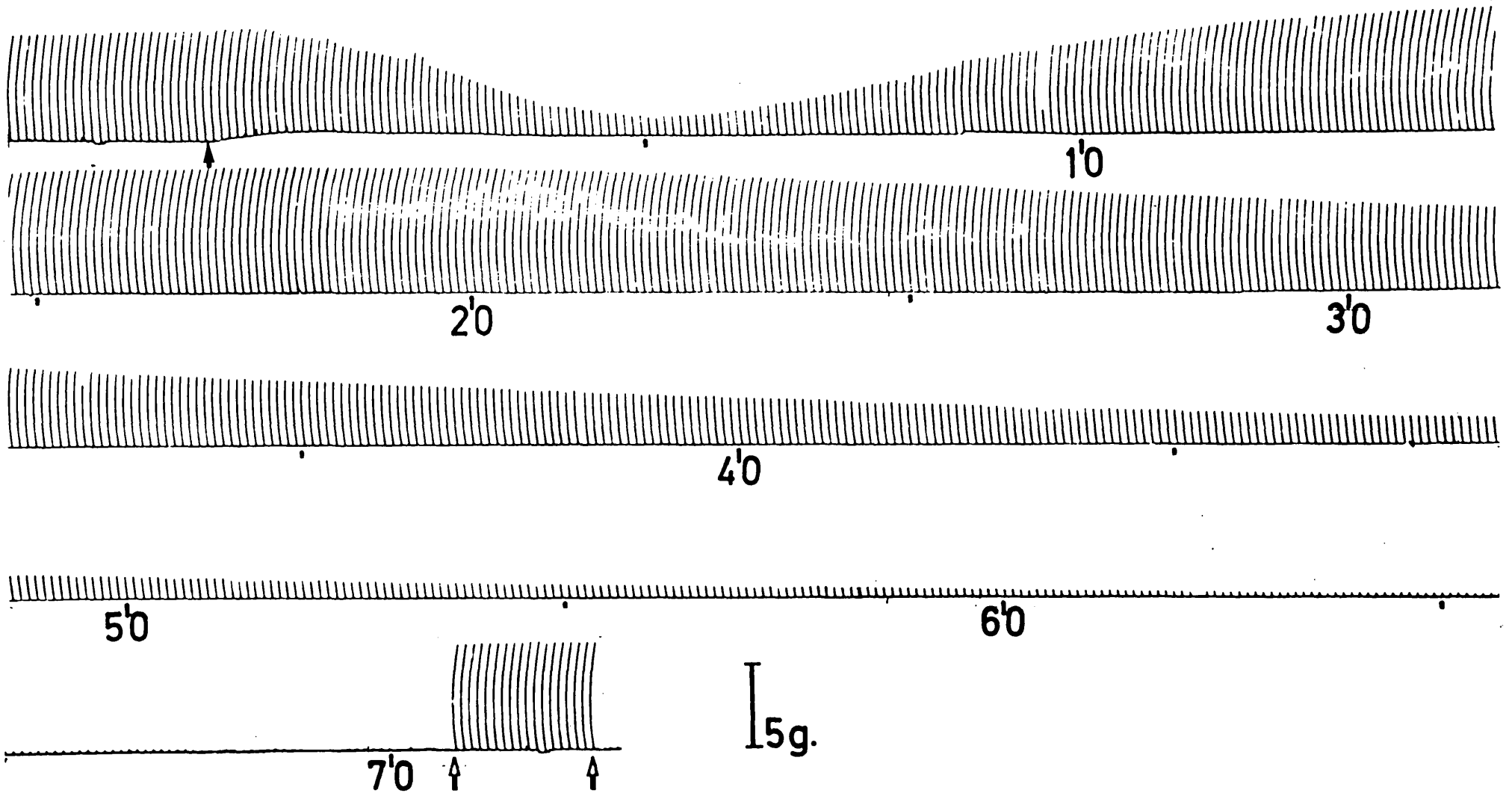

1'0
2'0
3'0
4'0
5'0
6'0
7'0
5g.

Table 3.1 Effect of 10 μM Decamethonium on Contractions

Initial Block as %	Time to Maximum Initial Block (min)	Maximum Recovery (% of Original)	Time to Maximum Recovery (min)	Time to Total Block (min)
80	4.3	86	16	60
83	5.4	113	18	70
85	4.6	100	21	75
92	5.8	91	20	80
94	4.4	97	19	64
94	5.6	98	24	80
100	5.5	67	27	95
		MEDIANS		
92	5.4	97	21	75

All times measured from point of addition of drug.

total absence of contractions occurred after 70 min. The muscle was directly stimulated at 71 min, yielding contractions of the same strength as those evoked indirectly before the drug.

Fig. 3.1 shows that the actions of decamethonium on guinea-pig diaphragm were similar to the effects on this muscle described by Jenden (1955).

Table 3.1 gives the results from 7 muscles showing the maximum recovery after initial block was 97% of the original value. Resting tension, measured at the end of the experiment by severing of the connection to the strain gauge, was found to have a median value of 4.2 g, from a range of 81 animals (95% confidence limits, 3.3 to 4.7 g).

(i) Dose-Response Relationship for Decamethonium

Fig. 3.2 shows the effect of different concentrations of decamethonium, displayed as a dose-response curve by using the initial block of contractions as the measure of effect. This was expressed as the percentage diminution of the original contraction height, and was referred to as % Initial Block.

All concentrations of decamethonium elicited a block of 2 phases, with a period of spontaneous recovery of contractions. 3.5 μM decamethonium gave a mean initial block of 13.5% ($n = 6$) followed by a small potentiation of contraction height before total block developed. This gave the first point for the curve.

Figure 3.2. Neuromuscular block produced by different concentrations of decamethonium Left-hand graph shows the mean value ($\pm$ SEM) for initial block, expressed as a percentage block, with drug concentration in μM on a logarithmic scale. Right-hand graph shows the same data linearized, with logits plotted against the log of concentration. At least five observations were made at each concentration.

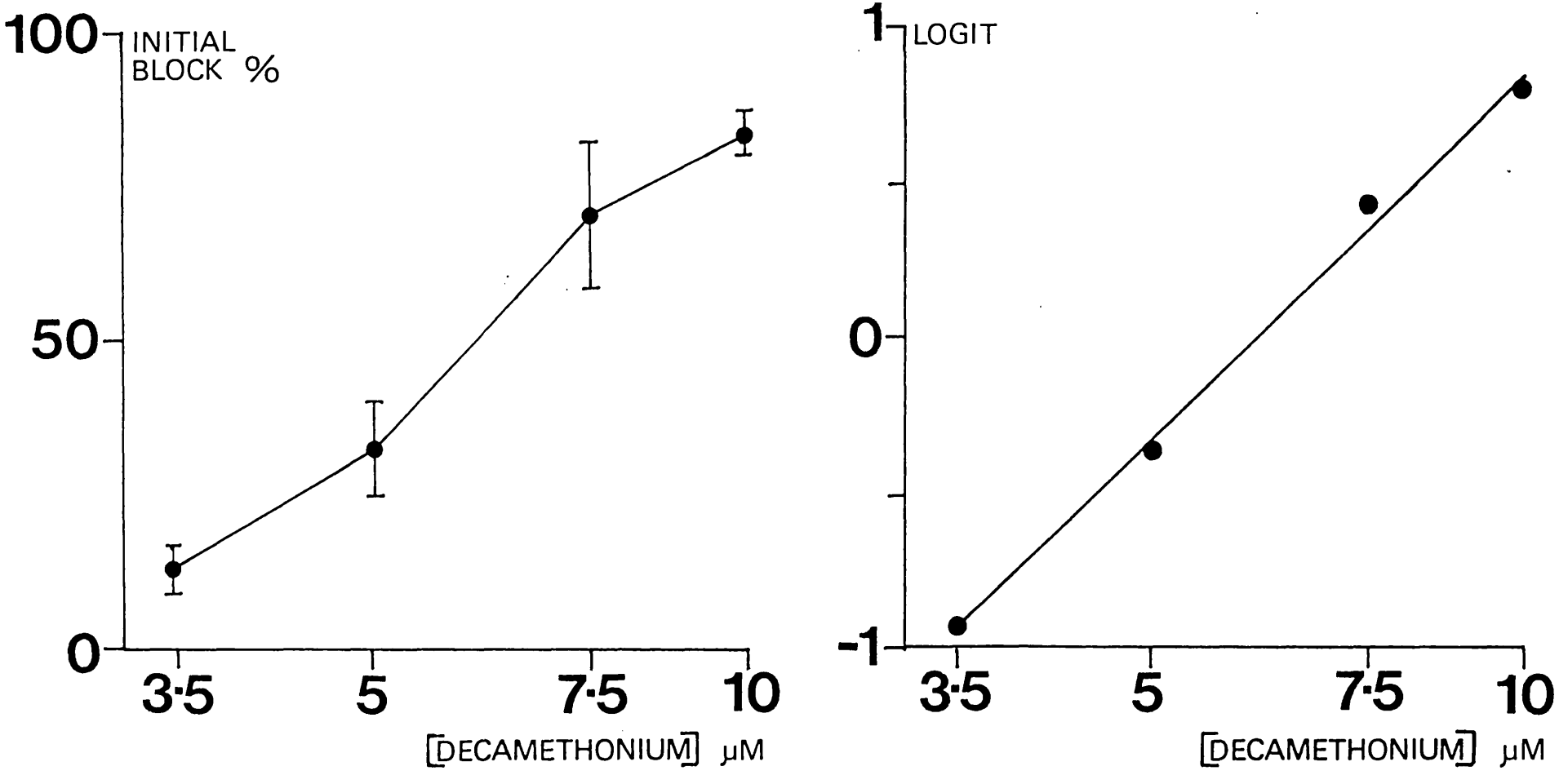

100
INITIAL
BLOCK %
50
0
3·5
5
7·5
10
[DECAMETHONIUM] μM
1
LOGIT
0
-1
3·5
5
7·5
10
[DECAMETHONIUM] μM

Table 3.2 Initial Block Produced by Decamethonium.

Conc (μM)	Log_{10} Conc	Mean Initial Block (f) (% $\pm$ SEM) (n)	Logit Function
3.5	0.544	13.5 $\pm$ 4.0 (6)	-0.929
5.0	0.699	33.0 $\pm$ 7.1 (5)	-0.354
7.5	0.875	70.4 $\pm$ 11.8 (5)	0.433
10.0	1.000	82.8 $\pm$ 3.8 (9)	0.786

Logits are from Fisher and Yates (1967, Table XI).

Logit transformation $Z = \frac{1}{2} \log_e (f/(1 - f))$

Slope from regression (unweighted) = 3.86 logit units per $\log_{10}$ μM.

Hill slope = 3.86/1.15 = 3.36 $\log_{10}$ units per $\log_{10}$ μM.

Median Effective Concentration = 6.1 μM.

The first graph in this Figure shows the mean value for % initial block plotted against decamethonium concentration on a logarithmic scale. Error bars show the standard error of the mean for 5 observations or more at each concentration.

The second graph shows a linearised dose-response curve with logits of mean values for % initial block plotted against concentration. The slope of the line was 3.86 logit units/ log unit and the median effect dose was calculated as 6.1 μM. (Table 3.2).

Actions of Other Depolarizing Drugs

Fig. 3.3 shows that carbachol, suberyldicholine and acetylcholine (in the presence of the anti-esterase physostigmine) all produced effects which were similar to those of decamethonium, namely an initial block which was followed by recovery, and then a secondary block. Concentrations were sought for future studies which produced a large but not complete initial block, followed by considerable recovery of contraction height.

(i) Carbachol

Concentrations in excess of 100 μM gave total initial block and less than 50% recovery of contractions. 80 μM carbachol proved to be a suitable concentration and produced a median block of 97% in 3 min (n = 6). In 1 muscle the initial block was only 42% (see Table 3.2). The recovery was 98% (median

Figure 3.3. Effects of other depolarizing drugs on indirectly elicited contractions, showing actions of carbachol (80 μM), suberyldicholine iodide (80 μM), and acetylcholine chloride (20 μM). The latter was added when a constant strength of contraction was established in the presence of physostigmine sulphate (3 μM). Each trace represents a single muscle. Ordinate gives tension developed at the strain gauge as a percentage of the initial value. Time in min after change over to drug-bearing solution.

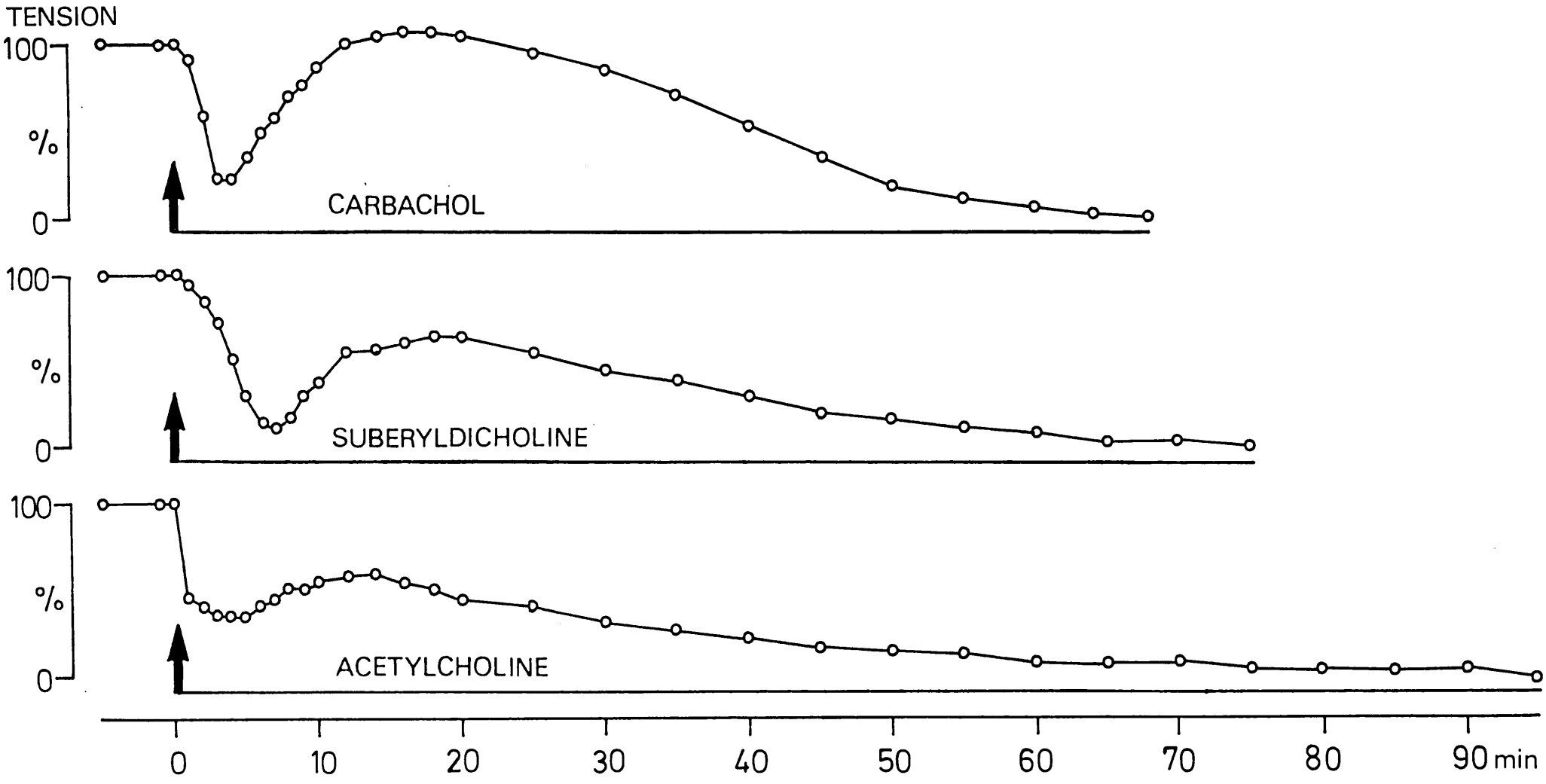

TENSION
100
%
0
CARBACHOL
100
%
0
SUBERYLDICHOLINE
100
%
0
ACETYLCHOLINE
0
10
20
30
40
50
60
70
80
90 min

Table 3.3 Effect of 80 μM Carbachol on Contractions

Initial Block as %	Time to Maximum Initial Block (min)	Maximum Recovery (% of Original)	Time to Maximum Recovery (min)	Time to Total Block (min)
42.5	2.1	98.6	11.5	43.0
79.6	3.4	106.8	15.8	78.0
97.2	4.0	101.4	22.5	71.0
98.3	5.0	80.7	22.0	75.0
100.0	2.4	79.3	26.5	61.0
100.0	2.5	98.3	21.8	53.0
		MEDIANS		
97.7	3.0	98.5	21.9	66.0

All times measured from point of addition of drug

Table 3.4 Effect of 80 μM Suberyldicholine Iodide on Contractions

Initial Block as %	Time to Maximum Initial Block (min)	Maximum Recovery (% of Original)	Time to Maximum Recovery (min)	Time to Total Block (min)
70.3	5.2	101.0	24.0	132.0
76.3	6.2	100.0	21.2	117.0
87.8	5.0	75.6	19.0	68.0
87.9	6.8	62.1	18.0	76.0
100.0	4.0	53.7	30.0	70.0
100.0	7.3	55.0	25.0	74.0
		MEDIANS		
87.9	5.7	68.8	22.6	72.0

All times measured from point of addition of drug

Table 3.5 Effect of 20 μM Acetylcholine Chloride on Contractions (after application of 3 μM Physostigmine Sulphate)

Initial Block as %	Time to Maximum Initial Block (min)	Maximum Recovery (% of Original)	Time to Maximum Recovery (min)	Time to Total Block (min)
60.5	4.3	68.6	15.2	128.0
61.8	3.2	53.9	7.4	65.0
63.6	3.2	56.8	13.0	96.0
70.3	3.0	89.2	14.2	126.0
74,1	2.6	51.9	13.2	98.0
76.8	3.1	48.4	12.5	-
		MEDIANS		
67.0	3.2	55.4	13.1	98.0

All times measured from point of addition of drug

of 6) and in 2 preparations there was potentiation of twitch height at maximum recovery.

Maximum recovery occurred at 22 min, after which the slower second block developed. Total block was complete in 66 min (Table 3.3).

A small brief potentiation of twitch height following the administrations of carbachol was observed in all experiments and was sometimes associated with an initial contracture.

(ii) Suberyldicholine Iodide

10 μM suberyldicholine produced no diminution of size of contractions but led to a slight potentiation. 100 μM produced almost 100% initial block with only 50% maximum recovery.

80 μM was the chosen concentration (Table 3.4), reducing the height of the contractions by almost 88% in just under 6 min (n = 6). 69% of original twitch tension was recovered in the presence of suberyldicholine in 23 min. The median time to total block was 72 min.

As with carbachol, all preparations displayed a potentiated height of contraction when suberyldicholine was added. There was usually a slight contracture which was not maintained beyond the first minute.

(iii) Acetylcholine Chloride

The response of mammalian skeletal muscle to the prolonged application of the natural transmitter, acetylcholine (Ach), was investigated. To prevent the hydrolysis of Ach in the synaptic cleft by the enzyme acetylcholinesterase, physostigmine sulphate was used as an inhibitor.

3 μM physostigmine potentiated the tension developed by the guinea-pig diaphragm in response to maximal indirect stimulation. Brown et al (1936) demonstrated in the cat that following the administration of physostigmine a single stimulus produced a short burst of muscle action potentials (repetition) and so each stimulus produced a brief tetanus. This is believed to account for the potentiation of the contractions.

In this preparation, physostigmine caused an increase in contraction height to 294% (median of 6). Acetylcholine was added when a constant twitch tension was established in the presence of the anticholinesterase, usually within 5 min of administration of the latter.

It proved not to be possible with Ach to associate a large initial block with a high percentage of spontaneous recovery of contractions. 50 μM for instance, caused 76% initial block but was followed by only 32% recovery. By contrast, there was 100% recovery of twitch height with 10 μM Ach, but only 25% initial block.

Fig. 3.3 and Table 3.5 show the effect of 20 μM Ach. There was an initial block of 67% in 3.2 min, followed by 55% recovery of strength of contractions in 13 min (median of 6 observations). Total block was complete in 98 min (n = 5).

Effect of Ouabain

In an attempt to determine the mechanism involved in the spontaneous recovery of contractions in the presence of a depolarizing compound, the action of ouabain was investigated. Any contribution of an active electrogenic mechanism to this recovery was likely to be inhibited (Thomas, 1972).

Fig. 3.4 shows the results obtained by this procedure. In trace A the action of decamethonium (10 μM) is depicted: there is an initial block, recovery and then a secondary block. Trace B shows the effect of ouabain (10 μM) added when recovery from the initial block had occurred. When the contractions exhibited maximum recovery in the presence of decamethonium (10 μM), the solution bathing the muscle was changed to one containing the ouabain in addition to the depolarizing drug. This action reduced the time taken for the development of total block with decamethonium. Trace C acts as control, ouabain (10 μM) causing a slow failure of contractions.

Figure 3.4. Effect of ouabain on spontaneously recovered contractions in the presence of decamethonium. 'A' shows action of decamethonium (10 μM) for comparison. 'B' shows administration of decamethonium (10 μM) followed by addition of ouabain (10 μM) at maximum recovery of contractions. 'C' acts as control, showing action of ouabain (10 μM) only. Ordinate gives tension developed at the strain gauge as a percentage of the initial value. Time in min after changeover to first drug-bearing solution. Note that the effect of ouabain in 'B' is more rapid than in 'C'.

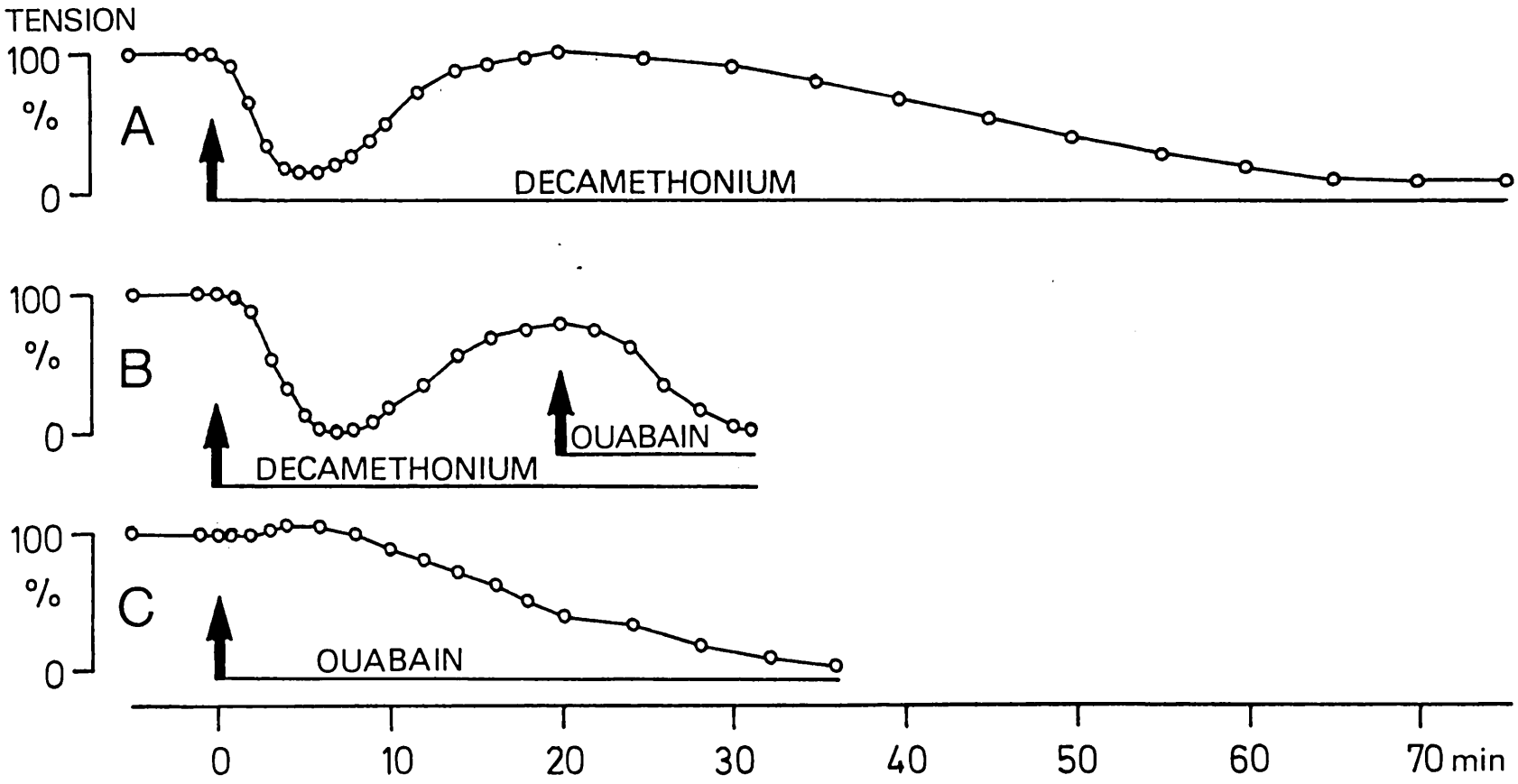

TENSION
100
%
0
A
DECAMETHONIUM
100
%
0
B
DECAMETHONIUM
OUABAIN
100
%
0
C
OUABAIN
0
10
20
30
40
50
60
70 min

Table 3.6 Effect of Ouabain on Contractions

GROUP B (with Decamethonium)		GROUP C (Control)
Time to Maximum Recovery After 10 μM Decamethonium (min)	Time to Absence of Contractions After Addition of 10 μM Ouabain (min)	Time to Absence of Contractions After 10 μM Ouabain (min)
18	7	34
14	8	35
10	9	35
10	10	37
20	10	37
16	12	43
	MEDIANS	
15	9.5	36

The median time taken for the development of total secondary block following maximum recovery with decamethonium was 54 min (n = 7, derived from Table 3.1. Trace A, Fig. 3.4). The addition of ouabain at the point of maximum recovery reduced this time to 9.5 min (n = 6, Table 3.6. Trace B, Fig. 3.4). Ouabain in the absence of decamethonium reduced the contractions to zero in 36 min (n = 6, Table 3.6. Trace C, Fig. 3.4). Thus the sensitivity to ouabain was higher after recovery from a depolarizing drug than in the normal state. These results are consistent with the concept that the recovery following initial block is partly sustained by a ouabain-sensitive process, and encouraged further efforts to demonstrate that recovery involved stimulation of the sodium potassium pump.

Summary

1. The actions of various depolarizing drugs on indirectly elicited contractions have been investigated, and all produced initial block followed by spontaneous recovery of contraction in the presence of the drug, and then a later secondary block.

2. With decamethonium (10 μM) there was an 80 to 100% initial recovery block of contractions within about 5 min. Maximum recovery (97%) occurred at 21 min, followed by complete block after 75 min (medians of 7 observations).

3. The median effective dose for the initial block of contractions by decamethonium was calculated as 6.1 μM.

4. Carbachol (80 μM) produced a response similar to that of decamethonium, with 98% initial block, maximum recovery at 22 min (99%), and total block after 66 min. (medians of 6).

5. Suberyldicholine iodide (80 μM) and acetylcholine chloride (20 μM ; after application of 3 μM physostigmine) also produced similar effects.

6. Ouabain (10 μM) reduced the contractions to zero in 36 min (6). If added at peak recovery in the presence of decamethonium (10 μM) the contractions were reduced to zero in 9.5 min, which was more rapid than in controls (6: $P < 0.01$).

CHAPTER 4

DEPOLARIZATION AND RECOVERY

DEPOLARIZATION AND RECOVERY

An investigation into the electrical events occurring at the cell membrane was performed by means of experiments employing microelectrodes. A single electrode was inserted at the end-plate and the time course of the action of a depolarizing compound was recorded. It was anticipated that these recordings would provide an insight into the mechanisms underlying the spontaneous recovery of strength of contractions in the continued presence of such drugs, as described in Chapter 3.

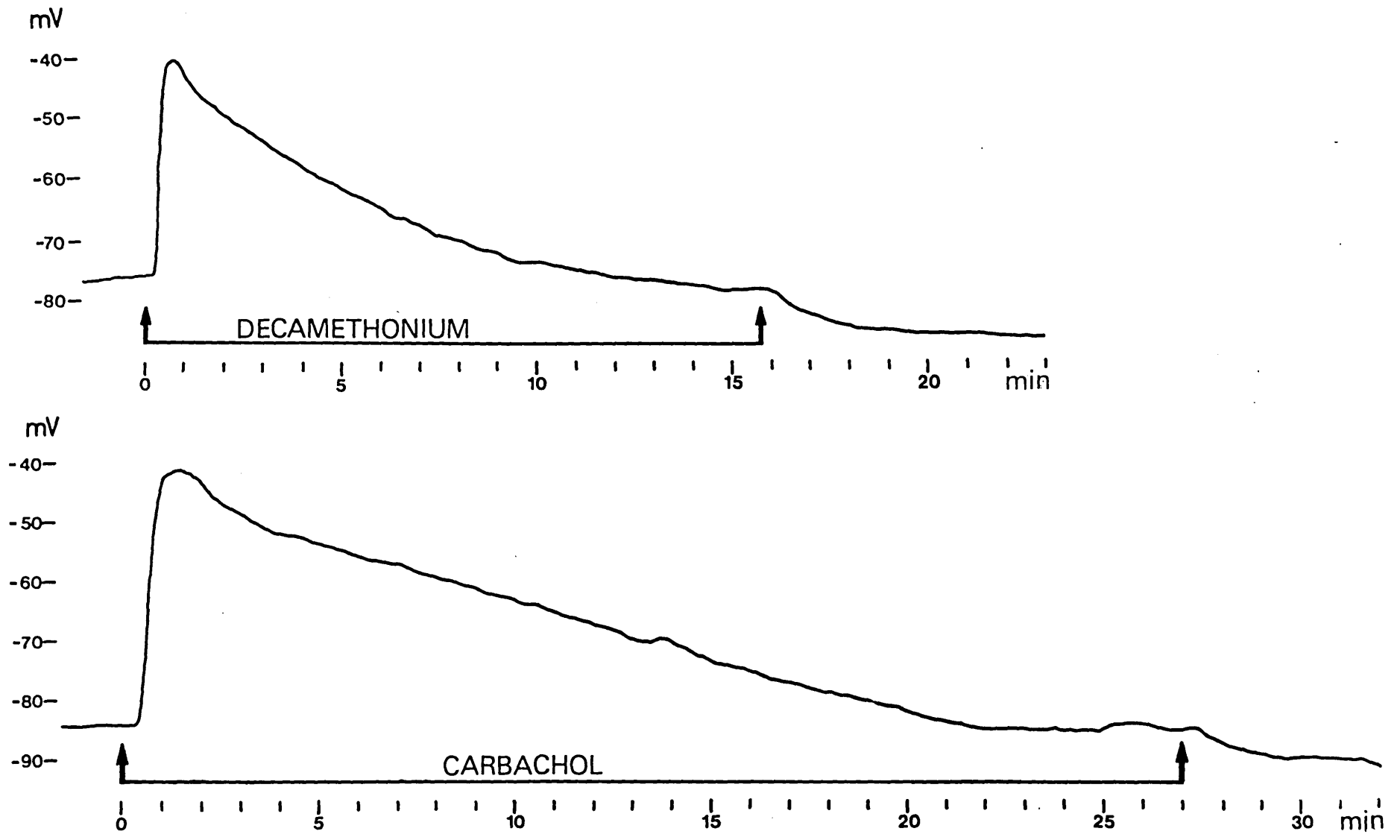
Effects of Decamethonium (10 μM)

The upper trace in Fig. 4.1 shows the action of decamethonium (10 μM) on membrane potential. The fibre had held a steady resting potential after the insertion of the microelectrode for 5 min prior to the administration of the drug, and a portion of this is shown. Miniature end-plate potentials (mepps) with a rapid rise time were apparent at this stage.

At the first arrow, the tap controlling the reservoir supply to the bath was turned and a solution containing decamethonium was admitted to bathe the muscle preparation. A brief delay due to the dead space in the tubes was recorded before depolarization began. The potential then rapidly became less negative, falling from an original value of -76 mV to a peak depolarized potential of -39 mV, at a maximum rate

Figure 4.1. Upper: Depolarization and spontaneous recovery with decamethonium (10 μM). Continuous recording made by microelectrode inserted at end-plate region of muscle fibre. Period of exposure to drug depicted by arrows and horizontal line. Ordinate in mV. Time in min after changeover to drug-bearing solution.

Lower: Depolarization and spontaneous recovery with carbachol (80 μM). Note hyperpolarization following restoration of drug-free solution at second arrow in both records.


mV
-40
-50
-60
-70
-80
DECAMETHONIUM
0
5
10
15
20
min
mV
-40
-50
-60
-70
-80
-90
CARBACHOL
0
5
10
15
20
25
30
min

of 160 mV/min. The last few millivolts of this depolarization progressed at a diminishing rate until, at about three-quarters of a minute after the solutions were switched, a brief peak was reached. By this time the mepps were no longer obvious.

Immediately following peak depolarization, the fibre showed spontaneous recovery of potential in the presence of decamethonium. The rate of this process was highest at the onset and diminished until a plateau at -77 mV was reached after about 15 min. Half of the potential was recovered within 4 min. The fibre was considered to have maximally regained its potential and was returned to normal physiological saline just before the 16th min.

Removal of the drug caused the end-plate to hyperpolarize to a plateau of -84 mV within 3 min of this change over. mepps again became apparent after 6 min and had regained their original amplitude by the 16th min in normal solution.

Table 4.1 lists 6 experiments using 10 μM decamethonium, including that which is described above. The drug caused a median depolarization of 28 mV from -82 mV to - 54 mV. The maximum rate of depolarization ranged from 25.9 to 160.0 mV/min, with a median value of 69.8 mV/min. In the presence of decamethonium, the lowest recovery of potential was 69% from peak depolarized potential, while the median was 80%. Median time taken from administration of the compound to a stable recovered membrane potential was 18 min.

Table 4.1 Effect of 10 μM Decamethonium on Resting Potential

Orig. Rest. Pot. (mV)	Peak Depol. Pot. (mV)	Change (mV)	Max. Rate of Depol. (mV/min)	Half-Time (sec)	Final Membrane Pot. (mV)	Time to Recovery of Stable Pot. (min)	% Recovery from Peak Depol. Pot. (%)
-76	-39	37	160.0	9.0	-77	15	103
-80	-56	24	25.9	41.0	-75	19	79
-82	-41	41	125.0	15.0	-74	18	81
-82	-51	31	60.0	25.5	-74	18	74
-83	-57	26	71.4	16.5	-75	12	69
-90	-64	26	68.2	16.2	-89	25	96
				MEDIANS			
-82	-54	28			-75	18	80

Table 4.2 Effect of 80 μM Carbachol on Resting Potential

(mV)	(mV)	(mV)	(mV/min)	(sec)	(mV)	(min)	(%)
-73	-47	26	31.6	28.5	-74	32	104
-74	-44	30	50.0	26.4	-74	40	100
-74	-50	24	15.8	82.5	-76	34	108
-79	-56	23	25.0	37.5	-92	30	157
-84	-41	43	113.2	19.5	-85	22	102
-84	-53	31	27.8	53.4	-83	29	97
				MEDIANS			
-77	-49	28			-80	31	103

Table 4.3 Hyperpolarization following withdrawal of carbachol (80 μM) and of Decamethonium (10 μM)

	Initial mV	Final mV	Change mV	Time in drug min
Decamethonium	-74	-80	6	24
	-77	-84	7	16
	-75	-83	8	19
Carbachol	-83	-87	4	32
	-85	-90	5	27
	-75	-82	7	30
	-77	-81	4	30
	-73	-83	10	30
Combined median	-76	-83	6.5	
n			8	
Range			4, 10	

Effects of Carbachol (80 μM)

The lower trace in Fig. 4.1 shows the action of carbachol (80 μM) on membrane potential. The resting potential was maintained at a steady -84 mV for over 8 min prior to the addition of carbachol. Mepps were observed with a rise time of 0.3 to 0.35 msec.

Carbachol, added at the first arrow, caused a depolarization from -84 mV to -41 mV at a maximum rate of 113.2 mV/min. Peak depolarization occurred 1.5 min after the tap was turned. At this stage mepps were absent. As with decamethonium, the membrane potential of the fibre in the presence of carbachol showed significant spontaneous recovery immediately after peak depolatization. The rate of recovery with this concentration of carbachol, however, was usually slower than with decamethonium, half the potential being regained in 10 min. The fibre in Fig. 4.1 took 22 min to attain a stable potential, which was hyperpolarized with respect to the original resting potential. Normal physiological saline at the 27th min (second arrow) caused a further increase in negativity to almost -90 mV. Hyperpolarizations following removal of drug are listed for both decamethonium and carbachol in Table 4.3, and the median was 6.5 mV (8).

Qualitatively, the actions of decamethonium (10 μM) and carbachol (80 μM) were similar. They varied quantitatively in some respects and comparison of Table 4.2 with Table 4.1 illustrates these points. Carbachol produced the same degree of depolarization (28 mV). As already related, the recovery process in carbachol was slower (31 min) than in decamethonium

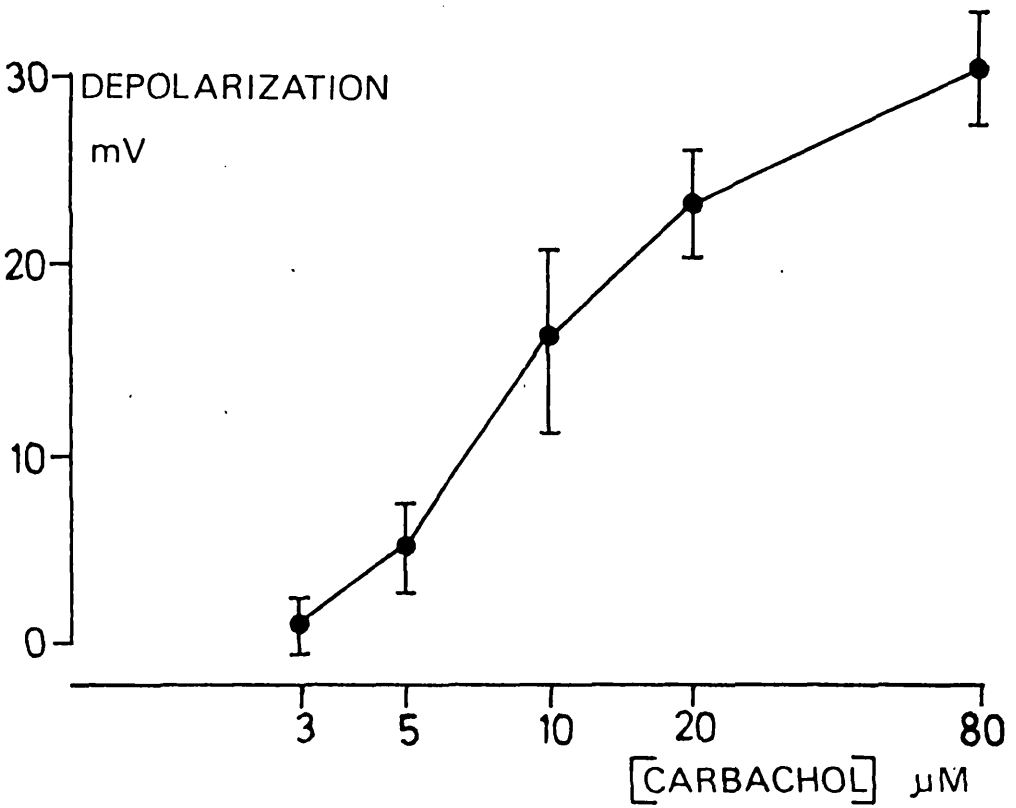
but the degree of recovery was greater. Only one out of six fibres in carbachol failed to regain its original potential, the recovery being 103% from peak depolarization.

(i) Dose-Response Relationship for Carbachol

The action of carbachol was recorded as the change in membrane potential from the original resting value to the peak depolarized potential. Concentrations of 80, 20, 10, 5 and 3 μM were investigated, and Fig. 4.2 shows the curve obtained by plotting the effect against concentration of dose on a log scale. Each point represents the mean (± SEM) of at least four observations. The median effective dose was estimated as 10.1 ± 0.88 μM (Table 4.4). This confirms that the concentration of 80 μM is likely to produce a maximal effect when recorded as depolarization: no correction has been made for the effect of voltage on conductance (Martin, 1955). The process of depolarization followed by recovery in the presence of the drug occurred at all concentrations except at 3 μM, where the degree of spontaneous repolarization was variable.

While 80 μM carbachol caused mepps to disappear during the period of rapid depolarization (as described above), other concentrations had different effects. With 20 μM carbachol the depolarization was 23 mV, 82% of the effect caused by 80 μM (Fig. 4.2), but at this concentration the mepps persisted for over 15 min, until more than half of the potential

Figure 4.2. Depolarization to different concentrations of carbachol. Left-hand graph shows the mean value ($\pm$ SEM) of the depolarization in mV, against drug concentration in μM on a logarithmic scale. Right-hand graph shows the same data with logit f (Table 4.3), where f is the depolarization as a fraction of the maximum, plotted against the log of concentration. At least 4 observations were made at each concentration. The Hill slope is 2.1 units, with median effective concentration 10 μM. The regression was obtained by a computer fit as described in the text.


30
DEPOLARIZATION
mV
20
10
0
3
5
10
20
80
[CARBACHOL] µM

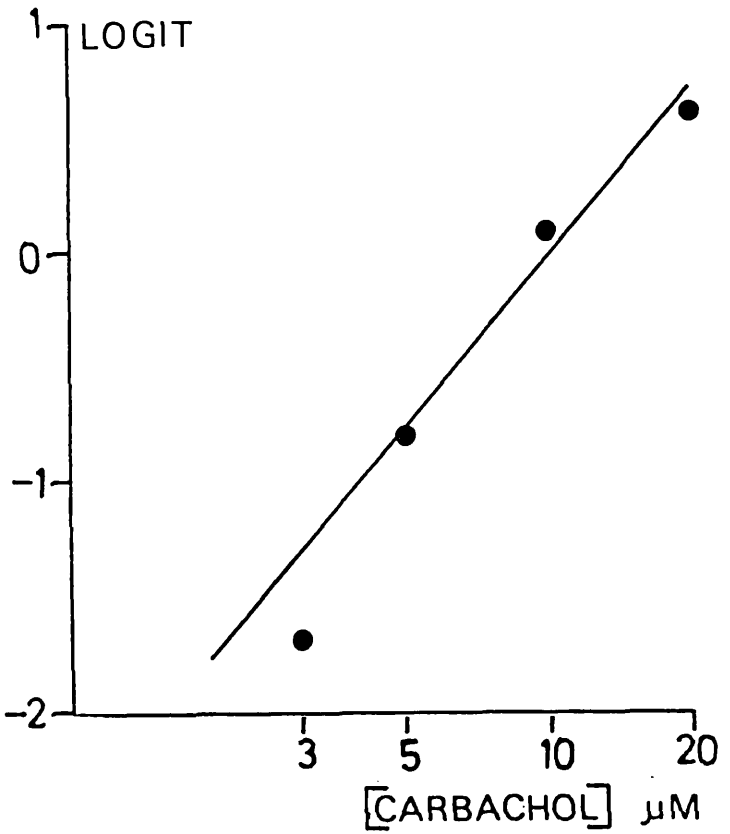

1
LOGIT
0
-1
-2
3
5
10
20
[CARBACHOL] µM

Table 4.4 Depolarization Produced by Carbachol

Conc (μM)	Log_{10} Conc	Mean Depol. (mV ± SEM)	(n)	Fraction f	Hill Function	Logit Function Fig. 4.2
3	0.477	1.0 ± 1.3	(4)	0.033	-1.467	-1.689
5	0.699	5.0 ± 2.2	(4)	0.167	-0.698	-0.804
10	1.000	16.0 ± 4.7	(4)	0.533	0.057	0.066
20	1.301	23.0 ± 2.8	(4)	0.766	0.517	0.596
80	1.903	30.0 ± 3.0	(8)			

Analysis of the curve was carried out using iterative techniques to fit the data to the hyperbola

$$D = \frac{D_m \times A^P}{A^P + ED_{50}^P}$$

where D is the depolarization, D_m is the maximum depolarizion, A is the concentration of carbachol, and P is the Hill slope (from plot of log_{10} (f/(1-f)) versus log_{10} concentration where f is the fractional depolarization). Analysis of the data using this function is described by Waud (1976).

From the analysis the following was obtained:

Maximum Depolarization = 30.0 ± 1.50 mV

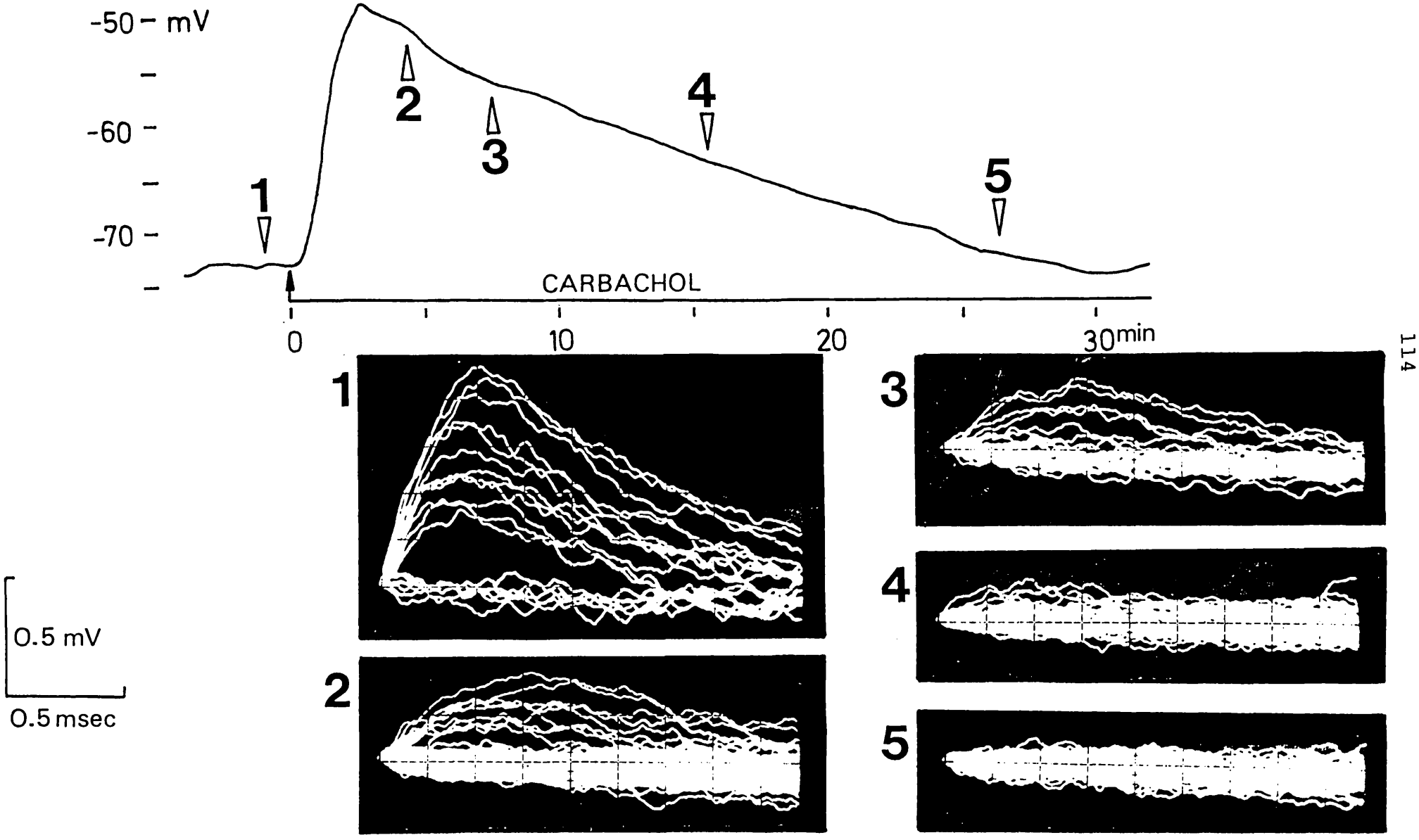
Median Effective Concentration = 10.1 ± 0.88 μM

Hill Slope = 2.15 ± 0.41 log_{10} units per log_{10} μM

Hence maximum depolarization was observed at 80 μM.

In Fig. 4.2 (right-hand side) the logit function has been plotted from Tables given by Fisher and Yates (1967, Table XI). In this presentation the logit function is used where logit f is ½ log_e (f/1-f) .

Figure 4.3. Miniature end-plate potentials recorded during prolonged exposure to carbachol (20 μM). Upper trace shows membrane potential during experiment, numbers indicating times at which mepps were recorded, and relating to lower plates. Photograph 1 was taken before the application of the drug and serves as control. Calibration bars at bottom left refer to photograph sequence. The mepps were absent by the 27th min.


-50 mV
-60
-70
1
2
3
4
5
CARBACHOL
0
10
20
30min
0.5 mV
0.5 msec

was spontaneously recovered (Fig. 4.3). At even lower doses (3 μM) the mepps remained indefinitely in the presence of the drug.

(ii) Extended Recordings in Carbachol (80 μM)

The stability of the spontaneously recovered membrane potential was investigated by means of microelectrode recordings made over prolonged periods. Three continuous records were obtained from muscle fibres for over 2 hr while in flowing solution containing carbachol (80 μM). Fig. 4.4 depicts a trace where the electrode remained successfully impaled in the membrane for 150 min. The figure shows excerpts from the continuous record for periods adjacent to the 60th, 120th and 150th min. The membrane potential appeared to be steady, ultimately attaining a slightly hyperpolarized plateau at -87 mV. No mepps were apparent following the initial rapid depolarization.

Table 4.5 lists results from the 3 continuous recordings in addition to those from intermittent recordings. This later technique was adopted to determine whether the two methods gave comparable results. The intermittent method involved recording the initial depolarization attributable to carbachol followed by the withdrawal of the electrode to a position just exterior to the cell membrane. Subsequent estimates of the potential were made by a single reinsertion at the same point. In some fibres the recorded potentials were stable but in others the potential had to be left for a few min to attain a stable value.

Figure 4.4. Extended recording of membrane potential with carbachol (80 μM), for over 150 min. Upper portion shows depolarization and spontaneous recovery, while lower half shows excerpts from the continuous record at later times. Application of drug is represented by horizontal line, oblique slashes indicating periods not shown. Oridinate in mV. Time in min after changeover to drug-bearing solution. The resting potential recovered and was steady at 2½ hr after application of the drug.

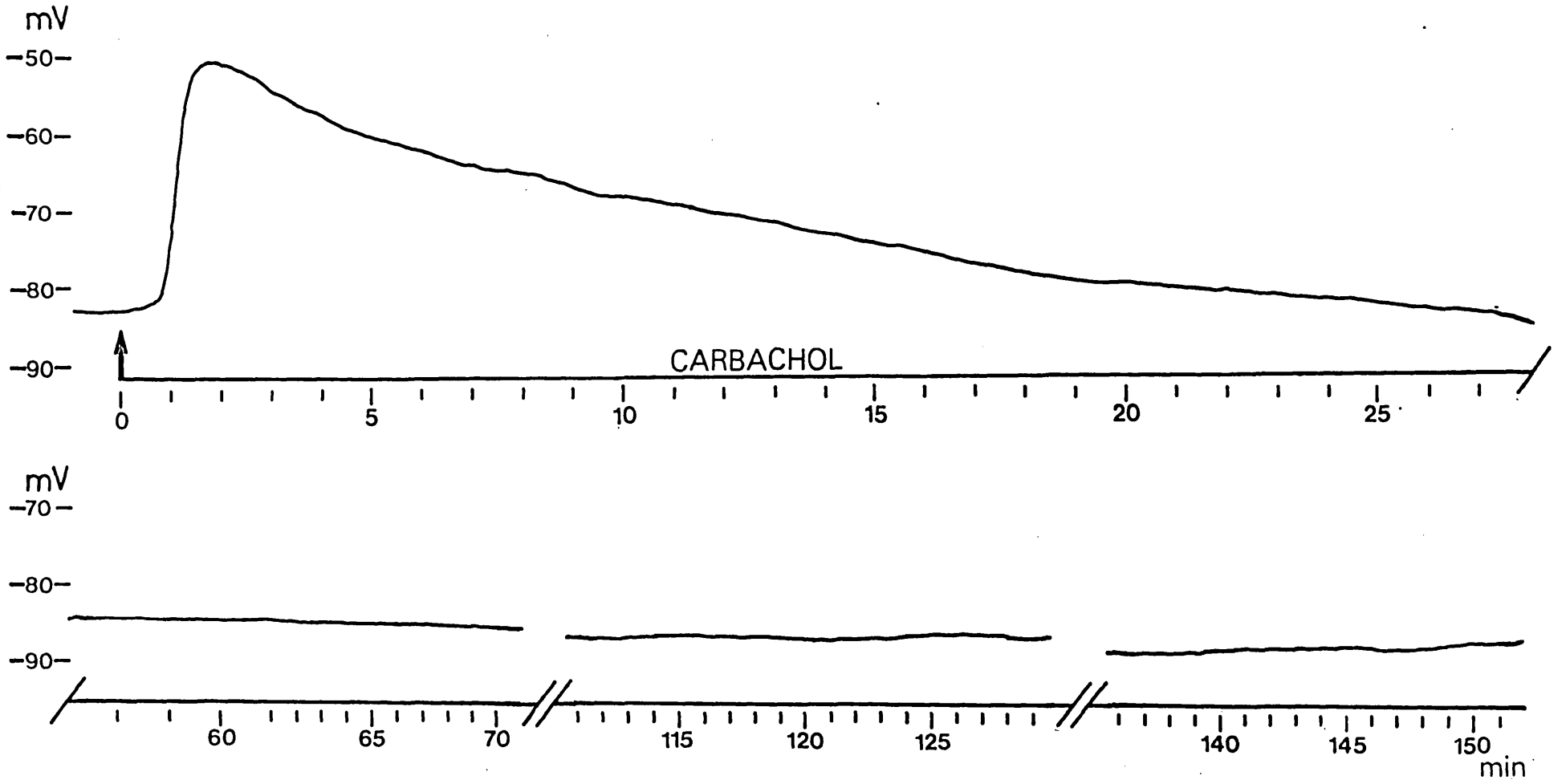

mV
-50-
-60-
-70-
-80-
-90-
CARBACHOL
0
5
10
15
20
25
mV
-70-
-80-
-90-
60
65
70
115
120
125
140
145
150
min

Table 4.5 Effect of Prolonged Exposure to 80 μM Carbachol on Resting Potential

Orig. Resting Pot. (mV)	Peak Depol. Pot. (mV)	Change (mV)	Max. Rate of Depol. (mV/min)	Half-Time (sec)	Time to Recovery of Stable Pot. (min)	Resting Pot. at 60 Min. (mV)	Resting Pot. at 120 Min. (mV)	Hyper-pol. (mV)
				CONTINUOUS RECORDINGS				
-79	-56	23	25.0	(Rep)	30	-92	-93	14
-83	-51	32	55.6	49.5	27	-85	-87	4
-86	-60	26	33.3	27.0	27	-88	-88	2
				INTERMITTENT RECORDINGS				
-76	-46	30	166.7	5.4	-	-74	-76	0
-78	-50	28	38.5	40.2	-	-69	-79	1
-83	-59	24	25.0	39.0	-	-85	-84	1
				MEDIANS				
-81	-54	27			27	-85	-86	

Intermittent recordings monitored the initial depolarization caused by the drug but subsequent measurements were made by reinsertion of the electrode at the same point after suitable intervals. (Rep. refers to data recorded in Table 4.2).

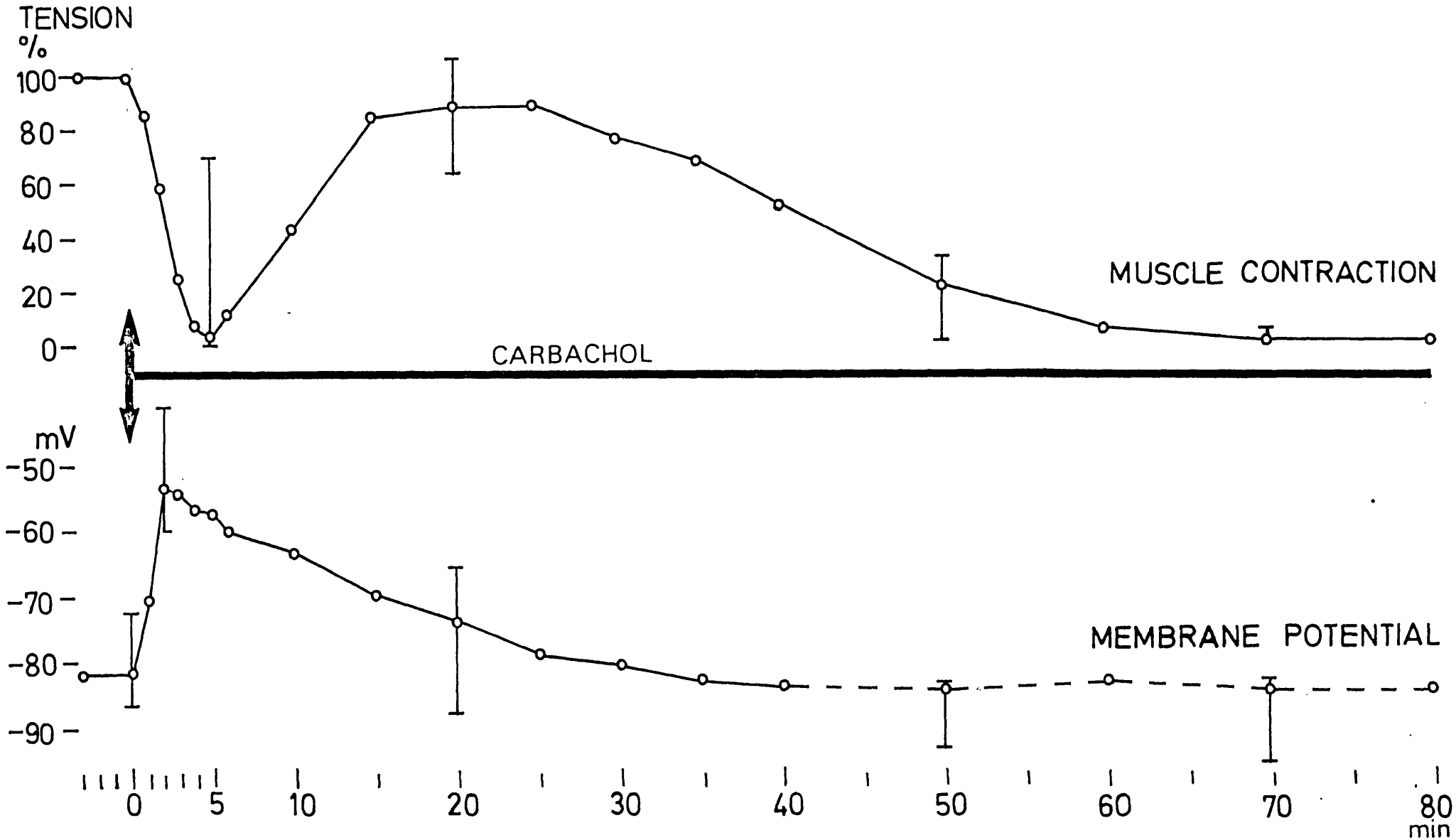
Both recording techniques showed substantial recovery and all 6 recordings are pooled to obtain the median values shown in Table 4.5. Prolonged exposure to carbachol (80 μM) revealed no further change following the spontaneous recovery of potential. The median potential at 2 hr was somewhat hyperpolarized with respect to the original potential (-86 mV and -81 mV respectively). Withdrawal of the electrode at the end of the experiment showed that the effects were not attributable to drift or to an altered tip potential.

When feasible, the recordings made continuously gave a reliable indication of electrical events occurring at the membrane. By its nature, the technique did not require the subjective interpretation that a repeat insertion may have necessitated. Conversely, the likelihood of success with a continuous recording was substantially lower than with an intermittent one.

Relation of Action of Carbachol on Contractions to Effect on Membrane Potential

Fig. 4.5 depicts the effect of carbachol (80 μM) on indirectly elicited muscle contractions (upper trace) and on membrane potential. The upper trace shows the effect of 6 observations (as referred to in Table 3.2) measured at suitable intervals. The error bars show the limits. The ordinate gives tension developed at the strain gauge as a percentage of the initial value.

Figure 4.5. Effect of carbachol (80 μM) on indirectly elicited muscle contractions (upper trace) and on membrane potential. The upper trace shows the median from 6 observations, error bars giving the range. The ordinate gives tension developed at the strain gauge as a percentage of the inital value. The lower trace shows the median from 8 observations (solid line) or from 3 observations (broken line); error bars give the range. Time in min after changeover to drug-bearing solution.


TENSION
%
100
80
60
40
20
0
MUSCLE CONTRACTION
CARBACHOL
mV
-50
-60
-70
-80
-90
MEMBRANE POTENTIAL
0
5
10
20
30
40
50
60
70
80
min

The lower trace shows the effect of carbachol (80 μM) on membrane potential from 8 experiments using continuous recording up to the 40th min. Subsequent points represent the median of 3 experiments. Error bars show 95% limits. The drug was applied to the preparations at the arrows, and the traces are aligned by these for the sake of comparison.

In Fig. 4.5 the electrical recording is from a surface fibre whereas the contractions are measured in the whole muscles so it is not surprising that the effects of the drug on muscle contractions should lag somewhat behind the electrical events. The onset of neuromuscular block appears to coincide with depolarization and the recovery is accompanied by restoration of the resting potential. However, the membrane remains polarized during the secondary slow block.

Summary

1. Carbachol (80 μM) and decamethonium (10 μM) both produced depolarization followed by spontaneous recovery of potential in their presence, recorded by microelectrodes inserted at the end-plate.

2. With decamethonium (10 μM), there was a depolarization from -82 mV to -54 mV, and recovery to -75 mV (medians of 6).

3. In carbachol (80 μM), the recovery was greater. Fibres were depolarized from -77 mV to -49 mV, and recovered to -80 mV (medians of 6).

4. The median effective dose for carbachol was estimated as 10 μM, indicating that the effect observed at 80 μM was likely to be a maximum.

5. The recovered membrane potential during the prolonged application of carbachol (80 μM) appeared to be steady for a further 2 hr. No mepps were apparent at this concentration following the initial rapid depolarization. At lower concentrations, however, mepps persisted for longer periods.

6. Electrical events at the end-plate have been related to the action of these drugs on contractions (previous chapter). The onset of block coincides with depolarization and recovery is accompanied by restoration of the membrane potential. The membrane remains polarized during the slow secondary block.

7. Removal of depolarizing drug following prolonged application of carbachol (80 μM) or decamethonium (10 μM) produced consistent hyperpolarization. The median effect was 6.5 mV (n = 8).

C H A P T E R 5

EFFECTS OF
POTASSIUM-FREE SOLUTION
AND OF OUABAIN

EFFECTS OF POTASSIUM-FREE SOLUTION AND OF OUABAIN

It was suspected that the spontaneous recovery of resting potential (as described in the previous Chapter) in the presence of depolarizing drugs might be partly attributable to the action of the sodium pump. Such a contribution would become apparent if the pump were to be inhibited. This was accomplished by 2 different methods: (i) by the removal of extracellular potassium, and (ii) by the addition of ouabain. Relevant section of this Chapter contain the results from both approaches.

Effects of Removal of Potassium

The effects of removal of potassium were recorded in preparation for experiments on the action of depolarizing drugs in potassium-free solution.

Fig. 5.1 depicts a typical trace showing the response to a period of potassium-free. All muscle fibres exposed to potassium-free bath solution responded by hyperpolarization of the membrane. Table 5.1 lists 10 observation showing a median increase in polarization of 15 mV, from -80 mV. A stable potential was established in potassium-free after 11 min (range 6 - 16 min). Returning the cells to normal solution caused the potential to return to a value which was always sightly more negative than the original potential (median

Figure 5.1. Effect of potassium-free solution on resting potential. Period of potassium-free represented by horizontal bar. Ordinate in mV. Time in min after changeover. Potassium-free solution produced hyperpolarization.

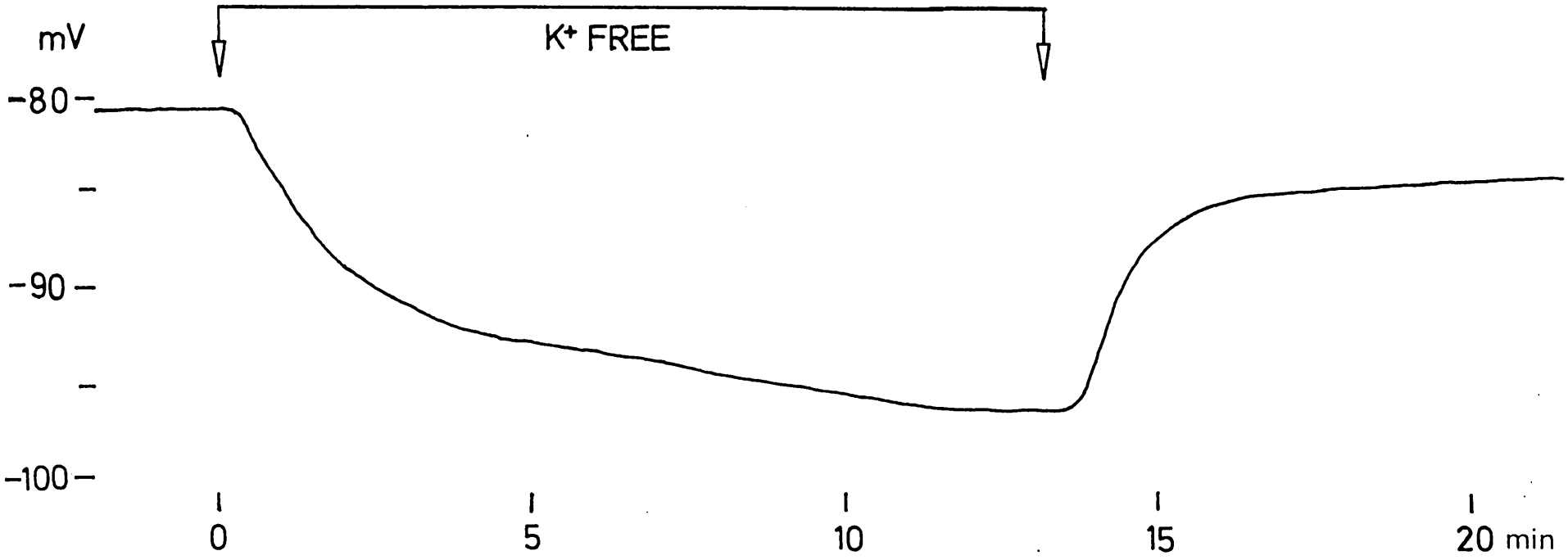

mV
K+ FREE
-80
-90
-100
0
5
10
15
20 min

Table 5.1 Effect of Zero Potassium on Resting Potential

Original Resting Potential (mV)	Peak Hyperpolarization in K^+-Free (mV)	Change (mV)	Time to Stable Potential in K^+ Free (min)	Recovered Potential in Normal Saline (mV)	Final Hyperpolarization (mV)
-75	-83	8	9	-76	1
-76	-84	8	6	-79	3
-76	-88	12	6	-81	5
-77	-92	15	14	-	-
-79	-93	14	12	-81	2
-81	-97	16	10	-85	4
-81	-102	21	13	-87	6
-81	-106	25	13	-	-
-83	-98	15	8	-84	1
-87	-99	12	16	-	-
			MEDIANS		
-80	-95	15	11	-81	3

difference 3 mV). Hyperpolarization of rat diaphragm muscle fibres in potassium-free Krebs' saline has also been reported (Hall, Hilton and West, 1972). Akaike (1975) using rat soleus muscle found that at 37°C the changeover to potassium-free saline caused hyperpolarization.

Mepps observed at a stable potential in the absence of potassium were of the order of 30% greater in magnitude than those seen in normal solution. This is probably attributable to the increased negativity of the membrane potential. Rise time appeared unaltered.

Effects of Decamethonium in Absence of Potassium

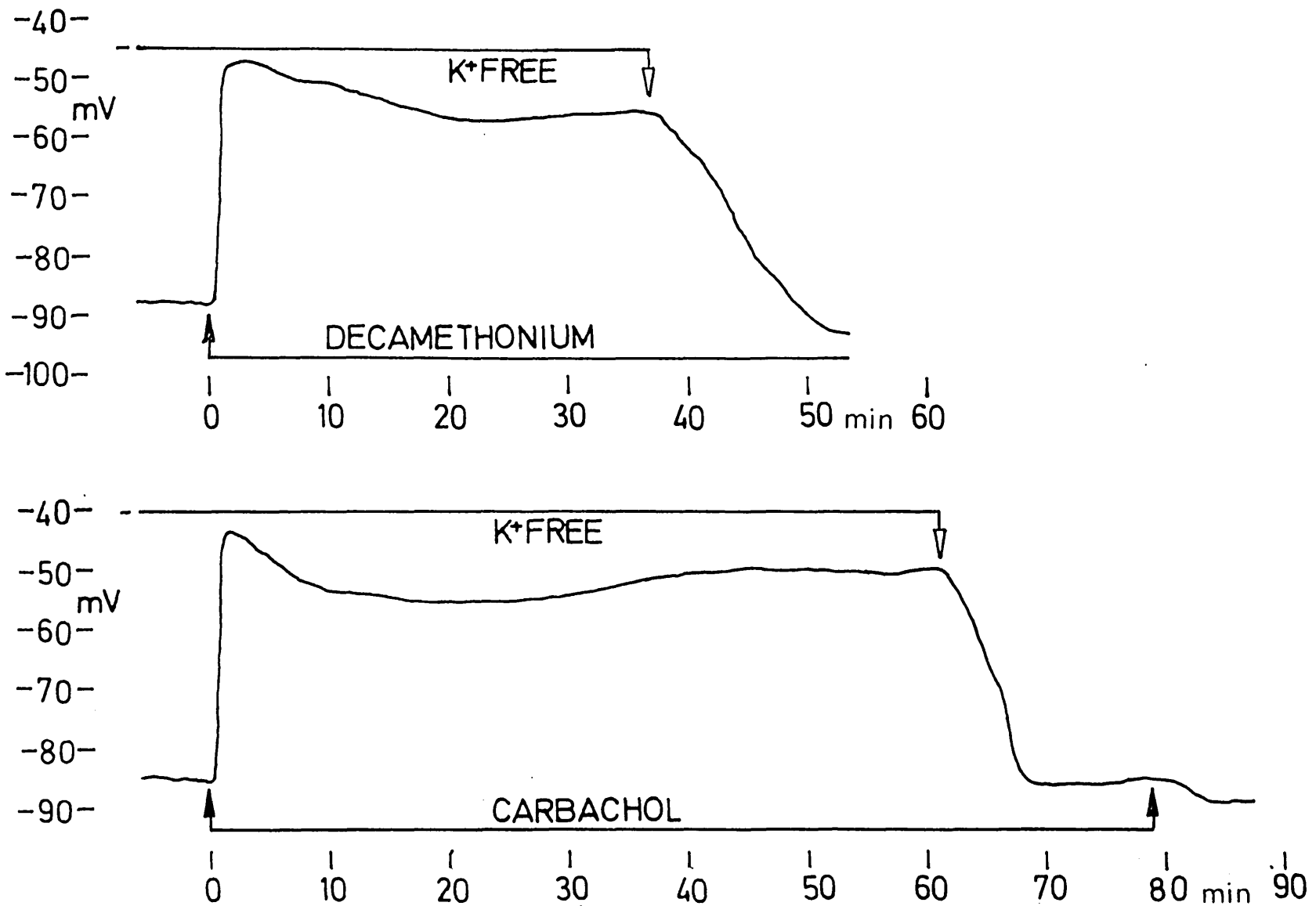
The involvement of electrogenic pump activity in the process of spontaneous recovery of membrane potential was investigated by the removal of extracellular potassium from the bathing medium. Muscle fibres were then exposed to decamethonium (10 μM) and the ensuing electrical events recorded.

Fig. 5.2 (upper trace) shows the membrane potential of a cell which had established a stable hyperpolarization in the absence of potassium. At the arrow, the solution was changed to one containing decamethonium, causing the potential to fall from -88 mV to a peak depolarized value of -47 mV, at a maximum rate of 95.2 mV/min. Peak depolarization occurred after 3 min. Following this there was a partial repolarization over a period of 20 min to a value of -57 mV, a percentage

Figure 5.2. Upper: Effect of decamethonium (10 μM) on membrane potential in absence of potassium. Fibre had established a steady potential in potassium-free (not shown). Period of drug application represented by solid arrow and horizontal bar. Exposure to potassium-free represented by line and open arrow. Ordinate in mV. Time in min after changeover to drug-bearing solution. The inital depolarization was followed by only a small degree of recovery (contrast Fig.4.1, upper).

Lower: Effect of carbachol (80 μM) on membrane potential in absence of potassium. (Contrast Fig. 4.1, lower).

Both muscles showed recovery of potential when potassium was returned to the bathing medium.


-40-
-50-
mV
-60-
-70-
-80-
-90-
-100-
K+FREE
DECAMETHONIUM
0
10
20
30
40
50 min 60
-40-
-50-
mV
-60-
-70-
-80-
-90-
K+FREE
CARBACHOL
0
10
20
30
40
50
60
70
80 min 90

recovery of 24%. This is in strong contrast to the observed effects of 10 μM decamethonium in the normal concentration of potassium (cf Fig. 4.1).

Table 5.2 shows that the recovery was 18% (median of 7), measured from peak depolarization in the absence of potassium. At a normal potassium concentration, 80% of the potential was regained (Table 4.1). Thus the recovery of membrane potential in the presence of decamethonium involves a process which requires the presence of potassium ions.

In 3 experiments from this series, the opportunity arose to return potassium to the bathing medium, while decamethonium remained in the solution. The open arrow in Fig. 5.2 indicates the point of replacement of potassium ions. In this instance the potential subsequently recovered, at a maximum rate of 4 mV/min. The potential reached a plateau at -93 mV, 15 min after the replacement of potassium. The end-plate was hyperpolarized by 5 mV with respect to the original resting value. The electrode was expelled just after the 53rd min, preventing any further recording. This fibre showed the largest degree of recovery following the return of potassium.

Effects of Carbachol in Absence of Potassium

A parallel series of experiments investigating the actions of a depolarizing drug in the absence of potassium were performed using carbachol (80 μM). The results confirmed the observations made with decamethonium.

Table 5.2 Effect of 10 μM Decamethonium in Potassium-Free Solution and Effect of Replacement of Potassium on Resting Potential

Resting Pot. in K^+-free Before Drug (mV)	Peak Depol. Pot. (mV)	Change (mV)	Stable Pot. in Drug (mV)	% Recovery from Peak Depol. (%)	Stable Pot. After K^+ Replacement (mV)	% Recovery After K^+ Replacement (%)
-85	-35	50	-53	36	-	-
-85	-55	30	-56	3	-	-
-88	-47	41	-57	24	-93	112
-89	-41	48	-49	17	-70	60
-89	-56	33	-68	36	-	-
-91	-37	54	-46	17	-	-
-96	-56	40	-63	18	-79	58
			MEDIANS			
-89	-47	41	-56	18	-79	60

Table 5.3 Effect of 80μ M Carbachol in Potassium-Free Solution- and Effect of Replacement of Potassium on Resting Potential

Resting Pot. in K^+-free Before Drug (mV)	Peak Depol. Pot. (mV)	Change (mV)	Stable Pot. in Drug (mV)	% Recovery from Peak Depol. (%)	Stable Pot. After K^+ Replacement (mV)	% Recovery After K^+ Replacement (%)
-81	-46	35	-62	46	-73	77
-83	-51	22	-51	0	-	-
-85	-43	42	-50	17	-86	102
-88	-42	46	-52	22	-60	39
-88	-45	43	-57	28	-104	137
-89	-47	42	-54	17	-79	76
			MEDIANS			
-87	-46	42	-53	20	-79	77

Recovery of membrane potential on replacement of potassium for eight complete experiments (Tables 5.2 and 5.3)

	Initial mV	Final mV	Change mV
Decamethonium	-57	-93	36
	-49	-70	21
	-63	-79	16
Carbachol	-62	-73	11
	-50	-86	36
	-52	-60	8
	-57	-104	47
	-54	-79	25
Combined median			23
n			8
Range			8, 47

The sequence of events of an experiment using carbachol is depicted in Fig. 5.2 (lower trace). The fibre had established a hyperpolarized membrane potential (-85 mV) in the absence of potassium. 80 μM carbachol (black arrow) caused a depolarization to a peak of -43 mV at a maximum rate of 57 mV/min. There ensued a spontaneous partial recovery to around -55 mV within 25 min, although this waned by 5 mV over the next 30 min.

After an hr in the drug there were no indications of further recovery and so the bathing medium was switched to one bearing potassium (open arrow). The potential subsequently recovered to a stable value of -86 mV at a maximum rate of 12 mV/min.

At the 79th min, the preparation was returned to normal physiological saline and the fibre hyperpolarized by a few millivolts.

Five other experiments confirmed these observation and the results are shown in Table 5.3. Carbachol in the absence of potassium caused a depolarization from -87 mV to -46 mV (median of 6), with a partial recovery to -53 mV, i.e. 20% from a peak depolarization. In all but one of the experiments it was possible to return to a solution containing potassium and this led to further recovery in all instances. Two fibres established a hyperpolarized potential with respect to their resting potential. The median value (of 5) was -79 mV, a recovery of 77%.

Results obtained using either decamethonium or carbachol at these concentrations are thus comparable both quantitatively and qualitatively. Both show that the large degree of spontaneous recovery of potential normally seen with this preparation is absent when potassium is omitted. This observation, in conjunction with the recovery which does occur after the replacement of this ion, indicates the activity of a mechanism which contributes to the polarity of the cell membrane and is dependent on the presence of extracellular potassium.

Removal of Potassium for Discrete Intervals

A different indication of the role of an electrogenic pump was given by experiments involving the removal of potassium for discrete intervals, at suitable times during the recovery from a depolarizing drug. It was anticipated that the experiments would contrast the activity of such a mechanism during the period of recovery of potential, with a diminished activity at a later time.

Two series of experiments employed decamethonium (10 μM) or carbachol (80 μM) to depolarize the cell membrane. The solution was subsequently changed to one lacking potassium but still containing drug, firstly for a period during the recovery of potential, and secondly for a period when a stable potential had been re-established.

(i) During Presence of Decamethonium

Fig. 5.3 depicts a trace where potassium was removed from the bathing medium for 2 discrete intervals during the presence of decamethonium (10 μM). The resting potential had been steady at -78 mV for over 5 min prior to the addition of 10 μM decamethonium. Administration of the drug caused depolarization to -38 mV at a maximum rate of 75 mV/min. At the 30th min, when 78% of the potential had been spontaneously recovered (-69 mV), the solution was changed to one lacking potassium. There ensued not just a depression of the recovery process but a depolarization to a potential of -56 mV. This action was maintained until potassium was returned at the 34th min, when the potential regained its repolarizing trend. Potassium was absent for 4 min and the depolarization associated with its removal was at a faster rate than that of the repolarization upon its replacement.

The membrane potential recovered to a hyperpolarized value (-86 mV compared with an initial R.P. of -78 mV). At the 60th min in this stage of full recovery, potassium was again removed. A hyperpolarization to -97 mV was observed, occurring over the 7 min for which potassium was absent. Its subsequent replacement caused a slight loss of negativity to -95 mV. The electrode was expelled just after the 79th min.

Table 5.4 lists the results obtained from 7 experiments including that in Fig. 5.3. Six muscles were exposed to potassium-free solution during spontaneous recovery in decamethonium (10 μM). With 4 of these muscles, the recovery

Figure 5.3. Effects of removal of potassium for discrete intervals during spontaneous recovery from decamethonium (10 μM). Period of drug application represented by solid arrow and horizontal bar. Individual periods of potassium-free represented by open arrows and shorter bars. Ordinate in mV. Time in min after changeover to drug-bearing solution. During early recovery, potassium-free produced depolarization, whereas at a later stage potassium-free solution produced hyperpolarization.

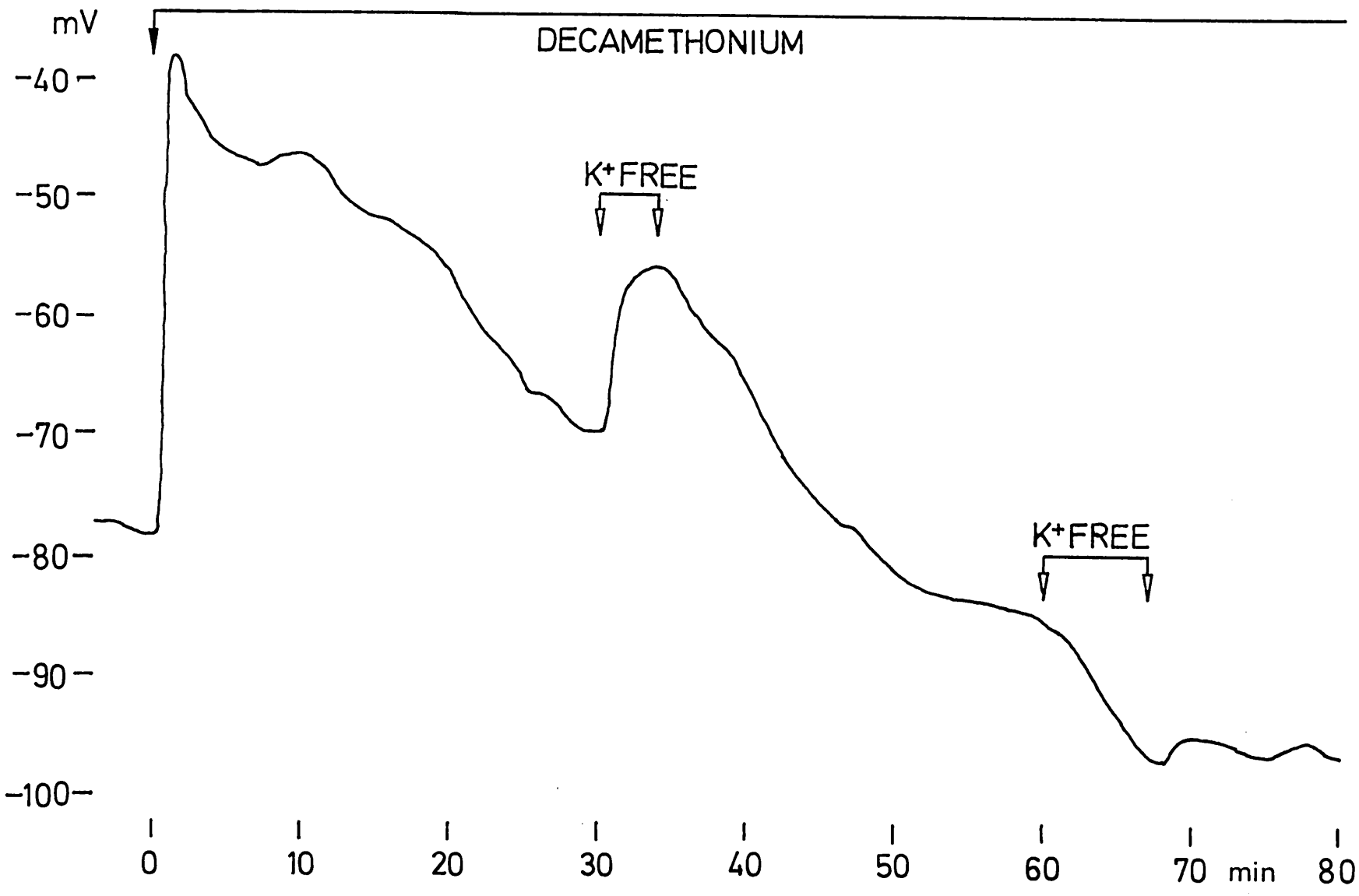

mV
DECAMETHONIUM
-40
-50
-60
-70
-80
-90
-100
K+FREE
K+FREE
0
10
20
30
40
50
60
70
min
80

Table 5.4 Effect of Removal of Potassium for Discrete Intervals During Recovery from 10 μM Decamethonium

					DURING RECOVERY OF POTENTIAL			DURING STABLE POTENTIAL		
Original Resting Potential	Peak Depol. Potential	Change	Max. Rate of Depol.	Half-Time	Pot. when K^+ Removed	Time when K^+ Removed	Pot. in K^+ Free	Pot. when K^+ Removed	Time when K^+ Removed	Pot. in K^+ Free
(mV)	(mV)	(mV)	(mV/min)	(sec)	(mV)	(min)	(mV)	(mV)	(min)	(mV)
-73	-49	24	28.6	45.0	-67	11.0	No Change	-91	60.0	-107
-75	-48	27	133.3	9.0	-63	10.0	-61	-	-	-
-77	-47	30	40.0	24.0	-66	13.0	-64	-	-	-
-77	-55	22	66.7	19.5	-	-	-	-92	60.0	-99
-78	-38	40	75.0	20.1	-69	30.0	-56	-86	60.0	-97
-80	-61	19	66.7	12.6	-68	10.0	-64	-	-	-
-83	-57	26	30.0	39.0	-69	6.5	-69	-84	31.0	-93
					MEDIANS					
-77	-49	26			-68	10.5	-64	-89	60.0	-98

Times measured from addition of drug.

Summary - 6 experiments on K^+ removal during recovery from decamethonium;
4 depolarized (reversed recovery process), 1 steady (recovery process halted),
1 no change in recovery process.
- 4 experiments on K^+ removal during stable potential in presence of decamethonium;
All 4 hyperpolarized.

process was reversed and the fibres depolarized. With 1 muscle the rate of recovery was diminished such that a steady potential was attained. These effects were maintained for the period of potassium-free, recovery continuing afterwards. In the remaining muscle, potassium-free solution had no effect on the recovery process.

Four muscles were exposed to potassium-free solution when the potential had established a steady value (Table 5.4); all 4 hyperpolarized. One of these had not previously been exposed to potassium-free during the recovery process.

(ii) During Presence of Carbachol

Results obtained from parallel experiments using carbachol (80 μM) resembled those where decamethonium had been employed. Fig. 5.4 represents a trace from an experiment where removal of potassium at different times in the presence of carbachol caused opposing effects.

The fibre depolarized from -72 mV to -48 mV due to the action of carbachol and when 54% of its potential had been regained, was bathed in a solution lacking potassium. This action caused the cell to depolarize from -61 mV to -56 mV, a stable potential being established within 5 min. The replacement of potassium led to a repolarization, at an increased rate to that of the original recovery process. At the 30th min, this rate waned to match the original one (see Figure 5.4).

Figure 5.4. Effects of removal of potassium for discrete intervals during spontaneous recovery from carbachol (80 μM). Period of drug application represented by solid arrows and horizontal bar. Individual periods of potassium-free represented by open arrows and shorter bars. Ordinate in mV. Time in min after changeover to drug-bearing solution. The effects are similar to those shown in Fig. 5.3.

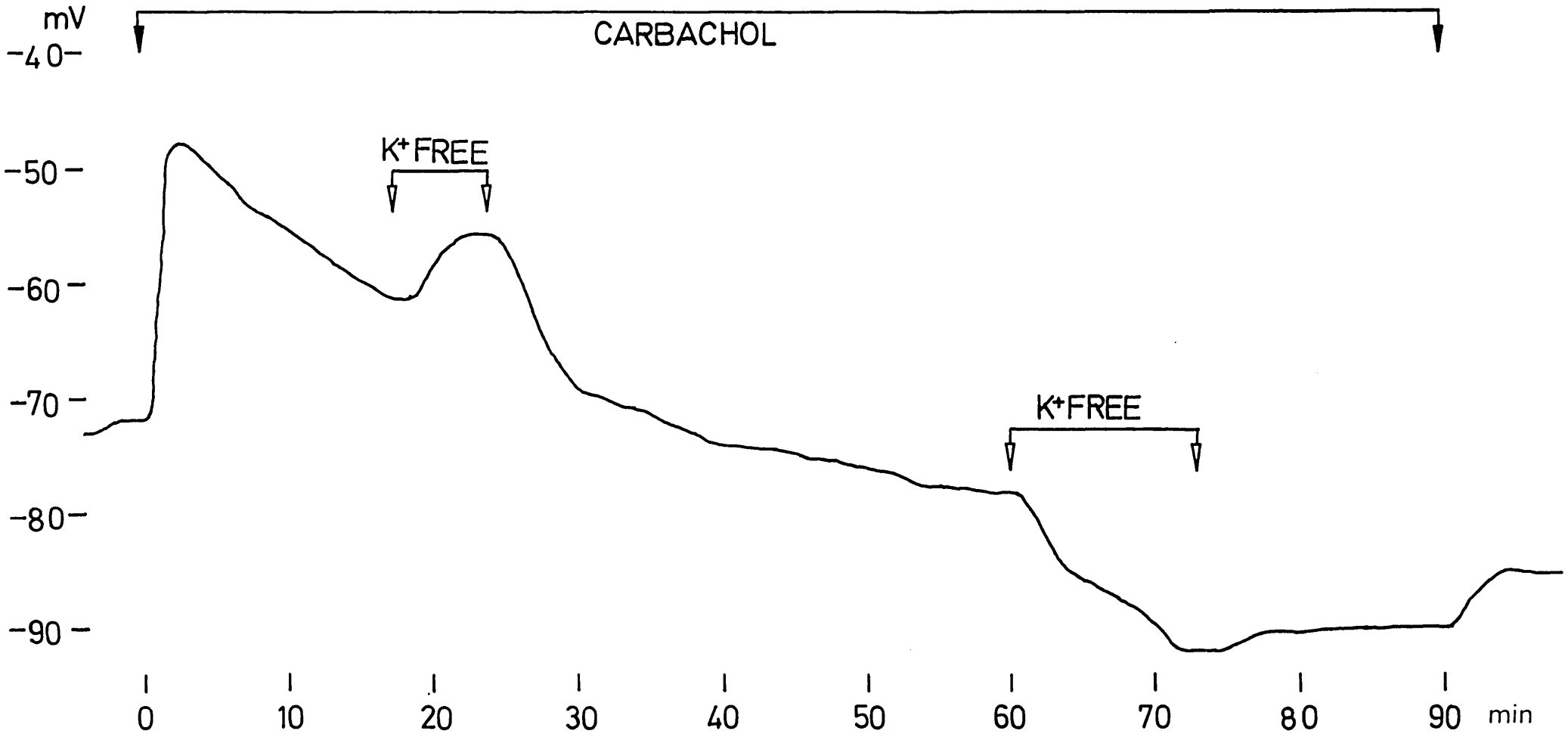

mV
-40-
-50-
-60-
-70-
-80-
-90-
CARBACHOL
K+ FREE
K+ FREE
0
10
20
30
40
50
60
70
80
90
min

Table 5.5 Effect of Removal of Potassium for Discrete Intervals During Recovery from 80 μM Carbachol

					DURING RECOVERY OF POTENTIAL			DURING STABLE POTENTIAL		
Original Resting Potential (mV)	Peak Depol. Potential (mV)	Change (mV)	Max. Rate of Depol. (mV/min)	Half-Time (sec)	Pot. When K^+ Removed (mV)	Time When K^+ Removed (min)	Pot. in K^+ Free (mV)	Pot. When K^+ Removed (mV)	Time When K^+ Removed (min)	Pot. in K^+ Free (mV)
-72	-48	24	24.0	43.5	-61	17.5	-56	-78	60.0	-92
-74	-57	17	17.6	34.5	-65	9.0	-65	-	-	-
-75	-43	32	100.0	15.0	-64	13.0	-55	-81	56.0	-88
-75	-48	27	61.5	21.0	-60	13.0	-57	-	-	-
-77	-46	31	61.5	24.0	-60	30.0	-60	-	-	-
-80	-49	31	37.5	27.0	-64	11.0	-52	-74	71.0	-84
-83	-51	32	55.6	(Rep)	-	-	-	-87	151.0	-100
-83	-59	24	25.0	(Rep)	-	-	-	-84	122.0	-94
					MEDIANS					
-76	-49	29			-63	13.0	-57	-81	71.0	-92

Times measured from addition of drug.

Summary - 6 experiments on K^+ removal during recovery from carbachol;
4 depolarized (reversed recovery process), 2 steady (recovery process halted),
- 5 experiments on K^+ removal during stable potential in presence of carbachol;
All 5 hyperpolarized.
(Rep. refers to data recorded in Table 4.4).

After 60 min in carbachol, the potential had established a slightly hyperpolarized value (-78 mV compared with initial R.P. of -72 mV). At this time the potassium was again removed for a discrete interval, causing the potential to become further negative, ultimately reaching a plateau at -92 mV. Two mV of this negativity were lost when potassium was returned to the bathing medium. (In contrast to earlier experiments where there was a slight gain in membrane polarization when the period of application of a depolarizing drug was ended (see Fig. 4.1), 5 mV depolarization was observed in this preparation upon a return to normal physiological saline).

Table 5.5 lists the results obtained from 8 experiments including that in Fig. 5.4. Six muscles were exposed to potassium-free solution during spontaneous recovery in carbachol (80 μM). Of these, 4 depolarized (the recovery process was reversed), and 2 became steady (the recovery process was halted).

Five muscles were exposed to potassium-free solution when the potential had established a steady value (also Table 5.5); all 5 hyperpolarized. Two of these had not been previously exposed to potassium-free during the recovery process.

(iii) Combined Results; Potassium Removal in Presence of of Decamethonium and of Carbachol

In total, 12 muscles were exposed to potassium-free solution during spontaneous recovery from a depolarizing drug. Eight of these depolarized, 3 attained a steady potential, and

1 exhibited no effect. Thus in 11 out of 12 experiments the recovery period was reduced or reversed ($P < 0.01$).

The results may also be combined for the effects of potassium removal when the membrane potential was stable. In all of 9 such experiments, potassium-free caused hyperpolarization ($P < 0.01$).

The effects of removal of potassium for a discrete interval during the presence of a depolarizing drug were dependent on the stage of recovery. The spontaneous repolarization process required the presence of potassium in the external medium, being reversed in most instances by potassium-free solution. The effect was quite different at a later stage. Removal of potassium when a stable potential had been established led to hyperpolarization.

Hyperpolarization due to potassium-free solution has already been described (Table 5.1, Fig. 5.1) and is attributable to a direct action on the membrane potential as predicted by the electrochemical equation. It is interesting to note that the median effect of potassium-free in the absence of any drug was a 15 mV hyperpolarization ($n = 10$) but that in the presence of a depolarizer, potassium-free led to only 10 mV hyperpolarization ($n = 9$).* Such an observation might indicate that a small proportion of the stable potential which is attained following spontaneous recovery is attributable to a process which is dependent on potassium ions.

* In the continued presence of drug, P_{Na}/P_K would be increased, and this might partly account for the smaller hyperpolarization seen on removal of potassium.

Such a dependency is far greater during the period of repolarization. Any hyperpolarization due to an electrochemical effect of potassium-free on the membrane potential is obscured at this stage by the inhibition of an electrogenic exchange mechanism. Hence the recovery process was in most experiments reversed, depolarization occurring.

Effects of Carbachol in Presence of Ouabain

Inhibition of an electrogenic pump mechanism by a reduction in external potassium creates a complication. A direct effect of zero potassium on the electrochemical potential (as already described) will tend to conceal the depolarization attributable to any decrease in electrogenic pumping.

Two series of experiments employing ouabain as a specific inhibitor of pump activity were performed to augment the observations made using potassium-free solutions. In the first, carbachol and ouabain were added simultaneously. In the second, ouabain was added only after spontaneous repolarization was complete in the presence of carbachol.

(i) Carbachol and Ouabain Added Simultaneously

Fig. 5.5 shows the action of carbachol (80μ M) and ouabain (10 μM) when added together. In this experiment, the simultaneous administration of these drugs led to a depolarization from a resting value of -74 mV to a peak depolarized potential of -38 mV at a maximum rate of 132.0 mV/min. There followed a small repolarization to - 44 mV over a period of about 20 min; such a slow drift is in strong contrast to the rapid and complete spontaneous recovery recorded in the presence of carbachol (Fig. 4.1). It proved possible to record intracellularly for over 1 hr in the experiment depicted in Fig. 5.5, and no subsequent recovery was detected. Table 5.6 lists the results from 6 experiments, including this one, and shows the median effects. Continuous recordings for over 1 hr were

Figure 5.5. The effect of carbachol (80 μM) and ouabain (10 μM) on membrane potential, when added simultaneously. Ordinate in mV. Time in min after change-over to drug-bearing solution. Rapid depolarization was followed by little recovery, the membrane remaining depolarized for the duration of the recording (over 1 hr). This is in contrast to the spontaneous repolarization with carbachol (Fig. 4.1) when ouabain was not present.

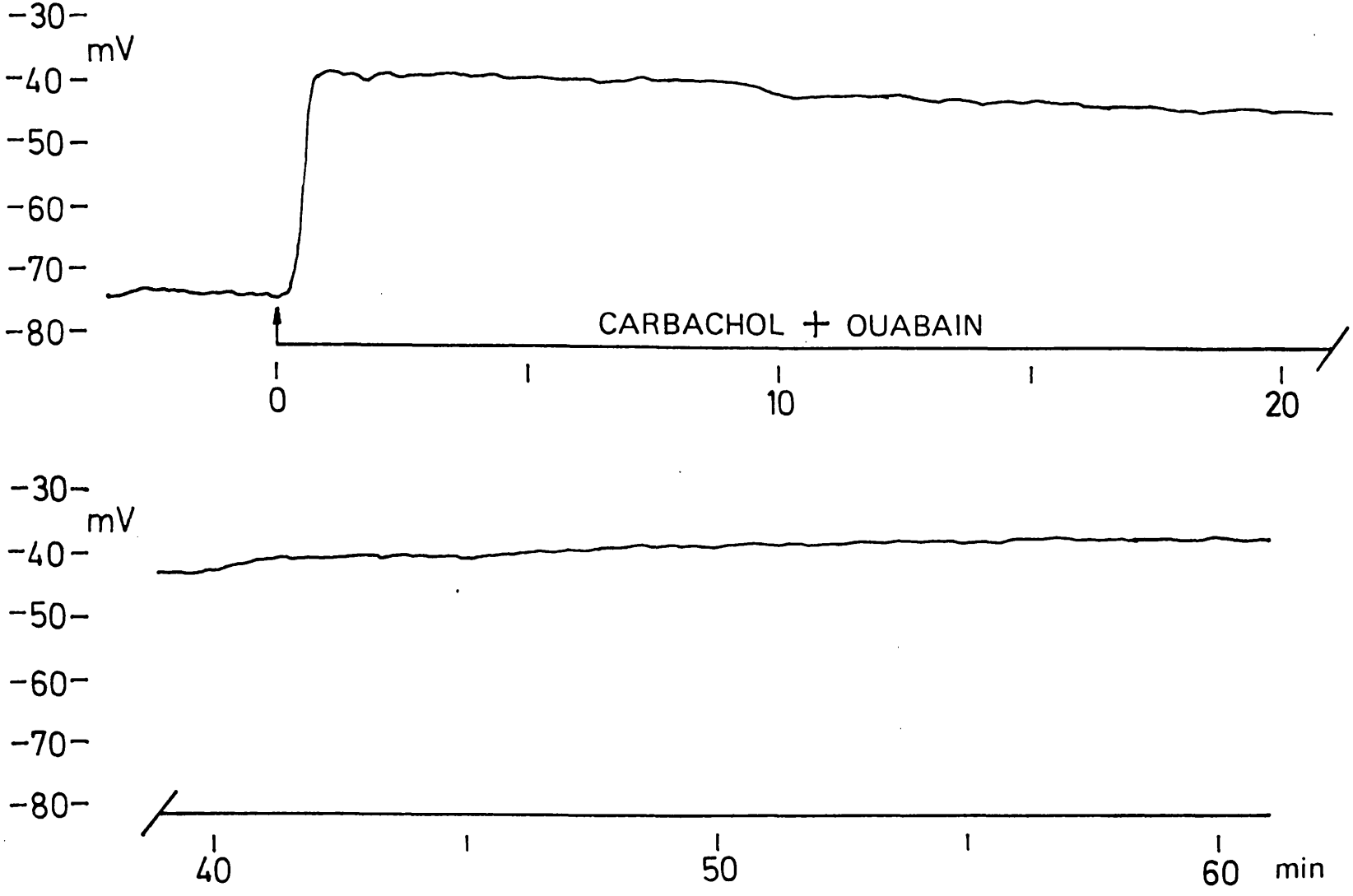

-30-
mV
-40-
-50-
-60-
-70-
-80-
CARBACHOL + OUABAIN
0
10
20
-30-
mV
-40-
-50-
-60-
-70-
-80-
40
50
60
min

Table 5.6 Effect of 80 μM Carbachol Plus 10 μM Ouabain on Membrane Potential

Rest. Pot. Before Drugs (mV)	Peak Depol (mV)	Change (mV)	Max Rate of Depol. (mV/min)	Plateau Pot. in Drugs (mV)	Pot. at 30 min (mV)	Pot. at 60 min (mV)
-74	-37	37	41.6	-37	-38	-40
-74	-38	36	132.0	-44	-44	-41
-75	-40	35	26.4	-40	-40	-30
-76	-50	26	86.8	-43	-43	-
-82	-38	44	74.0	-37	-38	-28
-86	-49	37	27.5	-50	-50	-46
			MEDIANS			
-75	-39	37		-42	-42	-40

Estimate of $g/(g+G)$ from $\Delta V/(V - 3.6)$.

$37/(74-3.6) = 0.526$
$36/(74-3.6) = 0.511$
$35/(75-3.6) = 0.490$
$26/(76-3.6) = 0.359$
$44/(82-3.6) = 0.561$
$37/(86-3.6) = 0.449$

Median 0.501 (6)

made in 5 out of 6 fibres, and none showed repolarization of more than 3 mV during this period.

(ii) Ouabain Added After Recovery in Carbachol

In these experiments, ouabain (10 μM) was added after spontaneous recovery in carbachol (80 μM) was complete; results obtained using this procedure constitute Group B. Another batch of experiments (Group A), where the effect of only ouabain on membrane potential was recorded, served as controls.

Group A

Trace A in Fig. 5.6 shows the action of ouabain (10 μM) on membrane potential, as recorded from the end-plate region. The addition of the drug caused a relatively slow depolarization, at a maximum rate of 0.93 mV/min, from a resting value of -87 mV to a plateau around -60 mV. In this instance it was possible to make a continuous recording for 50 min, and the potential remained constant.

The upper section of Table 5.7 lists the effect of ouabain (10 μM), as recorded from 6 different preparations. The rate of depolarization ranged from 0.51 to 1.45 mV/min, with a median of 0.86 mV/min.

Group B

Trace B in Fig. 5.6 shows the action of ouabain (10 μM) on membrane potential following complete recovery in the presence of carbachol (80 μM). Depolarization and recovery with carbachol have been described in the preceeding Chapter,

Figure 5.6. Effects of ouabain (10 μM) on membrane potential in presence of carbachol (80 μM). Trace 'A' (control) shows effect of ouabain (10 μM) on resting potential. Trace 'B' shows effect of ouabain on membrane potential following depolarization and spontaneous recovery in the presence of carbachol. Application of drugs is represented by relevant horizontal lines, oblique slashes indicating periods not shown. Both ordinates in mV. Time in min after changeover to first drug-bearing solution. In 'B' the depolarization due to ouabain has a faster rate than in 'A'.

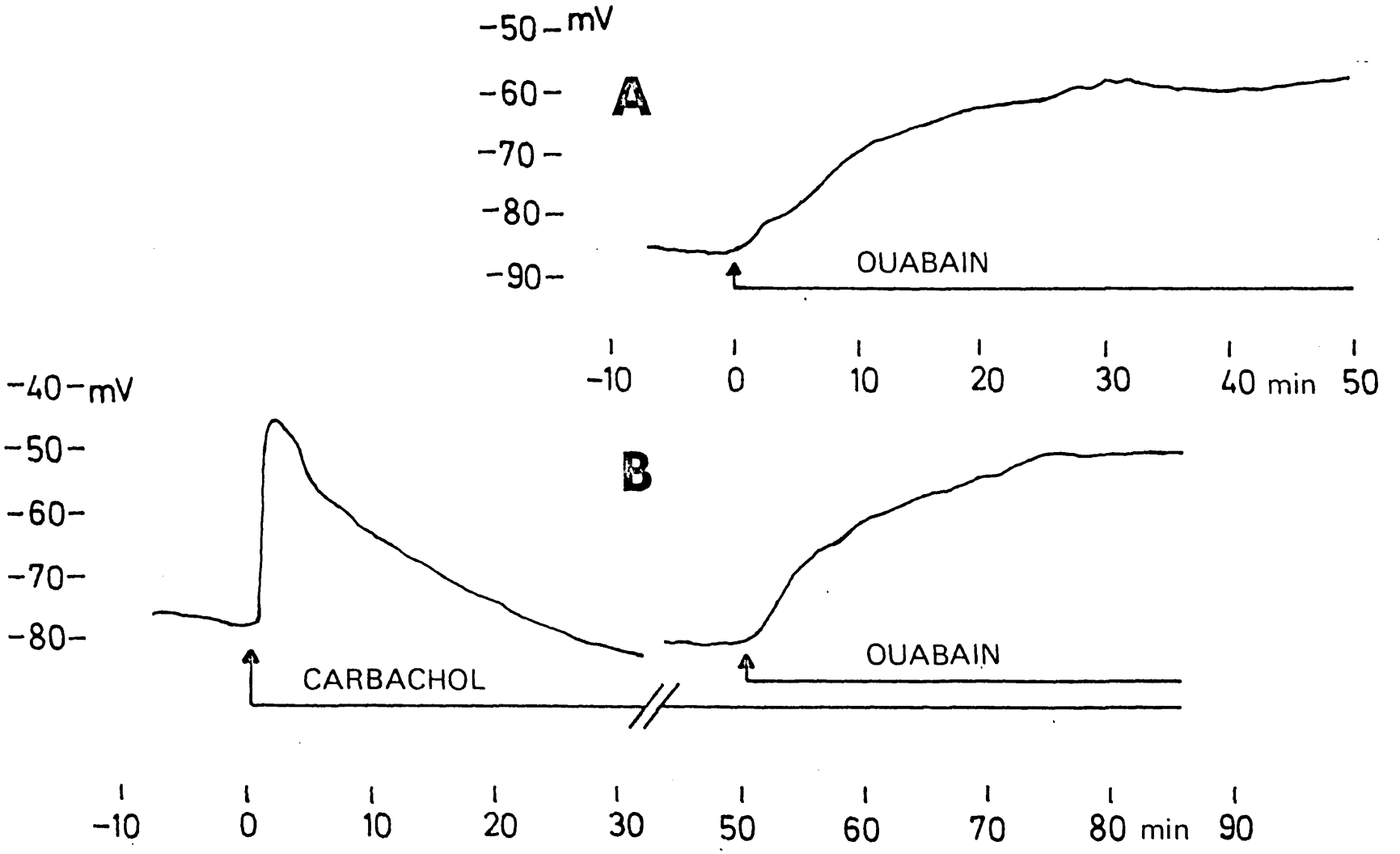

-50- mV
-60-
-70-
-80-
-90-
A
OUABAIN
-10
0
10
20
30
40 min
50
-40- mV
-50-
-60-
-70-
-80-
B
CARBACHOL
OUABAIN
-10
0
10
20
30
50
60
70
80 min
90

Table 5.7 Effect of 10 μM Ouabain on Membrane Potential Following Spontaneous Recovery in 80 μM Carbachol

GROUP A 10 μM OUABAIN ON RP (CONTROLS)

Original Resting Potential (mV)	Max Rate of Depol. (mV/min)	Plateau Potential (mV)	Change (mV)
-78	1.45	-59	19
-79	0.77	-60	19
-81	0.78	-60	21
-83	1.20	-51	32
-84	0.51	-61	23
-87	0.93	-60	27
	MEDIANS		
-82	0.86	-60	22

GROUP B 10 μM OUABAIN ON RP IN PRESENCE OF 80 μM CARBACHOL

Original Resting Potential (mV)	Time After Carb. (min)	Recovered Potential in Carb. (mV)	Max Rate of Depol. (mV/min)	Plateau Potential (mV)	Change (mV)
-74	60	-77	4.6	-56	21
-74	45	-85	3.7	no plateau	-
-74	60	-74	5.3	-54	20
-76	50	-80	3.7	-50	30
-78	60	-82	2.4	-62	20
-82	27	-76	3.2	-43	33
			MEDIANS		
-75	55	-79	3.7	-54	21

The second preparation in Group B showed a continuous fall in membrane potential after addition of ouabain and did not establish a plateau.

but the event is depicted to give an indication of the time course. Ouabain was added at the 50th min in the presence of carbachol, when the potential was -80 mV. Depolarization ensued at a maximum rate of 3.7 mV/min, to reach a plateau at -50 mV within 30 min.

The lower section of Table 5.7 lists the results obtained using this procedure in 6 preparations. All but one were performed after 45 to 60 min in the drug, when recovery was complete. The rate of depolarization with ouabain in the presence of carbachol ranged from 2.4 to 5.3 mV/min, with a median of 3.7 mV/min.

Ouabain did not produce a sudden change in potential in any of the fibres in Group A or Group B, but led to a steady fall in potential in each case. However, the rate of depolarization associated with the addition of ouabain was accelerated by a factor of about 4 when performed in the presence of carbachol. The decline in the control group (A) is attributable to a gradual loss of internal potassium and its possible accumulation on the outside of the cell membrane. The enhanced depolarization seen with the fibres in Group B indicate that the role of a sodium pump is greater in the continued presence of an agonist than in the resting situation.

Effect of Ouabain on Frequency of Miniature End-Plate Potentials

It was noticed while performing the control experiments with ouabain that there was an action on the frequency of mepps. Mepps were thus displayed on the oscilloscope screen at

suitable intervals and photographed. All such studies were made from continuous recordings at the end-plate.

The frequency of mepps at rest was in the order of 2 to 4 sec^{-1}. Ouabain (10 μM) increased the spontaneous release of transmitter such that there was an approximate 60 fold increase in mepp frequency within 30 min (4 observations). After such a time the depolarizing action of the drug caused the magnitude of the mepps to diminish and it became impractical to count them, but there appeared to be a waning of the frequency after longer exposures.

Fig. 5.7 shows a semilogarithmic plot of mepp frequency at intervals after the application of ouabain. The depression effect seen at 6 min was observed in 3 of the preparations where an analysis was made within the first 10 min. There was a sharp increase in the rate of rise of frequency between the 20th and 25th min in all muscles.

The facilitating action of ouabain on neuromuscular transmission in skeletal muscle has long been known and its two step action on mepp frequency has recently been reported (Branisteanu et al, 1979). In frog these workers described an initial phase in which sodium concentration played an important role in the slow increase of transmitter release. In the second phase of the response they attributed a much more rapid increase in mepp frequency to the calcium releasing effect of ouabain on the cytoplasmic sequestering sites. A decline in mepp frequency towards control values after prolonged exposure to another cardiac glycoside, digoxin, has also been reported (Birks and Cohen, 1968).

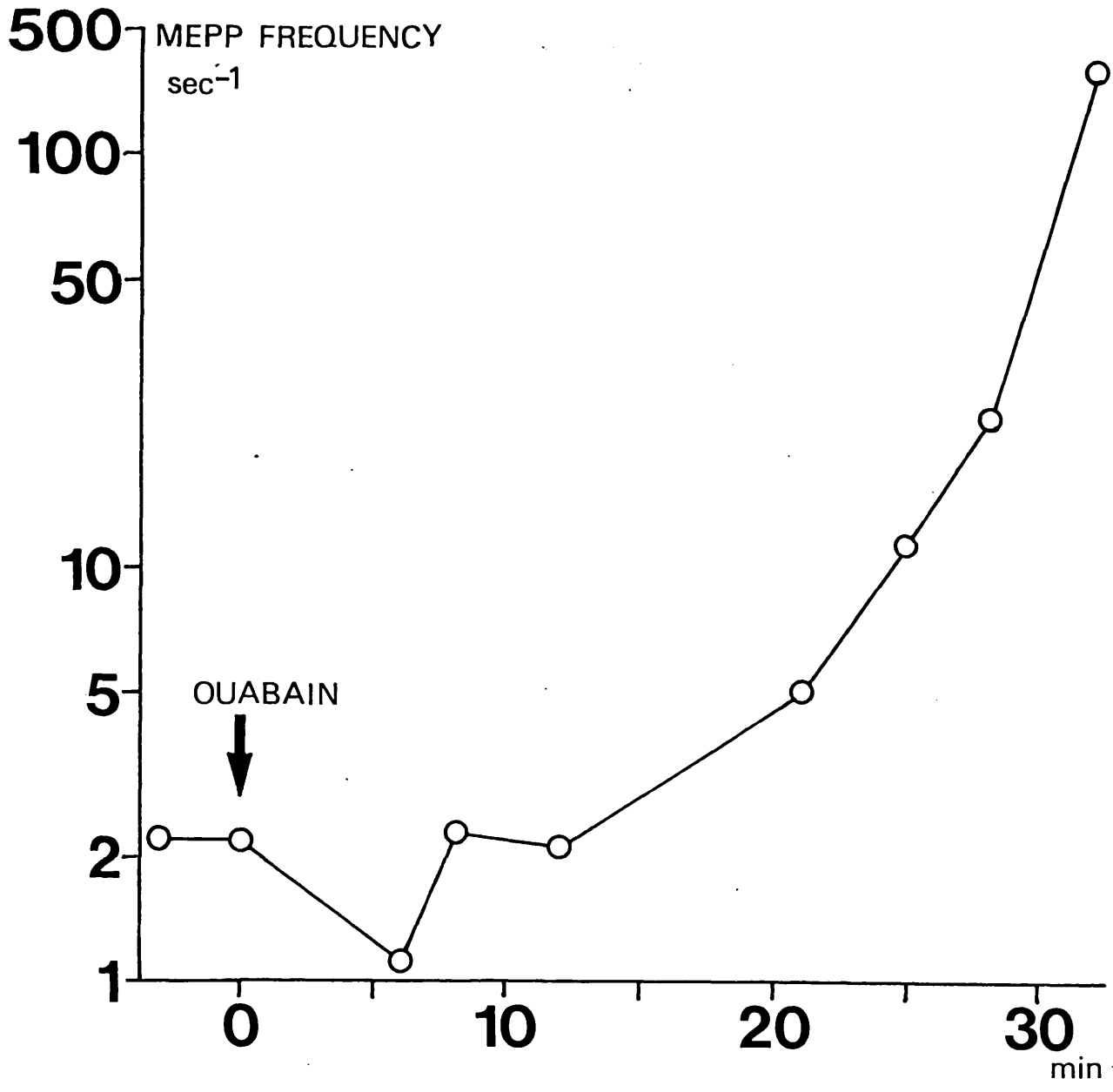


Figure 5.7. Effect of ouabain (10 μM) on mepp frequency. Arrow indicates point of changeover to drug-bearing solution. Ordinate shows mepp frequency on logarithmic scale in mepps/sec. Abscissa shows time after application in min.

SUMMARY

1. Exposure of muscle fibres to potassium-free saline led to a hyperpolarization of 15 mV (10: limits 8, 21 mV).

2. In the absence of potassium, depolarization due to decamethonium (10 μM) was followed by spontaneous recovery of only 18%. (This is in contrast to the 80% recovery which occurred at a normal potassium concentration).

3. The effects of carbachol (80 μM) in the absence of potassium were qualitatively similar to those of decamethonium.

4. Replacement of potassium after depolarization by agonist in potassium-free solution was followed by repolarization which was 23 mV (8 muscles, combined results with decamethonium and carbachol).

5. The effects of removal of potassium for a discrete interval during recovery from either decamethonium or carbachol were dependent of the stage of recovery. If the removal was performed during the period of spontaneous repolarization, then the recovery process was halted or reversed. If it was performed at a later time when a stable potential had been established, hyperpolarization resulted.

6. In the presence of ouabain (10 μM), spontaneous repolarization in carbachol (80 μM) was abolished.

7. If spontaneous recovery in carbachol was allowed to proceed, then the subsequent addition of ouabain produced a steady fall in membrane potential. The rate of this fall was about 4 times faster than that produced by ouabain in the absence of carbachol.

C H A P T E R 6

TWO-ELECTRODE RECORDINGS OF INPUT RESISTANCE

TWO ELECTRODE RECORDINGS OF INPUT RESISTANCE

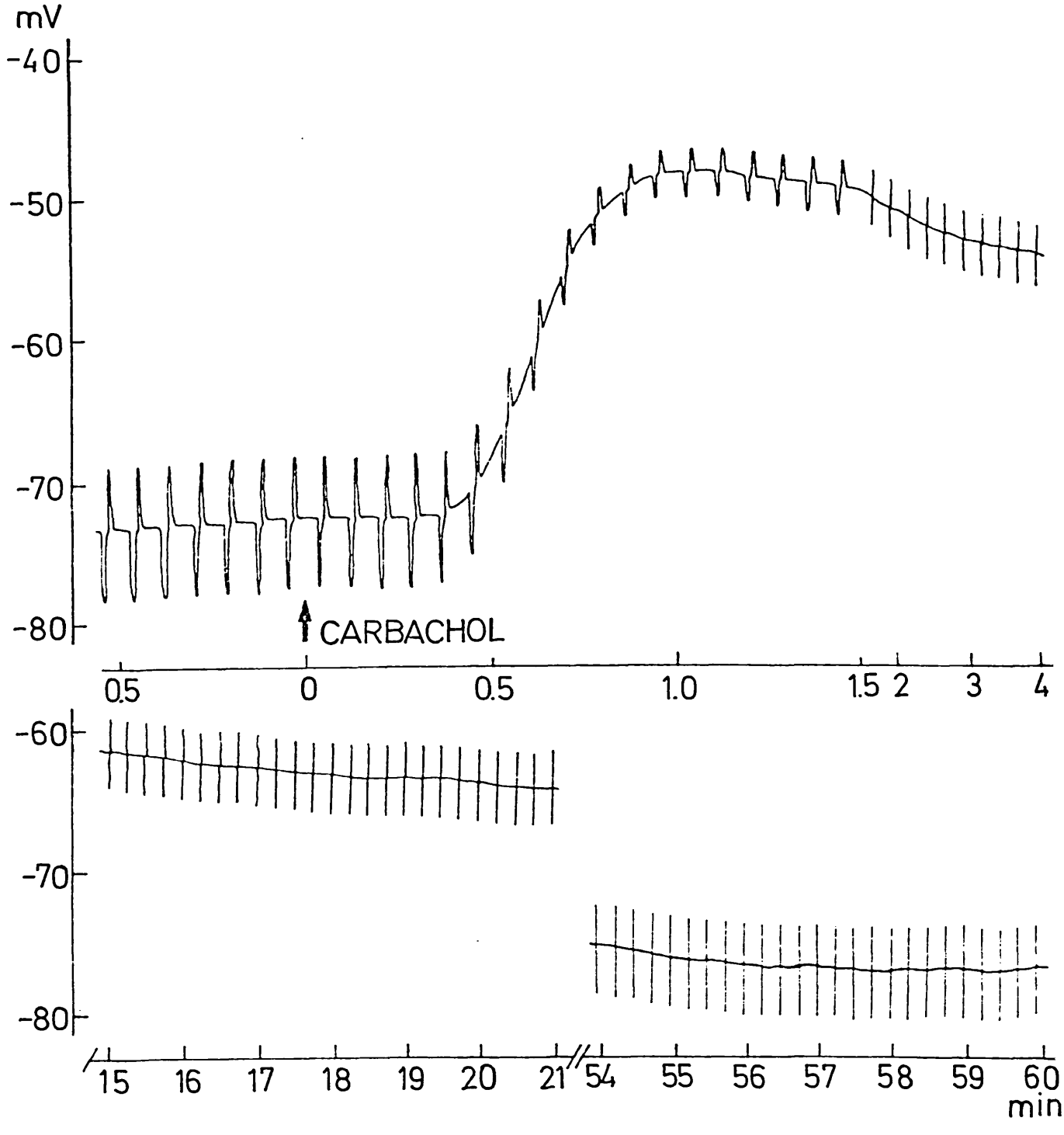
It was of interest to investigate the input resistance of guinea-pig diaphragm muscle fibres in the presence of agonist. A feature of this preparation was that membrane potential spontaneously recovered to initial resting values (Chapter 4), thus enabling direct comparison of input resistance values in the absence and presence of the drug at the same voltage. The measurement of input resistance is normally subject to a number of systematic errors (see Discussion), but it is possible to arrive at some definite conclusions.

The apparent input resistance of single muscle fibres was continuously measured by recording, with one internal microelectrode, the brief membrane depolarization or hyperpolarization repeatedly produced by measured current pulses delivered via a second closely-placed internal electrode (see Methods).

Due to the nature of the procedure there was a high proportion of unsuccessful experiments. Continuous two-electrode recordings from the end-plate at body temperature, with flowing solution, required anti-vibration techniques and also adequate shielding from electrical interference (described in Methods).

Three series of experiments were performed (Groups A, B and C). Preparations in Group A showed spontaneous recovery of potential in the continued presence of carbachol (80 μM).

Figure 6.1. Effect of carbachol (80 μM) on membrane potential and on electrotonic potentials. Drug was applied at the arrow. The speed of the recorder was decreased at 1.5 min, as was the frequency of the current pulses. Ordinate is potential in mV; abscissa is time in min after changeover. Excerpts show recovery of membrane potential in the presence of the drug, while the size of the electrotonic potentials remained less than the initial value.


mV
-40
-50
-60
-70
-80
CARBACHOL
0.5
0
0.5
1.0
1.5
2
3
4
-60
-70
-80
15
16
17
18
19
20
21
54
55
56
57
58
59
60
min

Preparations in Group B did likewise in decamethonium (10 μ M). Those in Group C were returned to physiological saline during the recovery process (the 'control' group).

Effects of Carbachol and of Decamethonium on Membrane Potential and Input Resistance

Group A

Figure 6.1 shows the effect of carbachol (80 μM) on membrane potential and on electrotonic potentials. 10 nA current pulses of 500 msec duration were injected into the muscle fibre at a rate set by a timing device and the resulting electrotonic potentials were detected by the recording microelectrode, superimposed on the membrane potential. Both the pulse rate and the rate of the recording paper-trace were increased for the duration of the period of rapid depolarization, so that a more detailed analysis of this event would be possible. The rate of the trace was reduced after about 1.5 min.

At the arrow, the preparation was bathed in a solution containing carbachol, which produced depolarization from -73 mV to -47 mV, at a maximum rate of 71.4 mV/min. There was an associated fall in the magnitude of the electrotonic potentials. Spontaneous recovery of potential was complete after 45 min, finally achieving a value of -76 mV. The size of the electrotonic potentials also showed some recovery but the final value in the presence of the drug remained low, being 69% of the initial value.

Figure 6.2. Effect of continuous exposure to carbachol (80 μM) on membrane potential (upper trace) and on apparent input resistance. Data for both traces comes from recording depicted in Fig.6.1. Ordinate for upper trace in mV, for lower in ohm x 10^5. The apparent input resistance, V/I, when membrane potential had fully recovered, remained low.

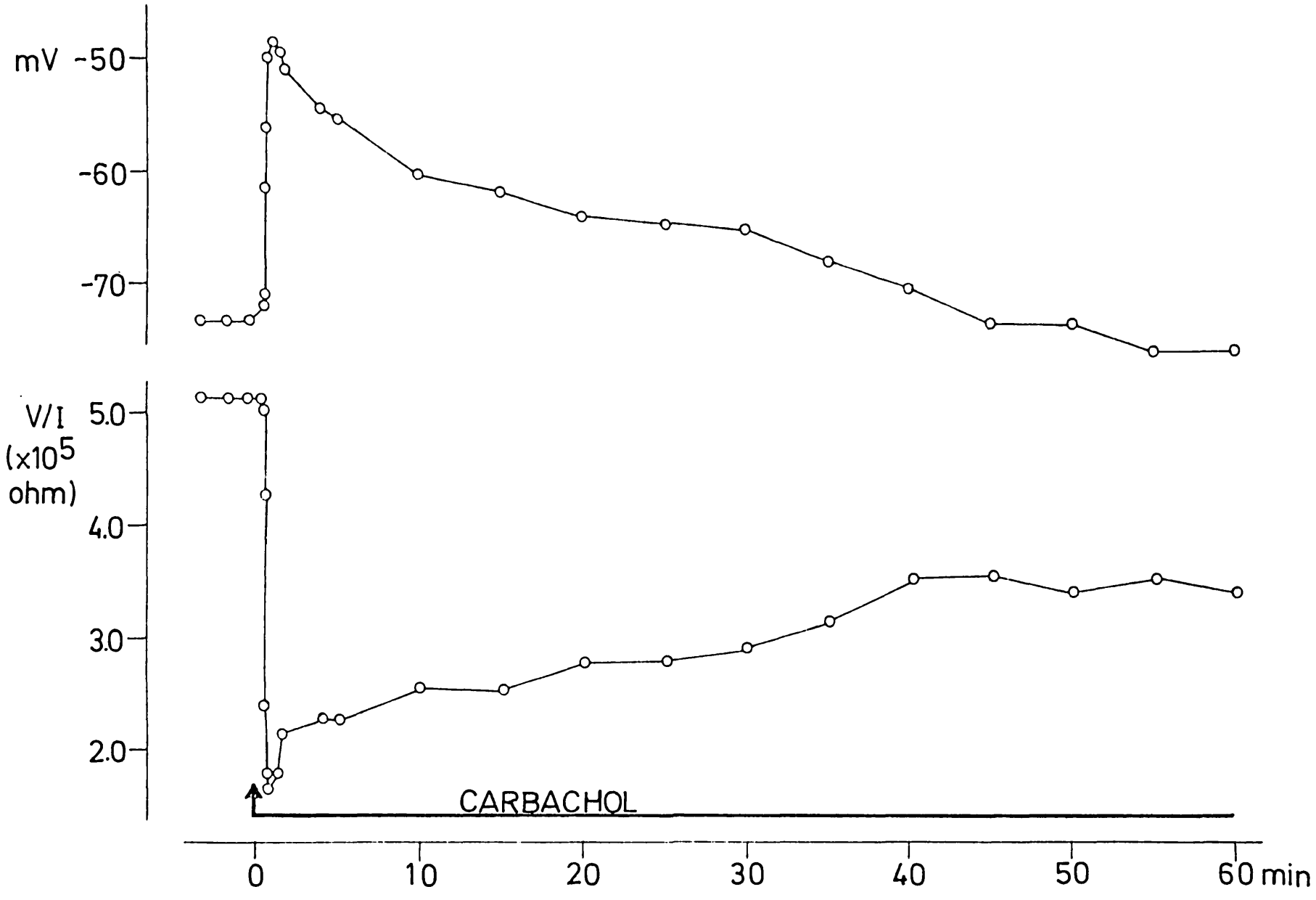

mV
-50
-60
-70
V/I
(x10^5
ohm)
5.0
4.0
3.0
2.0
CARBACHOL
0
10
20
30
40
50
60 min

Table 6.1 Effect of Carbachol and of Decamethonium on Resting Potential and Input Resistance

Orig. Pot. (mV)	Peak Depol. (mV)	Change (mV)	Max. Rate of Depol. (mV/min)	Orig. Res. (R= V/I) ($\times 10^5 \Omega$)	Min. Res. (R'= V'/I') ($\times 10^5 \Omega$)	Recovered* Res. (R'= V'/I') ($\times 10^5 \Omega$)	Recovered Res. as % of Orig. (%)
				A			
-75	-43	32	75.0	5.5	1.4	4.5	82
-71	-44	27	23.4	7.9	1.8	5.9	75
-75	-55	20	51.7	4.5	2.0	3.9	87
-76	-46	30	38.3	4.9	1.6	4.3	87
-73	-47	26	71.4	5.1	1.6	3.5	69
				B			
-72	-59	13	28.6	4.8	2.7	4.0	84
-73	-58	18	31.6	5.6	2.5	4.9	88
-73	-59	14	19.2	3.4	2.3	3.1	90
-75	-62	13	66.7	21.5	7.9	15.2	71
-82	-66	16	83.3	3.1	1.3	2.3	74
				Medians (10)			
				5.0		4.15	83
				C			
-73	-51	22	26.6	11.1	4.8	11.1	100
-74	-47	27	39.5	3.8	1.7	5.1	132
-76	-58	18	41.7	4.4	2.3	5.0	114
-76	-60	16	30.3	2.5	1.3	2.4	97
-77	-47	30	93.8	4.3	1.6	4.6	107
-77	-60	17	46.2	3.3	1.1	3.6	111
				Medians (6)			
				4.1		4.8	109

*Recovered input resistance was measured when the membrane potential had returned to its original value.
Preparations in group A showed recovery of potential in the continued presence of carbachol (80 μM). Muscles in group B showed recovery in the continued presence of decamethonium (10 μM). Muscles in group C were treated with decamethonium (10 μM), and were returned to solution without drug during the recovery period (controls).

In this Chapter the results pertaining to the electrotonic potentials will be described in terms of the apparent input resistance, R (= V/I, see Zaimis and Head, 1976, p373), as a basis for an interpretation in terms of membrane conductance in the Discussion. Fig. 6.2 is a graphical representation of the time course of the experiment depicted in Fig. 6.1, showing membrane potential in mV on the upper trace and V/I in ohms on the lower. Carbachol (80 μM) was added at the arrow. Apparent input resistance(V/I) fell from 5.1×10^5 ohm to 1.6×10^5 ohm. The decrease in resistance is related to the depolarization already described. The final value of input resistance was 3.5×10^5 ohm.

This diminished input resistance in the continued presence of carbachol measured at the point when membrane potential had just returned to its original value was observed in a total of five experiments. These constitute Group A in Table 6.1, where further details are given.

Group B

In this Group the measurements were repeated using decamethonium (10 μM) as the agonist. Fig. 6.3 depicts the trace from one such experiment and Fig. 6.4 is a graphical representation of the data.

Decamethonium produced depolarization from -82 mV to -66 mV, at a maximum rate of 83.3 mV/min. It was characteristic of decamethonium for the membrane potential to recover more rapidly than in carbachol (see also Fig. 4.1), and

Figure 6.3. Effect of decamethonium (10 μM) on membrane potential and on electrotonic potentials. Period of drug application represented by horizontal bar. The speed of the recorder was decreased 2½ min after the drug was applied and the pulse frequency was also decreased. Ordinate in mV. Time in min after changeover to drug bearing solution. The membrane potential recovered to -80 mV; the size of the electrotonic potentials were less than the initial value.

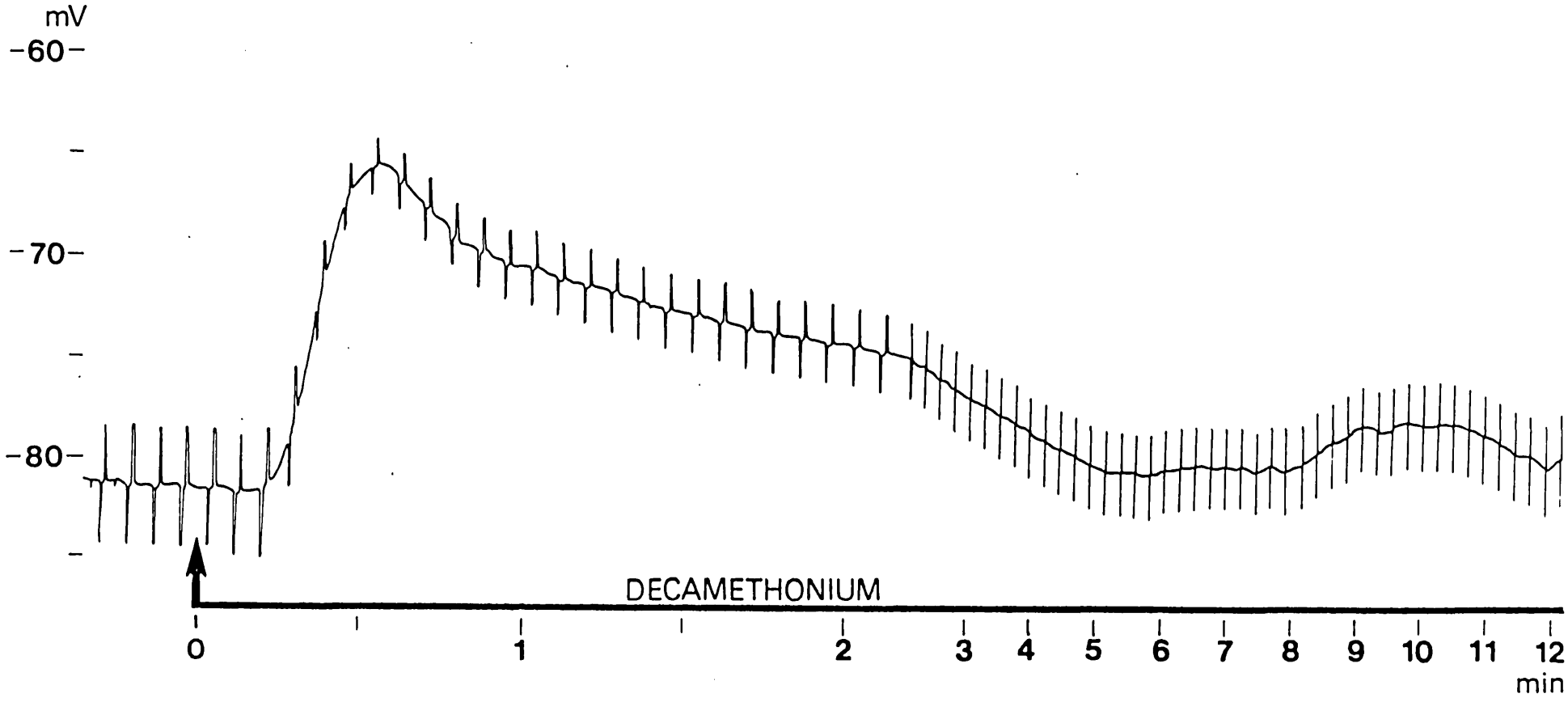
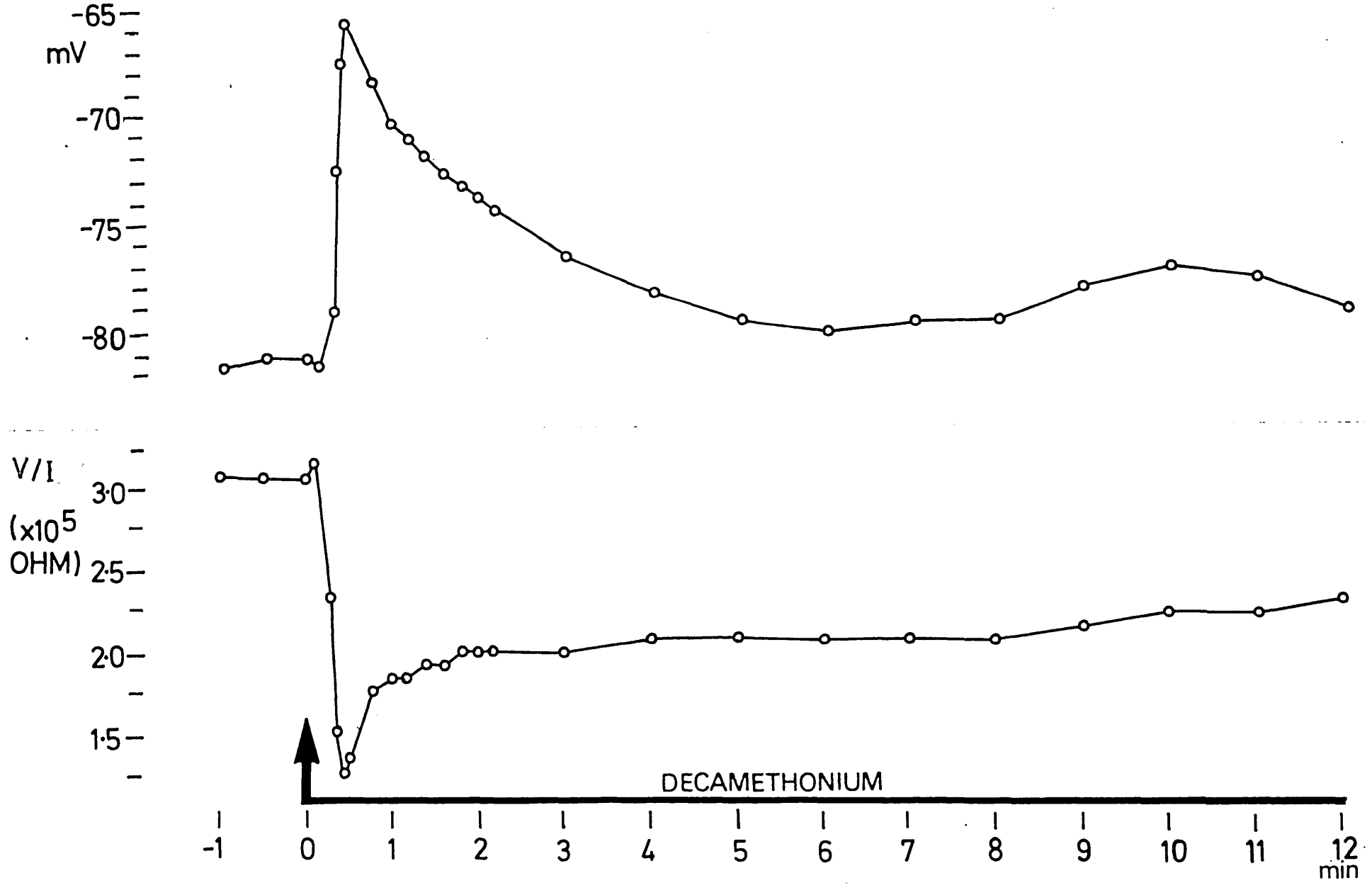

mV
-60-
-70-
-80-
DECAMETHONIUM
0
1
2
3
4
5
6
7
8
9
10
11
12
min

Figure 6.4. Effect of continuous exposure to decamethonium (10 μM) on membrane potential (upper trace) and on input resistance. Data for both traces come from recording depicted in Fig. 6.3. Period of drug application represented by horizontal bar. Ordinate for upper trace in mV, for lower in ohm x 10^5. Time in min after changeover to drug-bearing solution. The apparent input resistance, V/I, in the presence of decamethonium remained low despite the recovery of membrane potential.


-65
mV
-70
-75
-80
V/I
(x10^5
OHM)
3·0
2·5
2·0
1·5
DECAMETHONIUM
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
min

indeed spontaneous recovery was complete in this experiment within 6 min. A stable value of -80 mV was achieved, which was close to the original value of -82 mV. The size of the electrotonic potentials did not fully recover however, remaining at 74% of the initial value.

Input resistance (V/I), fell from a resting value of 3.1×10^5 ohm to a minimum of 1.3×10^5 ohm. The final value when membrane potential had recovered was 2.3×10^5 ohm. Five experiments in which decamethonium was used, including this one, comprise Group B in Table 6.1.

The results obtained using carbachol (Group A) and using decamethonium (Group B) have been pooled in Table 6.1 to obtain median values. From a total of 10 muscles, apparent input resistance (V/I) in the resting state was estimated as 5.0×10^5 ohm. In the presence of agonist, and at a time when membrane potential had fully recovered, apparent input resistance (V/I) was 4.15×10^5 ohm, 83% of the original value.

Group C

Experiments in Group C were intended to serve as 'controls' and to demonstrate that the diminished input resistance (V/I) seen in Group B was indeed attributable to the presence of depolarizing drug. To such an end, preparations in this series were exposed to decamethonium (10 μM) for only a limited period, being returned to normal physiological saline during the stage of spontaneous recovery of membrane potential.

Figure 6.5. 'Control' for experiment seen in Fig. 6.4. Decamethonium (10 μM) was applied for the period between the arrows. Ordinate for upper trace in mV, for lower in ohm x 10^5. Time in min from changeover to drug-bearing solution. Return of the tissue to normal solution during the recovery phase enhanced the rate of spontaneous repolarization. The apparent input resistance, V/I, also fully recovered, and this is in contrast to Fig. 6.4 where it remained low in the presence of the drug.

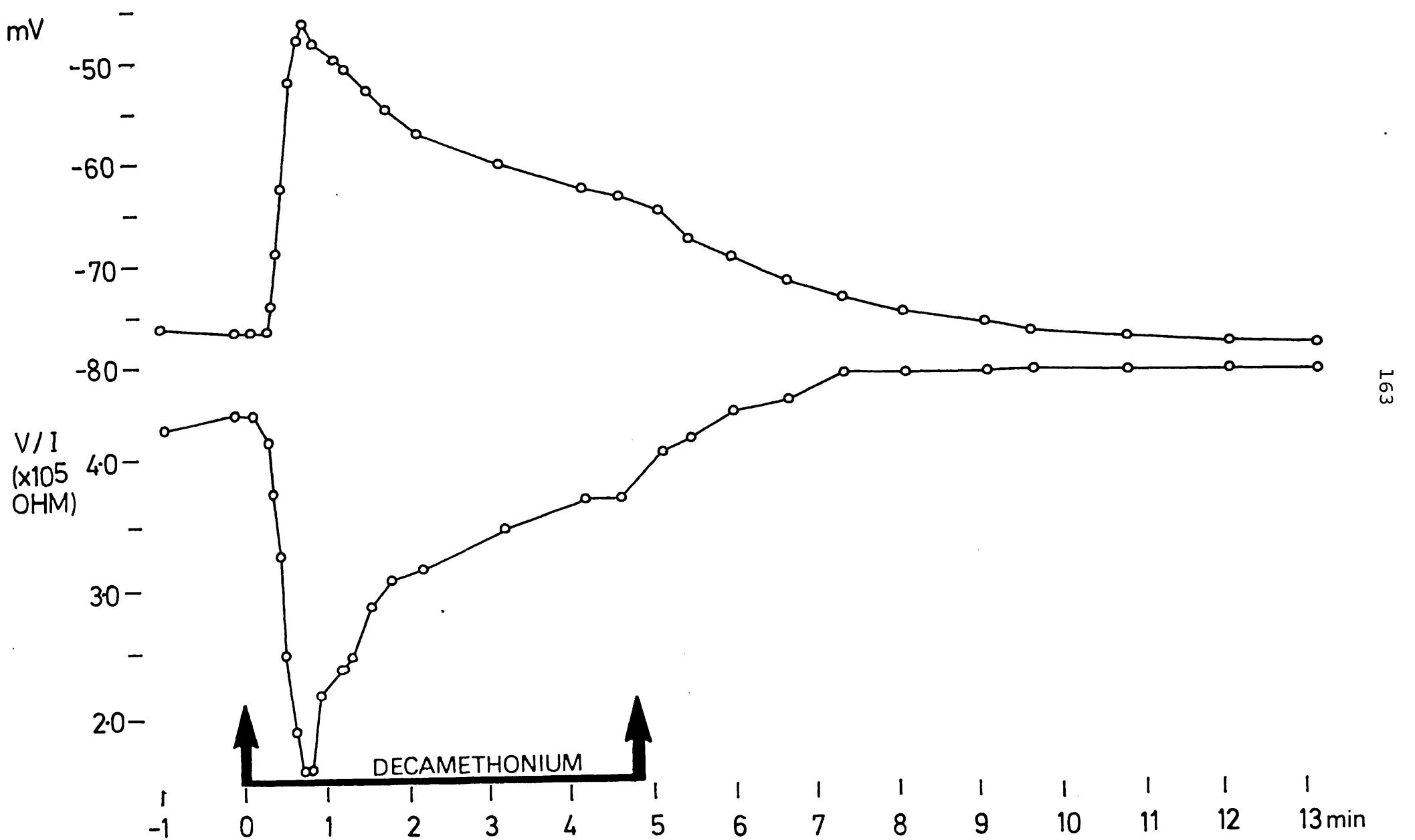

mV
-50
-60
-70
-80
V/I
(x10⁵
OHM)
4·0
3·0
2·0
DECAMETHONIUM
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13 min

Figure 6.5 is a graphical representation of the concurrent time courses of the effect of 10 μM decamethonium on membrane potential and on input resistance. The muscle was bathed in the drug for the period between the two arrows, and was returned to normal saline during the recovery phase. This led to a rise in the rate of spontaneous repolarization, the potential reaching a slightly hyperpolarized steady value. Analysis of the electrotonic potentials revealed a similar increase in the rate of recovery of apparent input resistance (V/I) upon reversion to saline. The resistance rose to reach a steady value in excess of the resting resistance.

Table 6.1 lists 6 'control' experiments, including the one described here, where muscles were exposed to decamethonium (10 μM) and then returned to drug-free solution during the recovery period (Group C). Median values are also given. The initial input resistance was 4.1×10^{-5} ohm, and the recovered resistance at the same membrane potential following return to normal saline was 4.8×10^{-5} ohm. The augmented recovery of input resistance in this group (109% recovery) contrasts with the diminished resistance in the continued presence of a depolarizing drug(Groups A and B; 83% recovery).

In Table 6.2, data regarding depolarization with carbachol (80 μM) are amassed for later consideration in the Discussion. An estimate of the change in input resistance as determined by the direct method of current injection may be compared with a value obtained by a calculation based on membrane potentials.

Table 6.2 Depolarization with Carbachol (80 μM)

Source	Rest. Pot.	R Initial	ΔV	R' at Peak Depol.	R'/R by Current	ΔV/(RP-17.5) or g/(g+G)	R'_m/R_m or 1-g/(g+G)
	mV	MΩ	mV	MΩ			
Table 4.2	-84		43			0.647	
	-73		26			0.468	
	-74		30			0.531	
	-74		24			0.425	
	-79		23			0.374	
	-84		31			0.466	
Table 4.5	-83		32			0.489	
	-86		26			0.380	
	-76		30			0.513	
	-78		28			0.463	
	-83		24			0.366	
Table 6.1	-75	0.55	32	0.14	0.25	0.557	0.44
	-71	0.79	27	0.18	0.23	0.514	0.49
	-75	0.45	20	0.20	0.44	0.348	0.65
	-76	0.49	30	0.16	0.33	0.513	0.49
	-73	0.51	26	0.16	0.31	0.468	0.53
Chapter 6	-73		30	0.39		0.541	
	-74		38	0.16		0.673	
	-77		33	0.20		0.555	
	-77		30	0.18		0.504	
	-73		27	0.45		0.486	
	-82		41			0.636	
	-74		31			0.549	
	-81		38			0.598	
	-77		28			0.471	
Median	-76	0.51	30	0.18	0.31	0.504	0.49
n	25	5	25	10	5	25	5
Limits	-74		27	0.16		0.471	
	-79		31	0.39		0.541	

The column for input resistance includes values from Chapter 6 in which the voltage was changed at peak depolarization. If g/(g+G) is f, then g/G is $(f^{-1}-1)^{-1}$.

To convert the last column to R'_{in}/R_{in}, use $(R'_m/R_m)^{\frac{1}{2}}$.

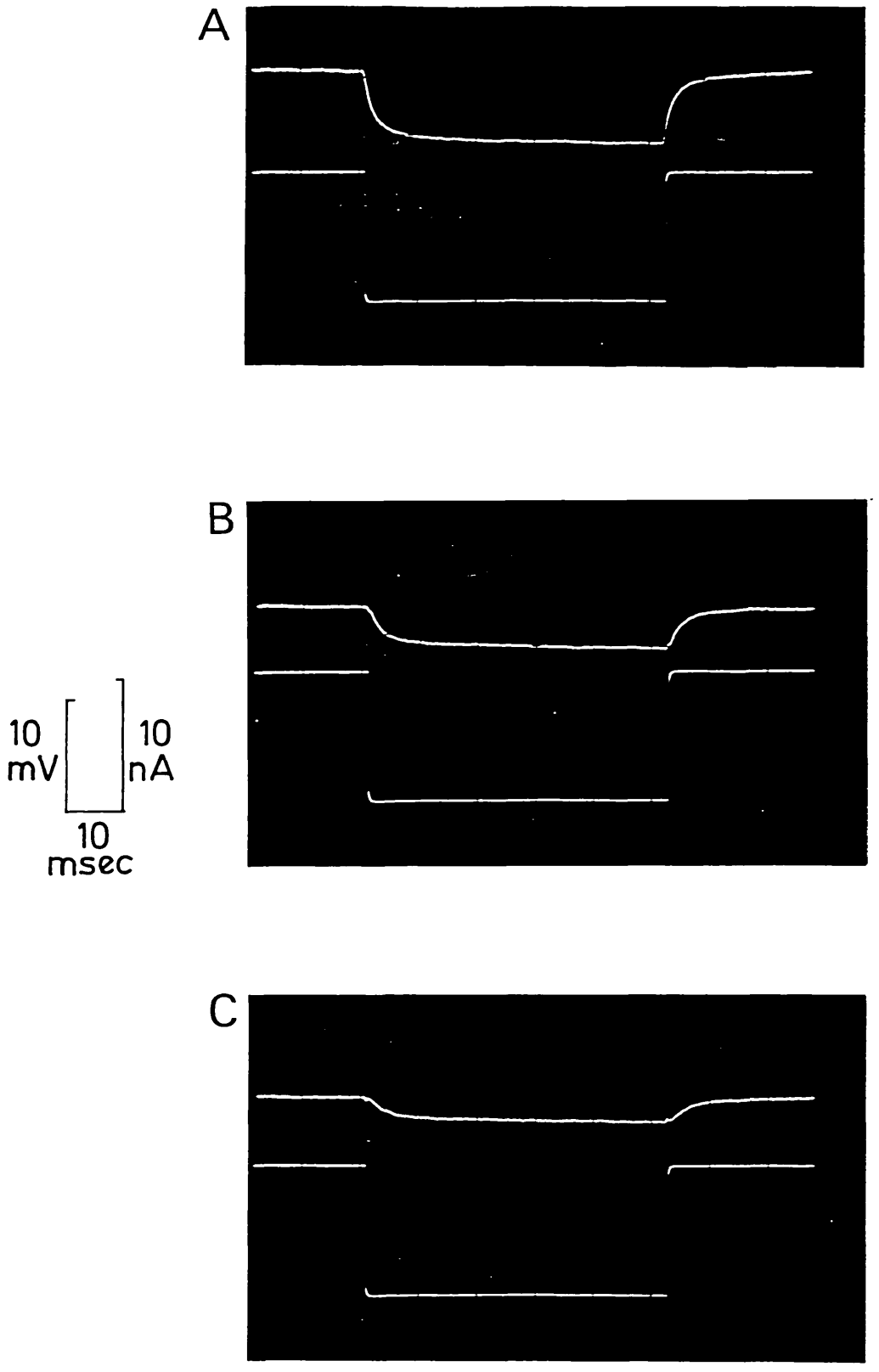
The resting potential and change in potential under the action of carbachol are listed for 25 muscle fibres. Most of the data comes from Tables already presented, (Tables 4.2, 4.5 and 6.1). The remainder comes from an incomplete series not described elsewhere in the text. In some of these, the input resistance at peak depolarization was determined by current injection, and these data are pooled with those from Table 6.1.

Membrane Space Constant

The magnitude of the electrotonic potential recorded by an intracellular electrode will vary with the distance of the electrode from the current source. Specifically, from cable theory (Hodgkin and Rushton, 1946) the potential change produced by a steady current across a membrane declines exponentially with distance. To aid interpretation of the results of the two-electrode experiments it was thus desirable to determine the membrane space constant, λ, in guinea-pig diaphragm muscle fibres at $37^{o}C$.

The space constant was measured by the technique of 'square pulse analysis' (see Methods). Fig. 6.6 shows current pulses, I, and electrotonic potentials, V, obtained from a fibre at three different electrode separations; A, 0.05 mm; B, 0.50 mm; C, 1.00 mm. The order of insertion of the current-passing electrode at different distances along the fibre was varied from fibre to fibre, and results were only accepted if the resting potential did not fall below -75 mV.

Figure 6.6. Electrotonic potentials produced by 10 nA current pulses at 3 different electrode separations in a fibre from guinea-pig diaphragm at 37^{o}C. Lower trace in each record is current pulse. Inter-electrode distances were, A, 0.05 mm; B, 0.50 mm; C, 1.00 mm.


A
B
C
10
mV
10
nA
10
msec

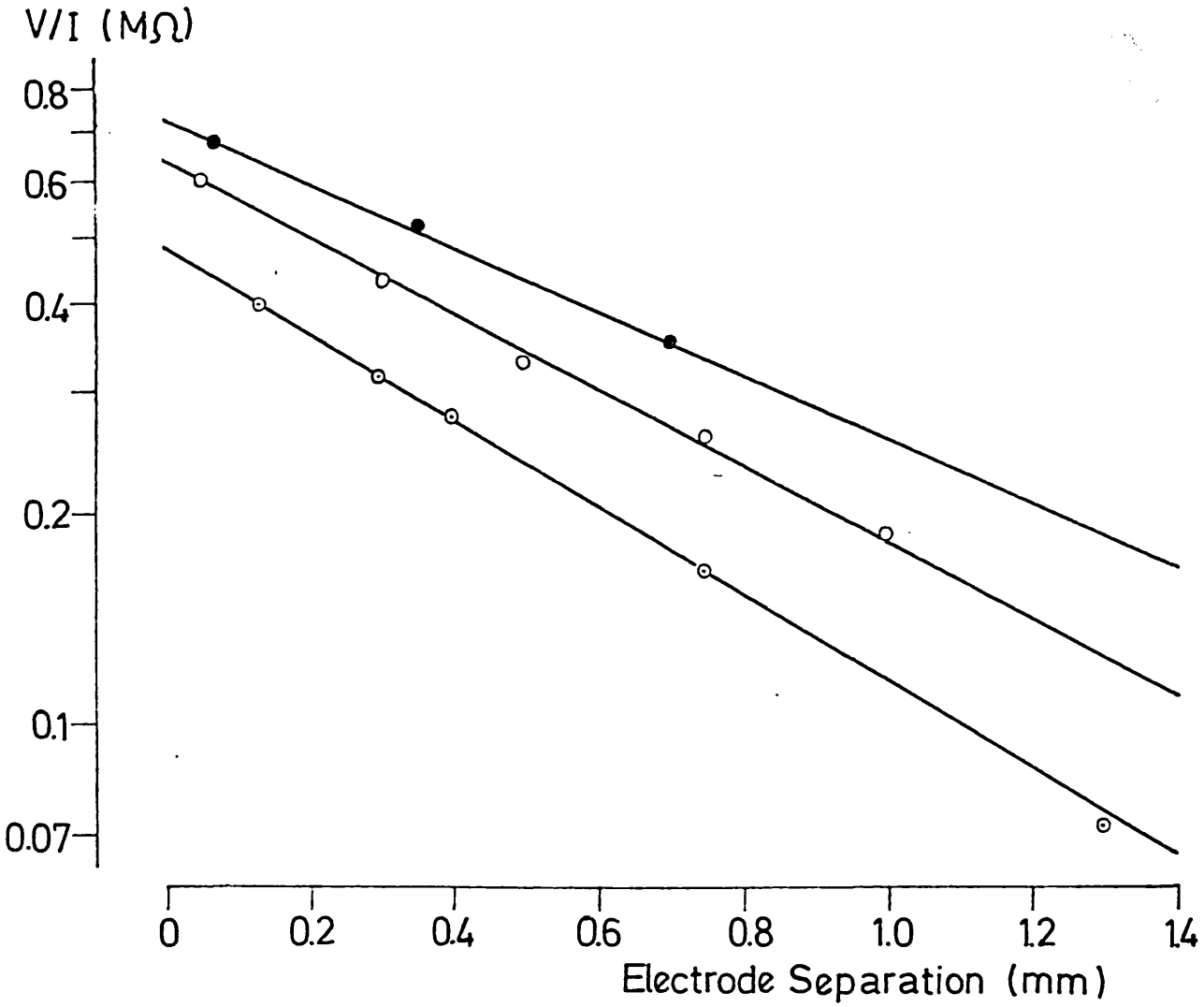


Figure 6.7. Semilogarithmic relation between V/I and micro-electrode separation for 3 different muscle fibres. Data from Fig. 6.6 provided 3 of the points for the line with open circles; the membrane space constant, λ , was 0.80 mm for this fibre. Extrapolation of the line to the vertical axis (x = 0) gives the input resistance, R_{in}; 6.4 x 10^5 ohm.

Table 6.3 Space Constants

	Input Resistance (R_{in}) ohm x 10^5	Space Constant (λ) mm
	5.2	1.14
	4.7	0.80
	4.5	0.77
	8.7	1.07
	7.2	0.98
	6.4	0.80
Median (6)	5.8	0.89

The six values of input resistance in normal muscle (R_{in}, above) were obtained by extrapolation (see Fig.6.7). Sixteen values of V/I have been taken from Table 6.1. R_{in} was obtained as (V/I)/0.9 where 0.9 is a correction factor for inter-electrode distance, and is taken as exp (-0.1) - see Table 9.3 and Discussion.

Median value	5.3 x 10^5 ohm
n	22
Limits	4.2, 6.4 x 10^5 ohm.

In Fig. 6.6 the electrotonic potentials rise in less than 10 msec to a steady value determined for a given current by the distance between the electrodes. The ratio V/I is plotted on a logarithmic scale against the separation of the micro-electrodes in Fig. 6.7. Results from this fibre are plotted as open circles, with two extra measurements at 0.30 mm and 0.75 mm. The experimental points fall on a straight line, as predicted by cable theory. Results from two other muscle fibres are also shown in Fig. 6.7.

The space constant, λ, may be obtained from the slope of the line (being the distance from any point on the fibre over which the electrotonic potential falls to 1/e of its value at that point) and is 0.80 mm for the fibre described above. Extrapolation of the line to the vertical axis (x = 0) gives the input resistance, R_{in}; 6.4×10^5 ohm. Table 6.3 lists these measurements from 6 muscle fibres, giving a median value for λ of 0.89 mm and for R_{in} of 5.8×10^5 ohm.

At the bottom of Table 6.3 the median value of R_{in} from 22 fibres is given as 5.3×10^5 ohm. This estimate includes the 6 fibres described here, and also 16 values of V/I taken from Table 6.1 which have been 'corrected' for an inter-electrode distance, x, of 80 μm (see Methods). The average value of the ratio x/λ is less than 0.1, and the factor for inter-electrode distance is $\exp(-x/\lambda)$ or 0.90 (see Discussion). In practice, the input resistance was taken as (V/I)/0.90 for the values described in Table 6.3.

SUMMARY

1. With carbachol (80 μM) and with decamethonium (10 μM) the initial depolarization was followed by full recovery. The apparent input resistance, R, measured as the ratio V/I, showed an initial fall with the depolarization and only partial recovery such that the value when membrane potential had recovered was less than the initial value.

2. Pooling results for both agonists gave an original 'resting' resistance of 5.0×10^5 ohm and a 'recovered' value of 4.15×10^5 ohm in the presence of the drug ($n = 10$).

3. Removal of decamethonium following peak depolarization (6 experiments) produced repolarization with recovered resistance (4.8×10^5 ohm) which was often above the initial value (4.1×10^5 ohm).

4. The space constant was measured in 6 fibres by 'square pulse analysis' and had a median value of 0.89 mm.

5. Input resistance, R_{in}, was measured in 6 fibres by extrapolation. In other fibres, R_{in} was estimated by a correction applied to measurements of V/I, with the term x/λ taken as 0.1. Results from 22 fibres gave R_{in} as 5.3×10^5 ohm.

C H A P T E R 7

RADIOACTIVE DECAMETHONIUM,

AND

FIBRE DIAMETERS

RADIOACTIVE DECAMETHONIUM AND FIBRE DIAMETERS

There is an indication from the results of the previous chapter that during the prolonged action of decamethonium some of the ionic channels at the end-plate continue to open. If this is so,then it should be possible to demonstrate by use of the appropriate marker that the end-plate region retains its special permeability, following prolonged application of decamethonium. Two labelled substances have been used in mammalian muscle to demonstrate increased uptake in the end-plate region: labelled sodium (Creese et al, 1977) and labelled decamethonium itself (Creese and England, 1970). It was decided to follow the uptake of tritium-labelled decamethonium in guinea-pig diaphragm in a concentration which is known to produce marked pharmacological effects.

Fibre diameters were also measured, and this enabled the uptake per end-plate to be calculated so that comparison could be made with rat muscle.

Radioactive Decamethonium in Guinea-pig Diaphragm

Fig. 7.1 shows the uptake of tritiated decamethonium (10 μM). The diaphragm was exposed for 60 min, and then washed for 10 min by passing through a succession of tubes of inactive saline. The muscle was frozen, sliced at intervals of 1 mm from tendon to rib, weighed, dissolved and counted.

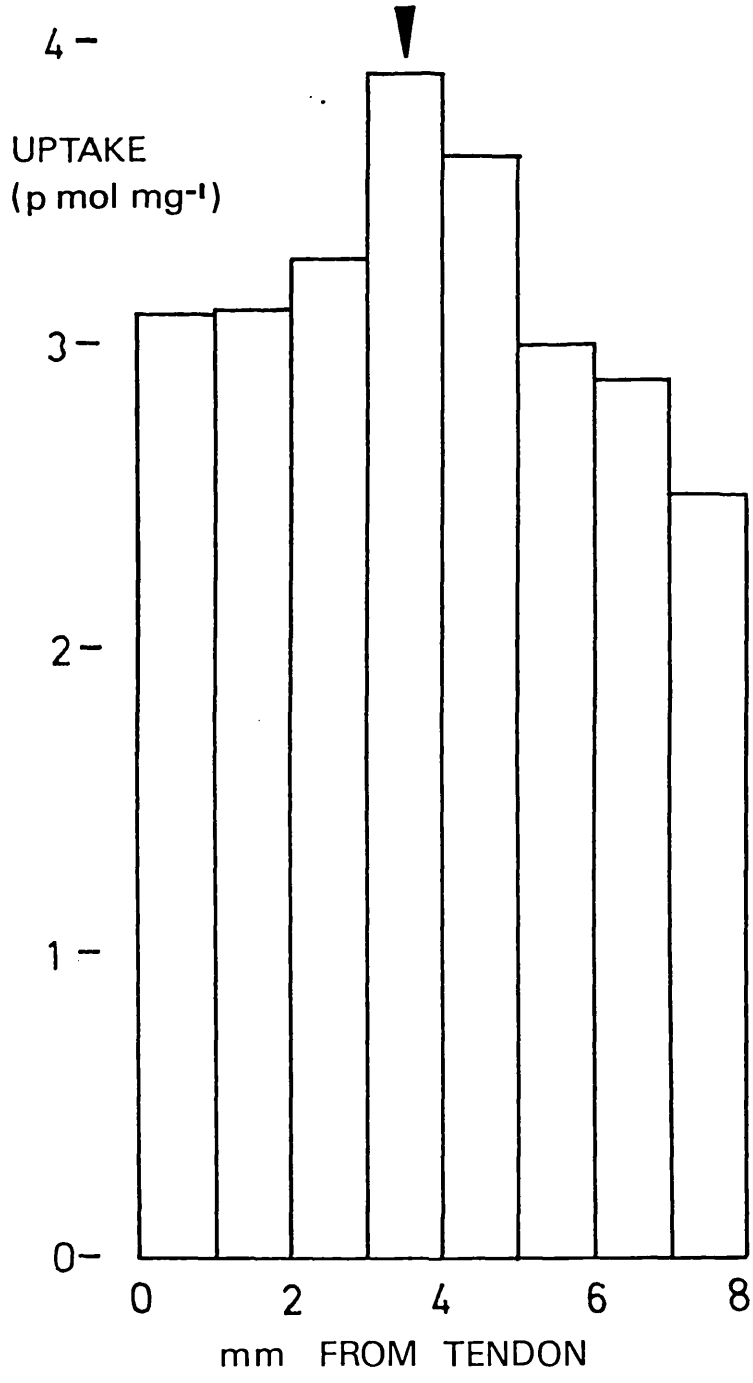


Figure 7.1. Uptake of labelled decamethonium (10 μM) following exposure for 60 min. Ordinate is in p-mol. mg^{-1}, abscissa is distance from the tendon in mm. There is increased radioactivity in the slice which contained the band of end-plates (arrowed).

Fig. 7.1 shows the distribution of radioactivity in consecutive slices from tendon to rib (left to right) in p-mole mg^{-1}.

There is a small peak of uptake in the slice which contains the band of end-plates (arrowed), as shown in the frozen muscle. The peak is much smaller than that found in rat muscle (Creese and Maclagen, 1970), but an increased uptake in the junctional region is detectable.

The histogram in Fig. 7.2 represents the uptake of tritiated decamethonium (10 μM) for various times, which are indicated in min below each histogram. The radioactive solution was changed every 30 min. Radioactive uptake is expressed in p-mol mg^{-1}, obtained from clearance (μl mg^{-1}) multiplied by concentration (μM). In Fig. 7.2 several muscles at each exposure time have been summed to give the histograms, with their peaks aligned: the rectangles give the medians from the muscles. Table 7.1 shows the procedure in the case of the muscles exposed for 60 min. The total uptake in Fig. 7.2 showed a steady increase with time, for a period of 2 hr.

The dashed lines transecting the histograms in Fig. 7.2 indicate the values assessed as non-junctional uptake at each exposure. Fig.7.3 depicts the summed uptake at the end-plate (in excess of the non-junctional uptake for each time interval). Abscissa gives exposure time in min. The line gives the regression through the origin and shows a steady uptake with time, indicating continuous entry of radiolabelled decamethonium.

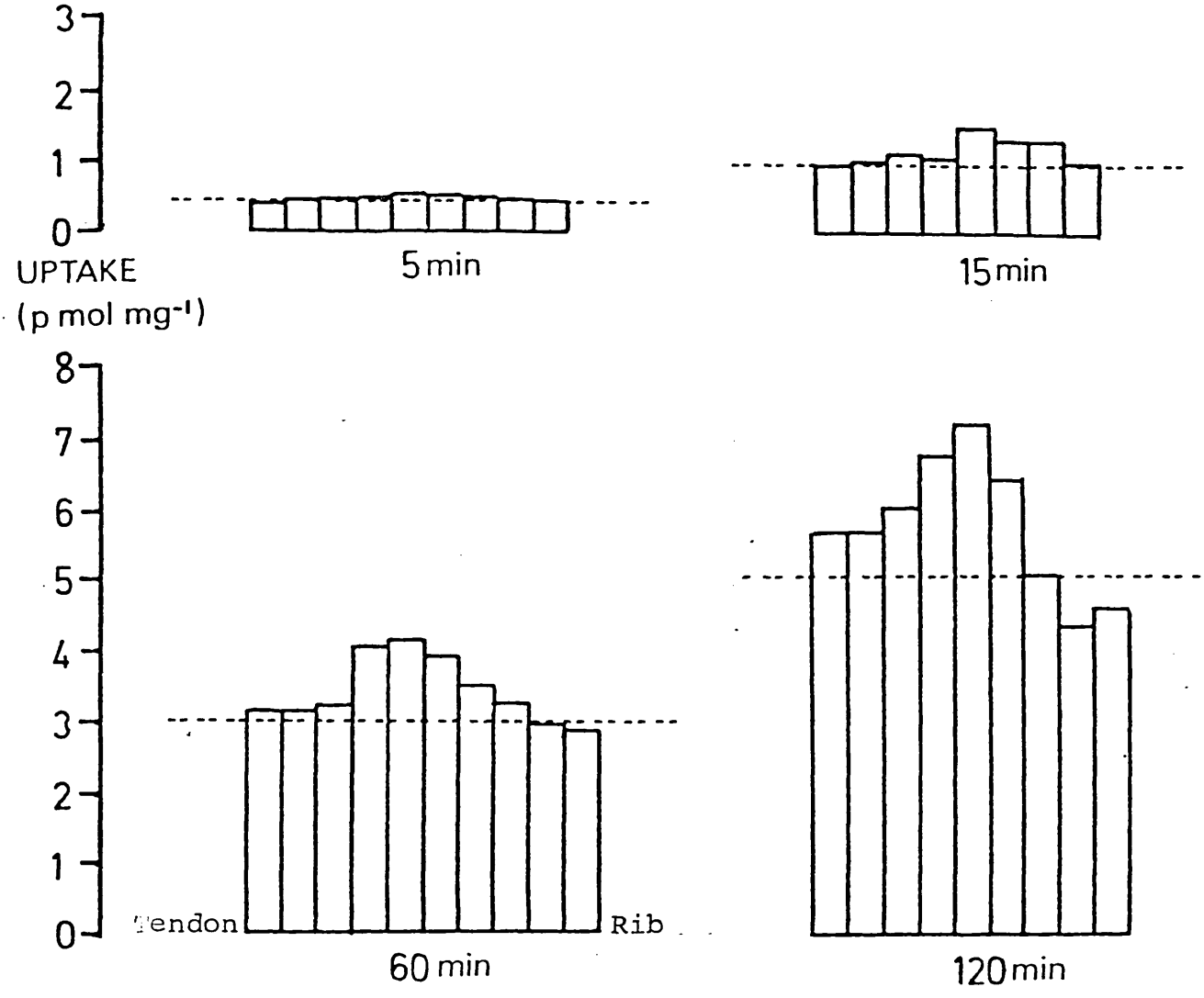


Figure 7.2. Effect of varied exposure on the uptake of labelled decamethonium (10 μM). Ordinate is in p-mol mg^{-1}, and the exposure time is indicated in min below each histogram. At least 12 muscles at each exposure time have been summed with their peaks aligned, each rectangle giving the median uptake for consecutive slices, so that the abscissa is in mm. The dashed lines transecting the histograms indicate the values assessed as non-junctional uptake at each exposure. The spread of radioactivity in the muscles is considered in the Discussion.

Table 7.1 Uptake of 10 μM ^{3}H Decamethonium. 60 min Exposure.

Aligned Muscles

Uptake Along Muscle (mm Intervals) in p mole mg^{-1}											
-5	-4	-3	-2	-1	M	1	2	3	4	5	6
	4.55	5.58	4.84	5.34	6.12	5.81	4.43	2.43	2.06	1.79	
2.13	2.61	2.90	2.56	2.22	3.34	3.66	3.73	3.52	3.13		
3.38	2.90	2.43	3.05	4.75	5.11	4.61	4.67	3.67			
		4.39	3.70	3.91	4.93	4.32	2.44	2.59	2.96	1.97	
		4.37	4.48	4.20	3.67	3.71	3.59				
	2.03	2.67	2.58	3.18	3.69	3.21	4.41	4.08	3.64		
2.82	2.71	2.71	2.91	3.22	3.85	3.51	1.86				
	3.69	4.03	3.47	4.03	4.59	4.46	2.30	3.42			
			4.71	4.67	4.72	4.27	4.69	5.87	4.47	4.70	3.82
		3.10	3.11	3.27	3.88	3.61	2.99	2.88	2.50	1.83	
	3.37	3.73	3.23	3.05	3.93	3.75	2.82	1.95	1.60		
3.45	3.30	2.65	2.97	4.06	4.21	3.99	3.04	1.85			
a	3.10	3.10	3.17	3.97	4.07	3.87	3.32	3.15	2.96	2.82	
b	8	11	12	12	12	12	12	10	7	9	
c	0.310	0.310	0.317	0.397	0.407	0.387	0.332	0.315	0.296	0.282	
d					0.304						
e			0.013	0.093	0.103	0.083	0.028				
f					0.320						

Key: a, median p mole mg^{-1}; b, number of muscles; c, μl mg^{-1}; d, non-junctional uptake (from 5 end columns); e, junctional uptake; f, summed junctional uptake, in $\mu l.mg^{-1}hr^{-1}$.

Muscles were aligned. In one diaphragm the high values at the ends of the muscle have been omitted. The value at m +5 was obtained from 9 values; 4 at +5, 4 at -5 and 1 at +6.

These results are shown in Fig. 7.2 as the histogram at 60 min.

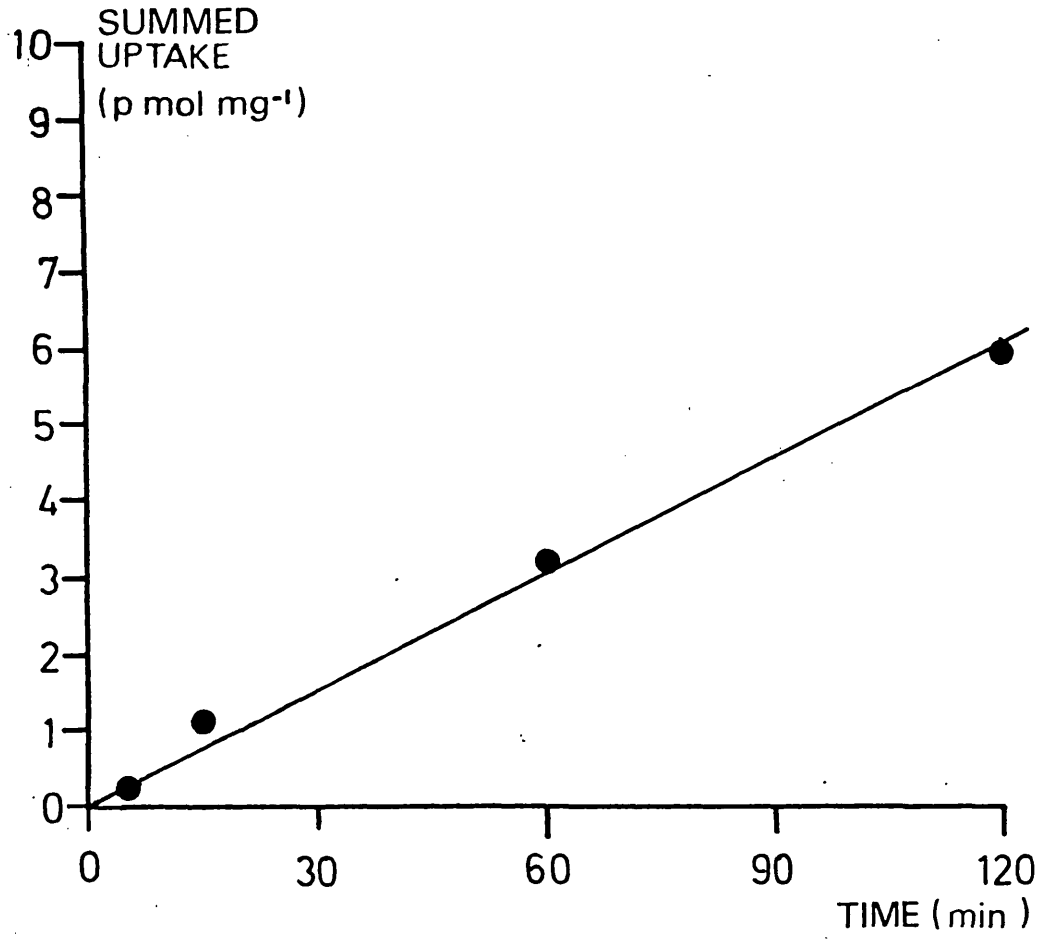


Figure 7.3. Effect of varied exposure to labelled decamethonium (10 μM) on summed uptake at the end-plate region (in excess of non-junctional uptake). Data for this graph are derived from observations depicted in Fig. 7.2. Ordinate is in p-mol mg^{-1}. Abscissa gives exposure time in min. There is continuous uptake of labelled decamethonium in the end-plate region for at least 2 hr.

Effect of Concentration on Uptake of Decamethonium

Fig. 7.4 shows histograms demonstrating the effect of concentration of labelled decamethonium on uptake at 1, 3, 10 and 100 μM. All muscles were immersed for 1 hr in the relevant concentration of drug and then washed for 10 min in a succession of tubes of saline. The uptake is given as a clearance ($\mu l\ mg^{-1}$) and the values are listed in Table 7.2.

In Fig. 7.4 the uptake of labelled decamethonium, expressed as a clearance, shows little change within the range 1 μM to 100 μM. There may be a small increase with concentration but the effect is slight. It is concluded that the results at 10 μM (Fig. 7.3) were at a concentration at which the clearance was near the maximum.

The results in Fig. 7.2 show continuous uptake of labelled decamethonium and are consistant with the concept that some channels continue to open with prolonged exposure to the drug.

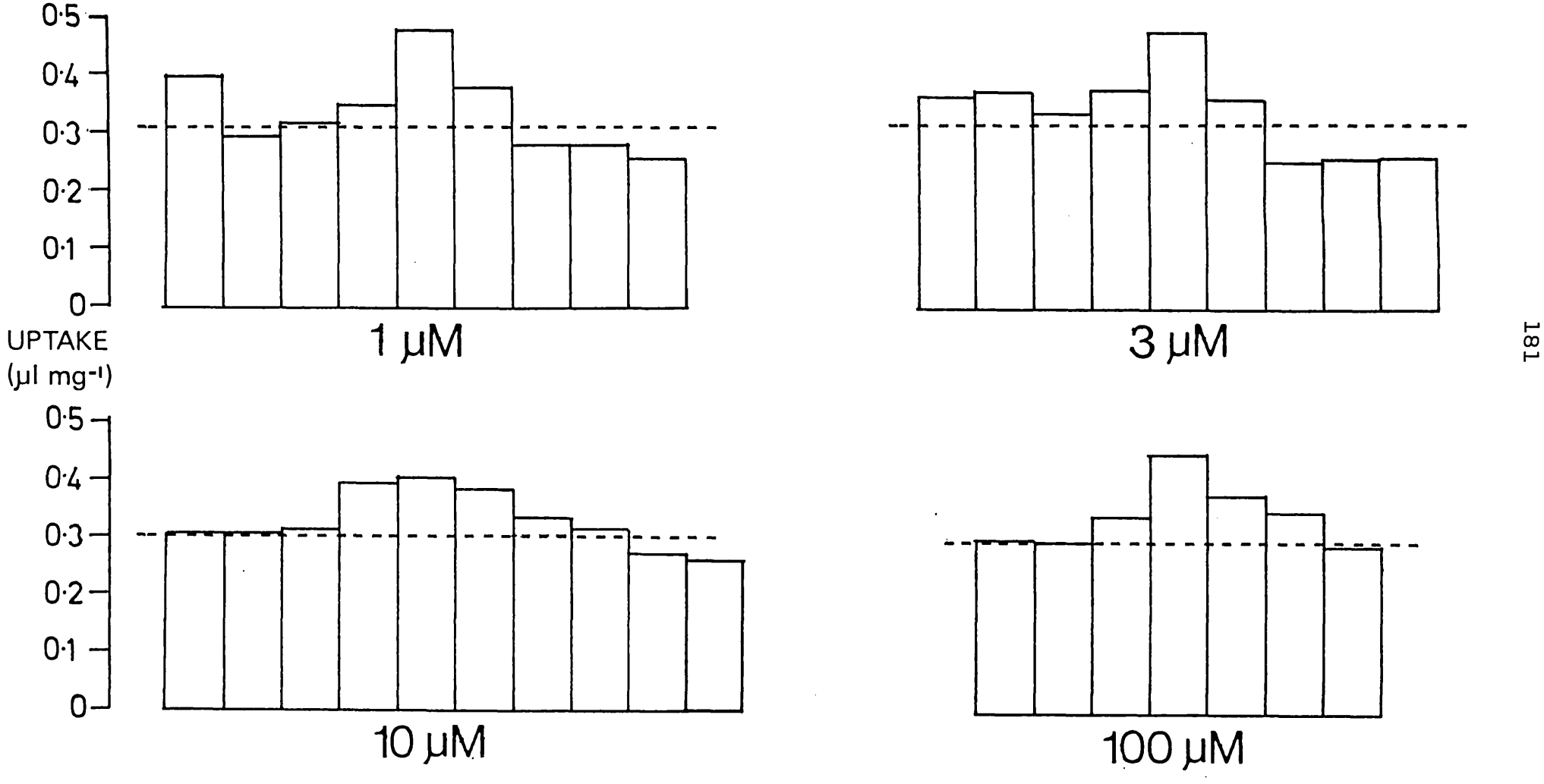
Table 7.2 Effect of Concentration on Uptake of ^{3}H Decamethonium.
60 Min. Exposure

^{3}H DECA. CONC	UPTAKE ALONG MUSCLE (mm INTERVALS) in $nl.mg^{-1}$										UPTAKE	
	-4	-3	-2	-1	M	1	2	3	4	5	NON JUNCTIONAL $nl.mg^{-1}$	SUMMED JUNCTIONAL
1 µM	400	296	309	348	474	378	281	280	256		308	
				40	166	70					(4)	276
3 µM	367	373	338	376	471	362	254	257	262		315	
			23	61	156	47					(4)	287
10 µM	310	310	317	397	407	387	332	315	296	282	304	
			13	93	103	83	28				(5)	320
100 µM		300	295	339	444	328	246	288			294	
			1	45	150	84	52				(2)	332

Muscles were aligned as in Table 7.1. The peak and two adjacent rectangles were used to obtain the junctional uptake; the end rectangles were used for the non-junctional uptake. (Uptakes listed above are in $nl.mg^{-1}$ for the sake of tabulation).

Concentration (µM)	Summed Junctional Uptake ($\mu l.mg^{-1}\ hr^{-1}$)	
1	0.276	estimated from 8 muscles
3	0.286	estimated from 8 muscles
10	0.320	estimated from 12 muscles
100	0.332	estimated from 8 muscles

Figure 7.4. Effect of varied concentration on uptake of labelled decamethonium for 60 min. Ordinate gives uptake expressed as a clearance ($\mu l.mg^{-1}$). Drug concentration is given in μM below each histogram. At least 8 muscles at each concentration have been summed with their peaks aligned, each rectangle giving the median uptake for consecutive mm slices. The dashed lines transecting the histograms indicate the values assessed as non-junctional uptake at each exposure. Over this range there is little change in the uptake when expressed in this way.


0·5
0·4
0·3
0·2
0·1
0
UPTAKE
(µl mg⁻¹)
1 µM
3 µM
10 µM
100 µM

Fibre Diameters

Fibre measurement was made by the use of two graticules, one in an eyepiece of the microscope and the other mounted on the front of the vertical bath. All observations were made at a magnification such that one eyepiece unit corresponded to 6.06 μm, (see Methods).

Table 7.3 lists the results of measurements made on 250 muscle fibres from 5 animals. The number of fibres within each range are shown as a percentage of the total number. The probit of the cumulant percentage and the log of the diameter are also given.

Fig. 7.5 shows that the distribution of muscle fibre diameters are asymmetrical, with a positive skew. Plotting the probit of the cumulant percentage against the log of fibre diameter gave a straight line (Fig. 7.6) indicating a log-normal distribution between 8 and 92% of the cumulant distribution.

The probit of 5 on the ordinate scale intercepts the line in Fig. 7.6 to give a median fibre diameter of 79.8 μm.

Table 7.3 Fibre Diameters in Guinea-pig Diaphragm

Units	µm Range	No	Cumulant	%	Cumulant %	Probit	µm	Log µm
4.2 - 7.5	27.3 - 45.5	20	20	8.0	8.0	3.60	45.5	1.66
7.5 - 10.5	45.5 - 63.6	44	64	17.6	25.6	4.34	63.6	1.80
10.5 - 13.5	63.6 - 81.8	64	128	25.6	51.2	5.03	81.8	1.91
13.5 - 16.5	81.8 - 100.0	53	181	21.2	72.4	5.60	100.0	2.00
16.5 - 19.5	100.0 - 118.2	27	208	10.8	83.2	5.96	118.2	2.07
19.2 - 22.5	118.2 - 136.4	23	231	9.2	92.4	6.43	136.4	2.13
22.5 - 31.5	136.2 - 190.9	19	250	7.6	100.0			

5 guinea-pigs used (median weight 195 g: range 190 - 210 g)

50 fibres measured in each preparation

The range 136.2 to 190.9 µm represents results pooled from 3 smaller ranges (see Fig. 7.5).

Figure 7.5. Distribution of muscle fibre diameters. Ordinate gives number of fibres; abscissa gives fibre diameters (μm) and also the eye-piece units. The distribution of diameters shows a positive skew.

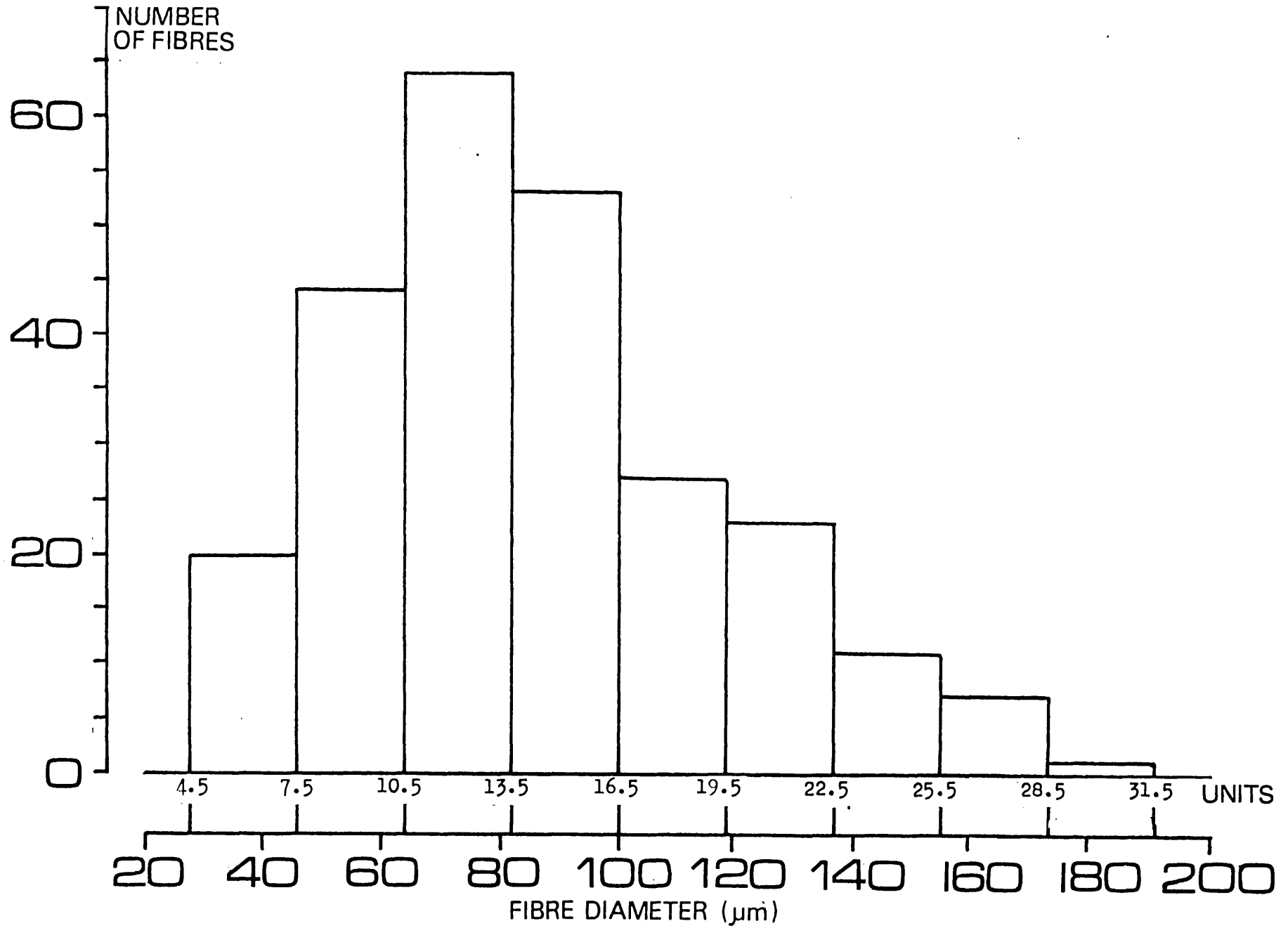
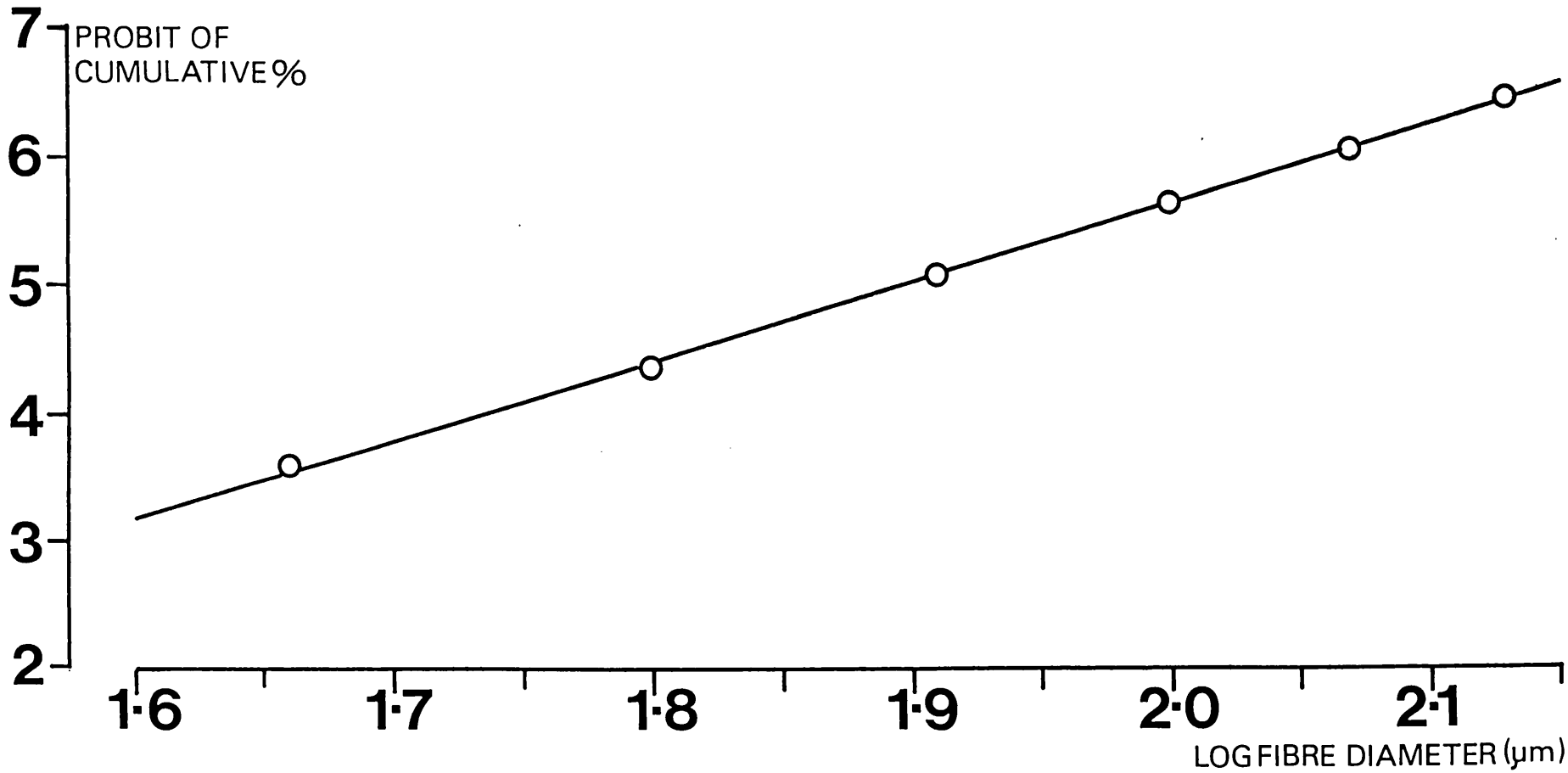

NUMBER
OF FIBRES
60
40
20
0
4.5
7.5
10.5
13.5
16.5
19.5
22.5
25.5
28.5
31.5
UNITS
20
40
60
80
100
120
140
160
180
200
FIBRE DIAMETER (µm)

Figure 7.6 Linearized plot of distribution of muscle fibre diameters. Ordinate gives probit of cumulative %, logarithmic abscissa shows fibre diameters in μm (Table 7.3). The straight line is the regression and indicates a log-normal distribution between 8% and 92% of the cumulative distribution.


7
6
5
4
3
2
PROBIT OF
CUMULATIVE %
1·6
1·7
1·8
1·9
2·0
2·1
LOG FIBRE DIAMETER (µm)

Fibres per mg Muscle, and Uptake per End-plate

The extracellular space of guinea-pig diaphragm is 0.30 by volume (Creese, Taylor and Tilton, 1963). The specific gravity of rat diaphragm is 1.07 (Klaus, Lullmann and Muscholl, 1960), and if this is applicable to guinea-pig muscle the volume of myoplasm is 0.70/1.07 or 0.654 ml g^{-1} or $\mu l.mg^{-1}$. If the fibres are treated as cylinders of 80 μm diameter, each fibre in a strip of 1 mm has a volume of $3.142 \times (40)^2 \times 1000$ or 5.027×10^6 cu μm. Each μl. is equivalent to 10^9 cu μm, so the number of fibres in each mg muscle is $(0.654 \times 10^9)/(5.027 \times 10^6)$ or 130 fibres mg^{-1}. A similar calculation in rats of 100 g gave 377 fibres mg^{-1} (Creese, Franklin and Mitchell, 1977).

The uptake of junctional decamethonium at 100 μM is 0.332 $\mu l.\ mg^{-1}hr^{-1}$ when expressed as a clearance (Table 7.2), and this is likely to be close to the maximum value. This is low in comparison with rat diaphragm, where the peak in histograms is much more prominent (e.g. England, 1970). The junctional uptake in rat diaphragm at 100 μM decamethonium is approximately 1.5 $\mu l.mg^{-1}hr^{-1}$ (Creese, Humphrey and Mitchell, unpublished observations), which is nearly 5 times that of guinea-pig diaphragm, when expressed per mg muscle. This is partly explained by the larger fibre diameter of guinea-pig muscle where the number of fibres per mg is approximately 1/3 of that in rat diaphragm.

Summary

1. In the guinea-pig diaphragm *in vitro* the maximum uptake of tritiated decamethonium was found to be at the end-plate region.

2. The uptake of decamethonium (10 μM) showed a steady increase with time and was measured for 2 hr.

3. The rate of uptake of labelled decamethonium expressed as a clearance (μl $mg^{-1}hr^{-1}$) showed little change with concentration between 1 - 100 μM.

4. Measurement of muscle fibre diameters revealed a log normal distribution with a median cell diameter of 80 μm (50 measurements from each of 5 guinea-pigs, wt 190 - 210 g).

5. Guinea-pig diaphragm showed a low rate of uptake of labelled decamethonium as compared with rat diaphragm (measured as $\mu l.mg^{-1}hr^{-1}$), and this is partly attributed to the difference in the number of end-plates per mg muscle.

CHAPTER 8

NERVE STIMULATION AND REVERSAL POTENTIAL

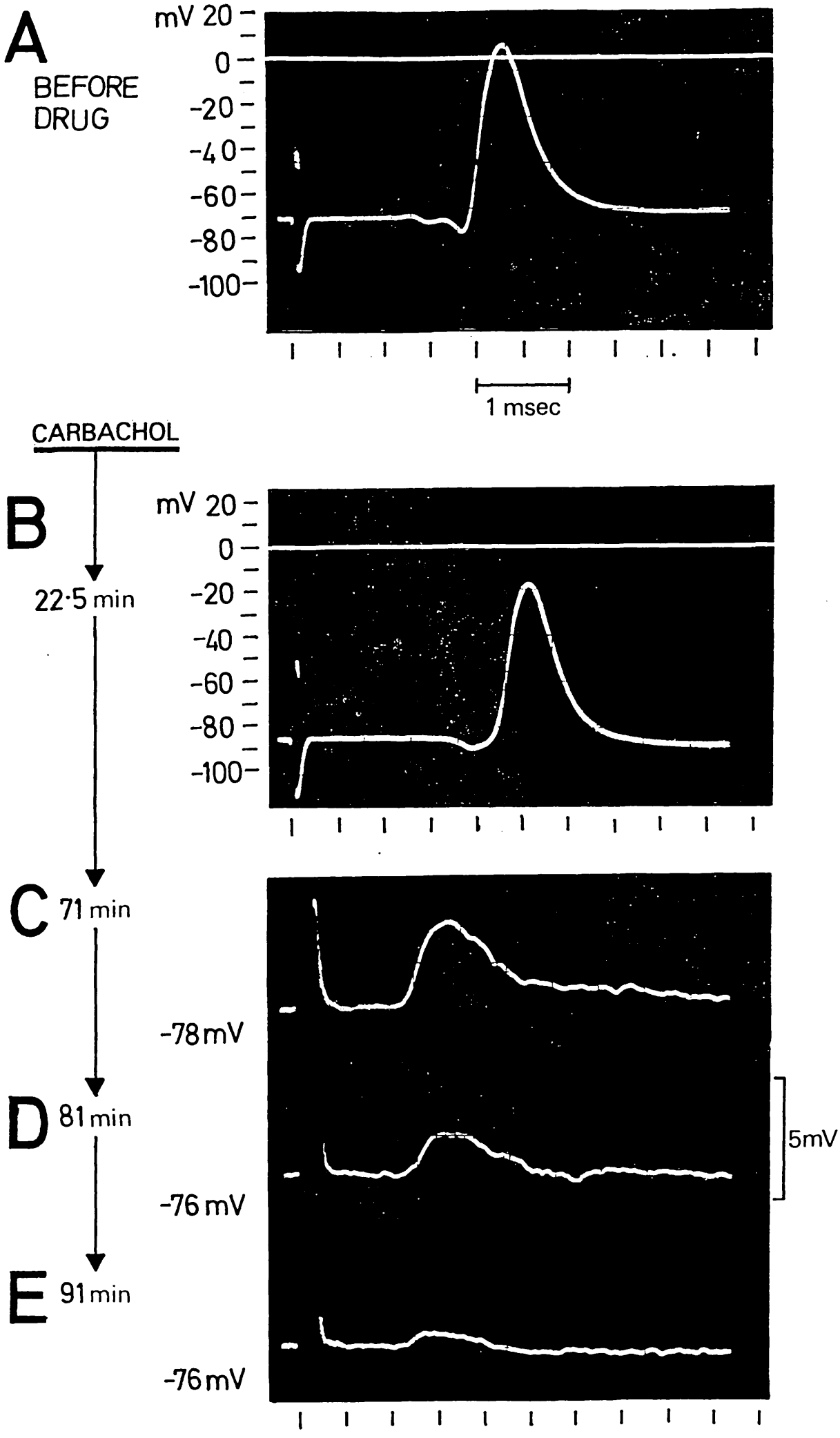
NERVE STIMULATION IN THE PRESENCE OF CARBACHOL

Analysis of the potentials generated at the end-plate in skeletal muscle, as a consequence of the interaction between acetylcholine and its receptor, allowed an examination of the transmission mechanism in isolated muscle. A supramaximal stimulus was applied to the phrenic nerve and the electrical events recorded by an internal electrode at the end-plate region. Carbachol (80 μM) was then added to the bathing medium and the nerve was stimulated again at suitable intervals. Prolonged recordings were often confounded by displacement of the electrode from the membrane, which usually occurred when neuromuscular blockade was incomplete with the contraction mechanism still coupled to neural events.

Sequence of Events

The effects of nerve stimulation which are described below can be considered in relation to parallel recording of contraction (Fig. 3.3) and resting potential (Fig. 4.1). Nerve stimulation without drug application resulted in an action potential recorded in the end-plate region (Fig. 8.1A). Application of carbachol (80 μM) produced depolarization, neuromuscular block and subthreshold end-plate potentials without spikes which are shown in Fig. 8.2. Spontaneous repolarization produced restoration of neuromuscular conduction and the reappearance of action potentials (Fig. 8.1B). Finally

Figure 8.1. Effects of nerve stimulation in the presence of carbachol (80 μM), as recorded from the end-plate via a microelectrode. Plate A was recorded in normal saline, all subsequent plates were taken after changeover to drug-bearing solution. Exposure time is given in min. Ordinate for plates A and B (action potentials) gives membrane potential in mV, calibration bar for C, D and E (end-plate potentials) represents 5 mV. Abscissa is the same for all plates, horizontal calibration below plate A represents 1 msec.


A
BEFORE DRUG
mV 20
0
-20
-40
-60
-80
-100
1 msec
CARBACHOL
B
22·5 min
C
71 min
D
81 min
E
91 min
-78 mV
-76 mV
-76 mV
5mV

neuromuscular conduction again failed and the action potentials were replaced by subthreshold end-plate potentials which progressively faded (Fig. 8.1 C, D, E: Fig 8.3).

Fig. 8.1 shows a sequence of photographs from the ocilloscope screen before and after the addition of 80 μM carbachol. In all of these photographs each time base division represents 0.5 ms. An action potential was recorded upon stimulation of the preparation in normal physiological saline and this is shown in picture A; the membrane depolarized from a resting potential of -72 mV to a spike value of +7 mV, and the electrode was ejected from the membrane by a contraction. Following its replacement, the bathing medium was changed to one containing carbachol. After 22.5 min in the presence of the drug the potential had recovered to -87 mV (B); on stimulating the nerve an action potential was recorded at the end-plate but the spike failed to traverse the zero line, reaching -17 mV. At this stage neuromuscular transmission was present as in Fig. 3.3.

Photographs C, D and E show consecutive traces, all obtained from a different fibre at later times. It was necessary to re-insert the electrode at another end-plate to obtain these observations, as it was not possible to obtain recordings from the same fibre. Location of another end-plate in the absence of mepps was accomplished visually with the aid of polarizing filters (see Methods). The calibration bar for the ordinate represents 5 mV (Fig. 8.1).

The end-plate potential (epp) in C shows a depolarization of only 3.8 mV from -78 mV, at 71 min. There was now no spike and neuromuscular transmission had ceased in this fibre (see Fig. 3.3). D and E were taken at subsequent 10 min intervals and show a progressive fall in response, with depolarization of 1.9 and 0.8 mV respectively.

Further experiments were performed to verify the observations made from this series of photographs, comprising the recording of action potentials before and after the addition of carbachol and the measurement of epps at varying stages during the recovery process.

Action Potentials

Twenty action potentials were recorded in normal physiological saline. The potential depolarized from a resting value of -74 mV (median of 20, 95% limits -68, -78 mV) to a spike potential of +12 mV (20, limits +11, +15 mV). All the potentials traversed the zero line.

By contrast, out of 9 action potentials recorded in the presence of carbachol (80 μM), only 1 gave a positive spike potential. The remainder failed to traverse the zero line. These action potentials were elicited from different preparations at varying times following the addition of the drug, ranging from 15 to 32 min. None was obtained after this later time. The partially recovered potentials varied

from -60 to -87 mV and the spike potential was -9 mV (9, limits -2, -18 mV).

End-plate Potentials

The failure to produce propagated action potentials following stimulation of the phrenic nerve occurred at two distinct periods after the addition of carbachol. At such times subthreshold end-plate potentials were obtained.

The first period coincided with peak depolarization as caused by the drug, at a time of 1 - 3 min after carbachol (80 μM) had been added. Fig. 8.2 shows an epp obtained upon nerve stimulation; the membrane potential was -45 mV.

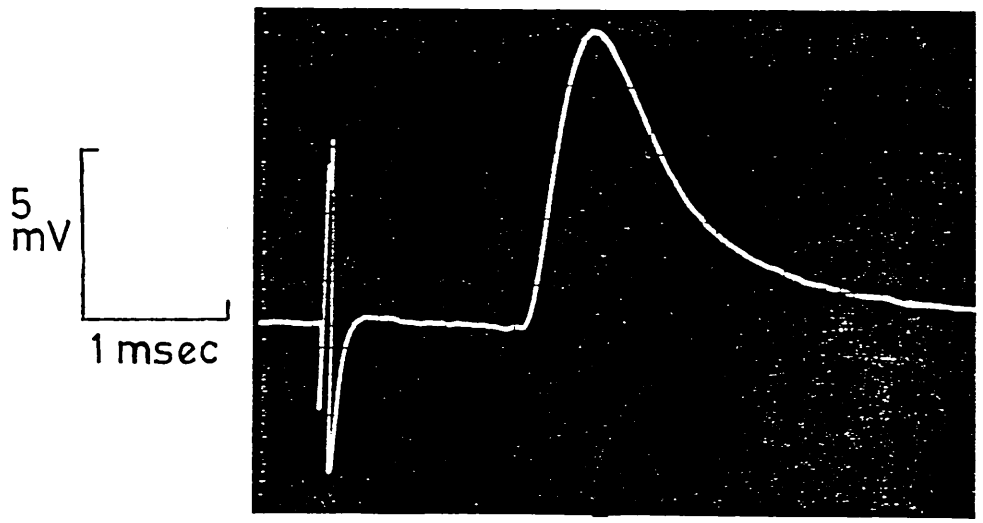


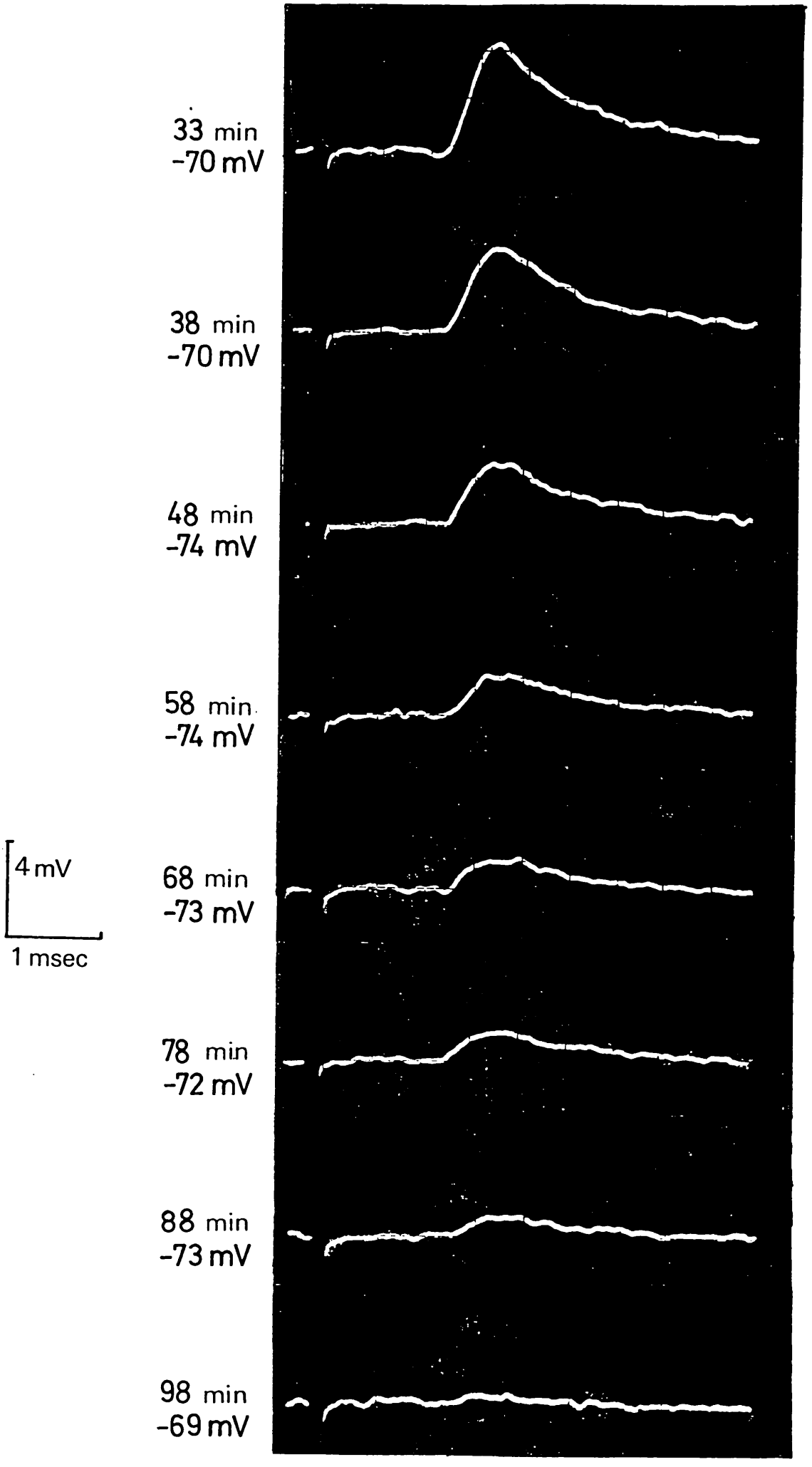
Fig. 8.2. End-plate potential recorded at peak depolarization with carbachol (80 μM) following nerve stimulation. Membrane potential was -45 mV. Epp is depolarizing in direction and 9 mV in amplitude. Latency 1.4 msec from stimulus artefact to beginning of spike.

In successful recordings the membrane potential spontaneously recovered and propagated action potentials (as described above) could be obtained upon stimulation. A second phase of subthreshold epps then occurred with the onset of secondary neuromuscular block. Fig. 8.3 shows a sequence of consecutive photographs of epps obtained upon indirectly stimulating the preparation. In this series of recordings the electrode was not dislodged by contractions of adjacent fibres. The calibration bars represent 4 mV on the ordinate and 1 msec on the abscissa. The amplitude of the epp diminished from 4.6 mV, after 33 min in carbachol to a barely apparent effect at 98 min. The membrane potential had fully recovered and showed little fluctuation over this period. Displayed graphically the relation between epp and exposure time proved to be exponential (Fig. 8.4; correlation coefficient 0.98) with a half-time of 20 min.

One-hundred and ten photographic analyses of the potentials generated at the end-plate were made, following neural stimulation of 23 diaphragm preparations. In all of the preparations but one, no epp could be evoked after 95 min in the presence of 80 μM carbachol. When technically possible, the waning of the epp with time was recorded on film, such sequences being akin to that depicted in Fig. 8.3.

When end-plate potentials were recorded (Fig. 8.2, Fig. 8.3), the fibres showed neuromuscular block. The latencies in these results were less than 1.4 msec, measured from the stimulus artefact to the start of the epp. Longer delays were sometimes associated with small irregular disturbances which were attributed to effects from neighbouring end-plates (Fatt and Katz, 1951) or from contractions in other fibres which were not blocked. In practice it was found that consistent results were obtained with latencies not exceeding 1.4 msec, and so this was accepted as a criterion.

Figure 8.3. Diminution of epp amplitude in the prolonged presence of carbachol (80 μM), following a sequence of nerve stimulations. Plates were taken at intervals after changeover to drug-bearing solution, and the times are given with each trace (min). The membrane potential in mV is also shown. Calibration bars apply to all plates, showing 4 mV on the ordinate and 1 msec on the abscissa. The end-plate potentials declined with time and were barely detectable at 98 min.


33 min
-70 mV
38 min
-70 mV
48 min
-74 mV
58 min
-74 mV
4 mV
1 msec
68 min
-73 mV
78 min
-72 mV
88 min
-73 mV
98 min
-69 mV

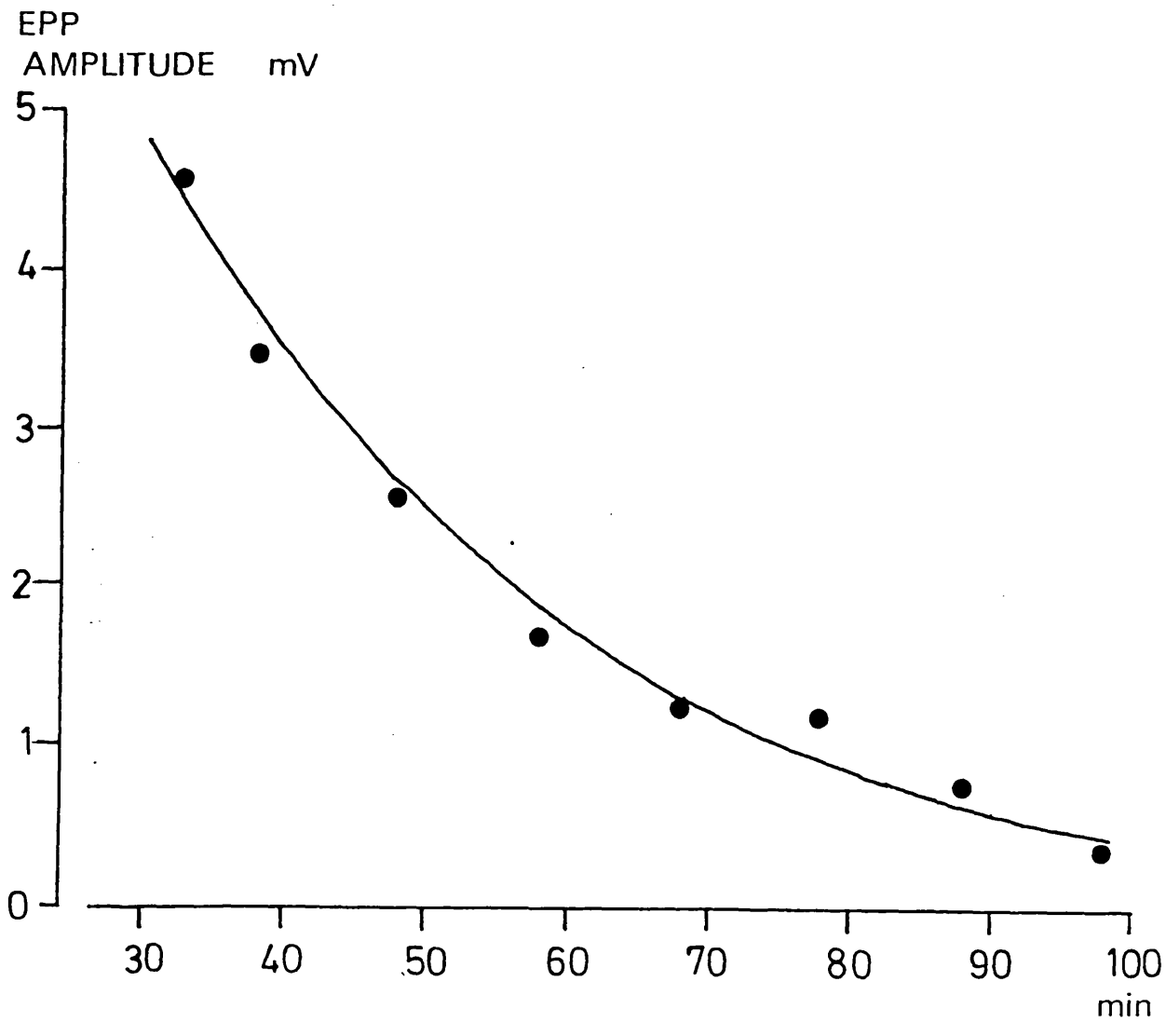


Figure 8.4. Exponential decay of epp amplitude in the prolonged presence of carbachol (80 μM). Data come from traces in Fig. 8.3. Ordinate is in mV, abscissa shows exposure time in min. Half-time calculated as 20 min.

REVERSAL POTENTIAL

The reversal potential, V_{rev}, of control muscles and of muscles bathed in carbachol was examined. V_{rev} is the null-point for acetylcholine-induced end-plate current flow, and in the control muscles the amplitude and direction of miniature end-plate potentials (mepps) were used to make an estimate. In guinea-pig diaphragm exposed to carbachol there are no mepps, despite the recovery of membrane potential (Fig. 4.3). Stimulation of the nerve, however, gives rise to subthreshold epps for a time (see previous section and Fig. 8.3) and use was made of these for examining V_{rev}.

'Control' Reversal Potential Using Mepps

One micro-electrode was inserted at the end-plate and mepps detected according to the techniques described on p80. A second electrode for passing current was inserted in the same fibre within 80 μm. Figure 8.5 shows mepps recorded from the end-plate at a resting potential of -73 mV, as stored on the Racal tape-recorder. The mean mepp amplitude was 0.73 mV.

Current was passed through the second electrode to displace the membrane potential to -51 mV for a period of 15 sec, and the mepps again recorded at this holding voltage. This procedure was repeated with increasing depolarization steps until the membrane potential was beyond zero. At

Figure 8.5. Miniature end-plate potentials at different membrane potentials, used to determine 'control' reversal potential. The membrane potential was changed to different holding voltages for 15 sec periods by passing current through a second micro-electrode. Mepps were recorded on tape and were found to have changed sign at inside-positive values of membrane potential. Mean mepp amplitude was determined by computer; data from this experiment are listed in Table 8.1 (Expt. D) and plotted in Fig. 8.6.

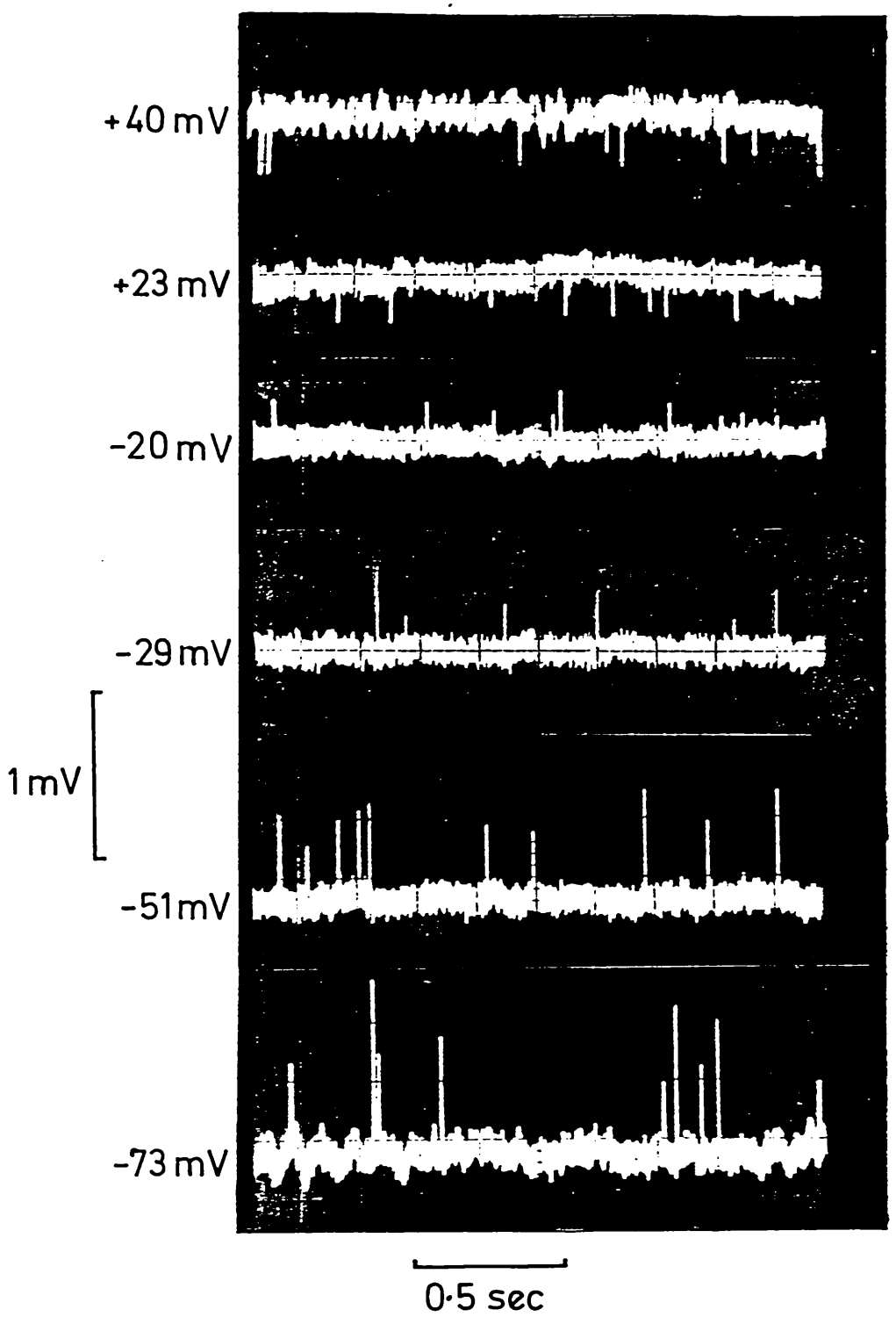

+40 mV
+23 mV
-20 mV
-29 mV
1 mV
-51 mV
-73 mV
0·5 sec

Figure 8.6. Effect of membrane potential on direction and amplitude of mepps using data from Fig. 8.5. Mean mepp amplitude, as determined by computer, is plotted against the potential at which the membrane was being held. At inside-positive values of membrane potential the mepps changed direction; these are given a positive sign in the Figure. The curve was fitted according to a procedure described in the Appendix, and crosses the line of zero mepp amplitude at a membrane potential of -2.2 mV.

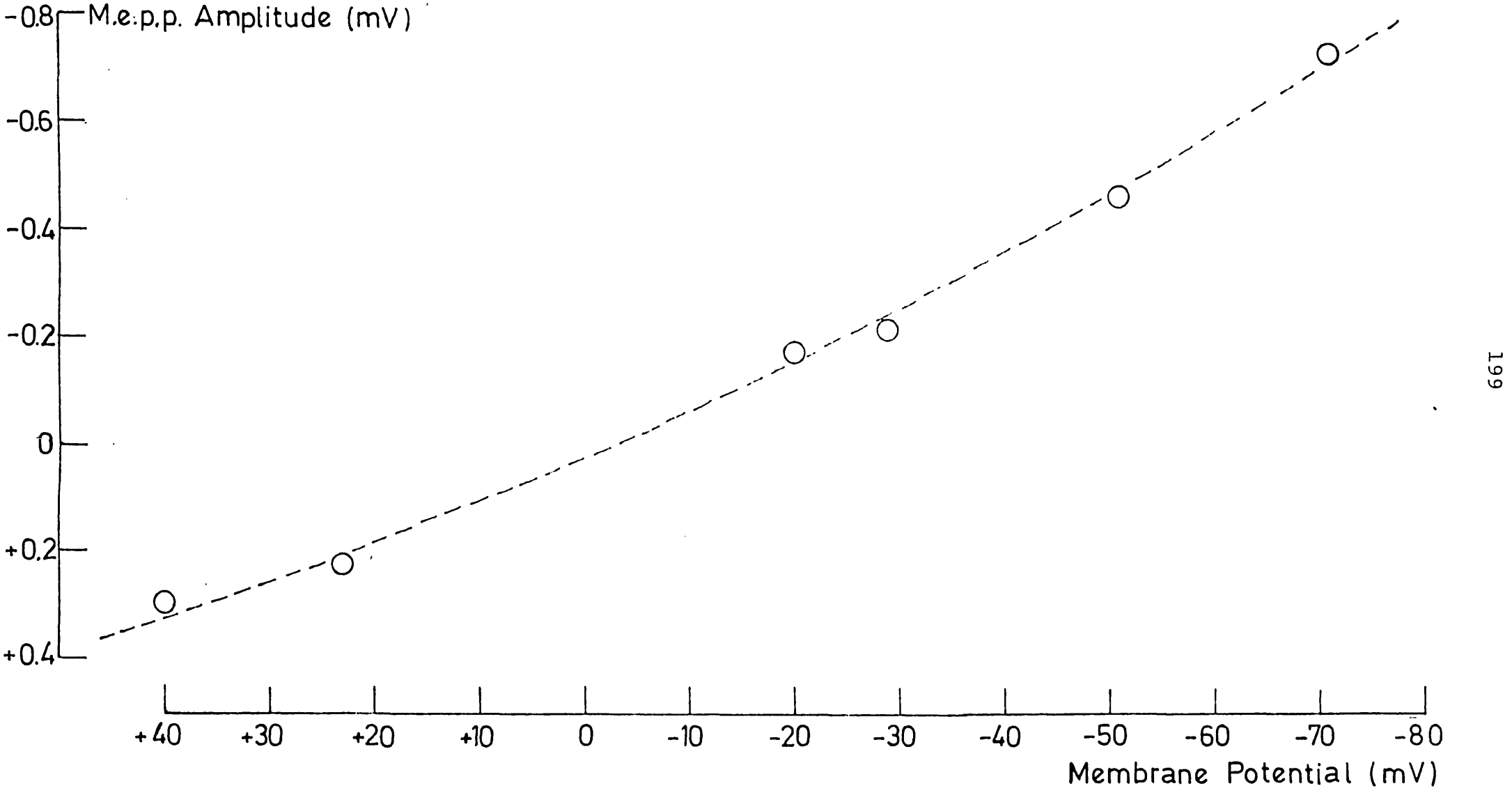

M.e.p.p. Amplitude (mV)
-0.8
-0.6
-0.4
-0.2
0
+0.2
+0.4
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
Membrane Potential (mV)

Table 8.1 Reversal Potential (Control) Measured Using Miniature End-Plate Potentials

Expt	Resting Potential mV	n	Mean Mepp Amplitude mV	SD	V_{rev} mV
A	-60	35	-0.82	0.19	
	-26	19	-0.24	0.05	-5.0
	+20	7	+0.25	0.03	
B	-74	34	-0.74	0.18	
	-51	23	-0.45	0.10	-4.2
	-34	7	-0.26	0.04	
	+32	20	+0.23	0.04	
C	-75	12	-0.74	0.12	
	-54	15	-0.53	0.20	-0.7
	-34	5	-0.31	0.03	
	+24	6	+0.21	0.03	
D	-73	17	-0.73	0.17	
	-51	26	-0.47	0.15	
	-29	16	-0.22	0.06	-2.2
	-20	20	-0.18	0.04	
	+23	26	+0.22	0.06	
	+40	66	+0.29	0.06	
E	-72	20	-0.53	0.08	
	-40	24	-0.31	0.07	-3.0
	+32	28	+0.29	0.06	
F	-77	51	-0.64	0.13	
	-57	29	-0.41	0.06	-10.9
	-39	52	-0.25	0.06	
	+ 1	31	+0.15	0.04	
				Median	-3.6 (6, limits -0.7, -10.9)

The duration of the depolarizing current was 15 seconds. Miniature end-plate potentials were recorded on tape and analysed later. Results from experiments D are shown in Fig. 8.5 and Fig. 8.6. Curves were fitted to the results shown in this table as described in the text, except for the results of experiment E which were fitted by linear regression.

inside-positive values the mepps were found to have changed sign (+23 and +40 mV, Fig. 8.5). At holding potentials close to zero the amplitude of the mepps decreased to noise level. When the current was switched off, the membrane potential returned to within a few mV of the resting value and then recovered in a matter of min. Full recovery between holding potentials was a requisite for data to be accepted.

Mepps were analysed later on a PDP 11/10 Digital computer to determine mean mepp amplitude ($\pm$ SD), according to a program described by Head (1983). Results from 6 fibres are listed in Table 8.1, where the data from Fig. 8.5 is referred to as Experiment D. Mean mepp amplitude and direction are plotted against membrane potential using these data in Fig. 8.6. Mepps which were hyperpolarizing in direction have been given a positive sign. The line has been fitted according to a procedure used by Mallart,Dreyer and Peper (1976), which is described in the Appendix; the curvature of the plot is considered in the Discussion. By the process of interpolation, mepps would be reduced to zero amplitude at a membrane potential of -2.2 mV. The median V_{rev} from 6 experiments was -3.6 mV.

Reversal Potential in Carbachol Using Epps

During prolonged exposure to carbachol (80 µM) the membrane potential fully recovers (Chapter 4). Action potentials may be elicited from the preparation in the presence of the drug for only a limited period however (see

Figure 8.7. End-plate potentials at different membrane potentials in the presence of carbachol (80 μM), used to determine reversal potential. The muscle had been exposed to drug for 125 min and the membrane potential had spontaneously recovered to -74 mV. Stimulation of the nerve resulted in an epp 1 mV in amplitude. Current was passed to change the membrane potential, and a series of epps obtained. At a holding-potential of -20 mV there was no apparent response to nerve stimulation; at -5 mV the epp had changed direction. Data from this experiment are listed in Table 8.2 (Expt. G).

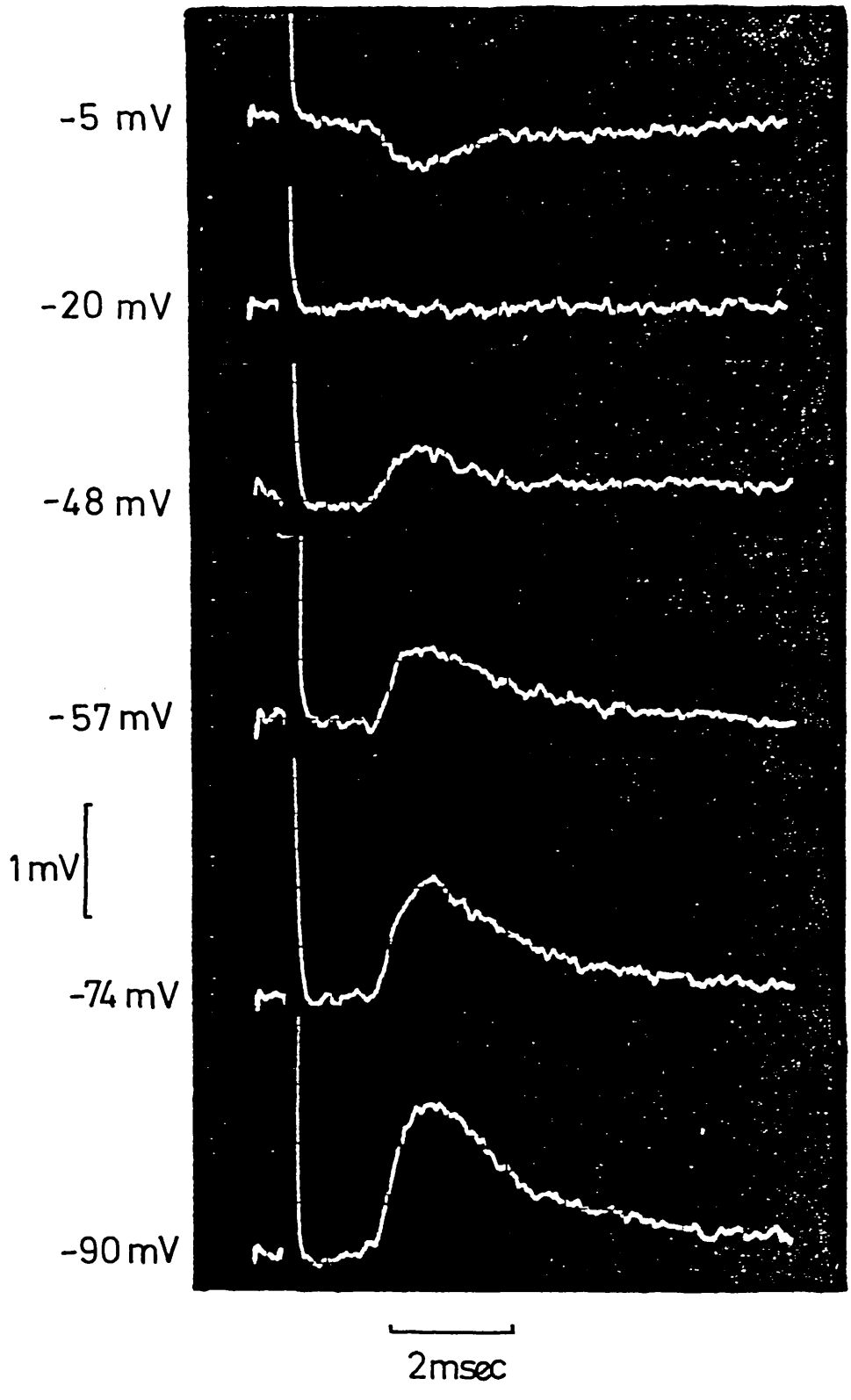

-5 mV
-20 mV
-48 mV
-57 mV
1 mV
-74 mV
-90 mV
2 msec

Figure 8.8. Effect of membrane potential on direction and amplitude of epps in the presence of carbachol (80 μM) using data from Experiment L (Table 8.2). Epps changed sign at an inside-negative value of membrane potential, found by curve-fitting (see Appendix) and interpolation to be -16.3 mV. This is in contrast to the result of Fig.8.6, obtained in drug-free solution, where the reversal potential was close to zero (-2.2 mV).

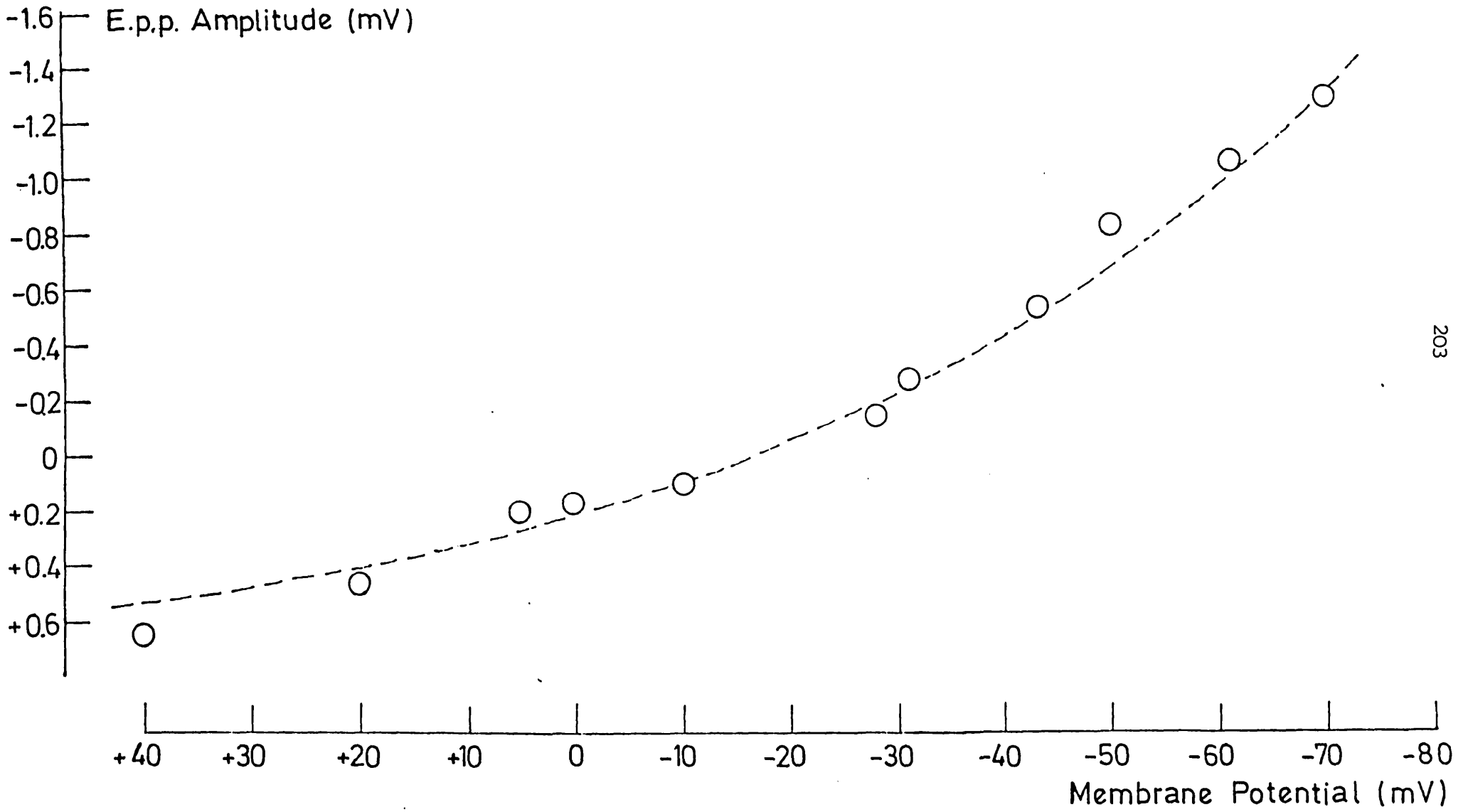

E.p.p. Amplitude (mV)
-1.6
-1.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0
+0.2
+0.4
+0.6
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
Membrane Potential (mV)

Table 8.2 Reversal Potential in the Presence of Carbachol (80 μM) Measured Using End-Plate Potentials

Expt	Time in Drug min	Membrane Potential mV	Epp Amplitude mV	V_{rev} mV
G	125	÷90	-1.30	
		-74	-1.00	
		-57	-0.60	-23.3
		-48	-0.50	
		-20	0	
		-5	+0.40	
H	53	-100	-0.57	
		-70	-0.50	
		-44	-0.24	-18.5
		÷15	+0.07	
		-10	+0.06	
		-10	+0.09	
I	76	-74	-1.05	
		-40	-0.31	
		-33	-0.14	-16.4
		+10	+0.11	
		+20	+0.20	
J	46	-81	-1.56	
		-56	-1.08	-12.8
		-42	-0.48	
		0	+0.17	
K	110	-70	-0.77	
		-60	-0.71	
		-50	-0.37	-21.4
		-40	-0.28	
		-30	-0.09	
		+11	+0.20	
L	70	-70	-1.31	
		-61	-1.08	
		-50	-0.85	
		-43	-0.54	
		-31	-0.28	
		-28	-0.15	-16.3
		-10	+0.10	
		0	+0.17	
		+5	+0.20	
		+20	+0.45	
		+40	+0.65	
			Median	-17.5 (5, limits -12.8, -23.3)

Carbachol (80 μM) produced initial depolarization followed by recovery of resting potential, as shown in Fig. 4.1. The membrane potential was altered by passing current through one electrode, and end-plate potentials were recorded via the second electrode following nerve stimulation. The end-plate potentials were small in these desensitized muscles. Fig. 8.7 shows epps from fibre G, and Fig. 8.8 is a graph using the results of fibre L. Curves were fitted to the data, except for the results of fibres G and H where linear regression was used.

earlier in this Chapter), none being seen after 32 min. A phase of subthreshold epps then occurs with the onset of secondary neuromuscular block.

It was decided to take advantage of this situation and determine V_{rev} in the presence of an agonist. Electrodes were inserted within 80 μm of each other, as for the previous section (mepps), and the fibre exposed to carbachol. After at least 45 min the measurements were made, the series of observations being performed as quickly as possible knowing the effect of time on epp amplitude (i.e. Fig. 8.3).

In Fig. 8.7 the effect of 'holding potential' on direction and sign of the epp is shown. The membrane potential before any current was applied was -74 mV, and the response to nerve stimulation was an epp with amplitude of 1 mV. Current was then passed to vary the potential, and a series of epps obtained. There was no detectable change in membrane potential at -20 mV upon stimulation of the nerve. At -5 mV the epp had changed sign. These data are listed as Experiment G in Table 8.2 and a plot (not shown) of epp amplitude against potential gave a value for V_{rev} of -23.6 mV by interpolation. The most complete series of observations were made in Experiment L (Table 8.2) and it is these that are shown in Fig. 8.8.

Curves were fitted to the data for 4 fibres, linear regression being used for Experiments G and H. The median value for V_{rev} in the presence of carbachol, determined by interpolation was -17.5 mV (n = 6). This value is very different from that in control muscles and the two series do not overlap; this is considered in the Discussion.

SUMMARY

1. Nerve stimulation before drug application resulted in an action potential which was recorded in the end-plate region. All such potentials had a positive overshoot.
2. A stimulus at peak depolatization in carbachol (80 μM) led to a subthreshold end-plate potential.
3. Following spontaneous repolarization in the presence of the drug, a further stimulus yielded an action potential. Such potentials contrasted with those observed in normal saline by failing to have a positive overshoot.
4. With later stimulation the action potentials again disappeared and were replaced by a second phase of subthreshold end-plate potentials, the amplitude of which waned ultimately to zero.
5. In recordings of end-plate potentials, consistent results were obtained when the latency from the stimulus artefact was 1.4 msec or less.
6. The reversal potential was estimated as -3.6 mV (n = 6) in control muscles using miniature end-plate potentials.
7. The reversal potential was also examined in muscles exposed to carbachol (80 μM) after the recovery of membrane potential. Neurally-evoked end-plate potentials were used, and the reversal potential estimated as -17.5 mV (n = 6).

C H A P T E R 9

DISCUSSION

DISCUSSION

Evidence for Channel Opening in Desensitized Muscle

Prolonged action of depolarizing drugs leads to the onset of desensitization in which the stimulant drugs now produce a diminished response, and this action has been considered in the Introduction. The initial action of stimulant drugs is to induce an inward current at the end-plate region which is explicable in terms of the opening of channels for cations (Anderson and Stevens, 1973). This is followed by a fall in the inward current (Jenkinson and Terrar, 1973), with presumably a diminished frequency of channel openings. It is likely, however, that some channels continue to open, and this has been observed in frog muscle by the "patch clamp" technique in which the currents produced by a single channel can be measured: prolonged action of acetylcholine was found to produce bursts of current which indicate that some channels continue to open in the desensitized state in this preparation (Sakmann, Patlak and Neher, 1980).

In guinea-pig muscle there are 2 pieces of evidence which support this concept. The input resistance was measured in the presence of decamethonium and of carbachol. The interpretation is hindered by the depolarization produced by the drug, but in most cases the membrane potential

returned to the previous value and this enables direct comparison of input resistance with and without the presence of the drug. It was found in this small series that with prolonged application of decamethonium the final input resistance was less than in muscle without the drug (and in muscle in which drug had been applied and then withdrawn), and this implies that the drug was still producing an increase in conductance and that some channels continued to open. This conclusion also gains support from the experiments with labelled decamethonium (Chapter 7). In rat muscle decamethonium has been found by autoradiographic means to enter at the end-plate region (Creese and Maclagen, 1970) and the entry is likely to be via cation channels (Creese, Franklin and Mitchell, 1977). In guinea-pig muscle the continued entry of labelled decamethonium for a period of at least 2 hr is consistent with the view that channels continue to open.

In frog muscle decamethonium is a weak agonist and the depolarization is substantially less than that induced by acetylcholine (del Castillo and Katz, 1957), and it can also produce channel block (Adams and Sakmann, 1978a, 1978b). In mammalian muscle there seems no good evidence for regarding decamethonium as a partial agonist (Zaimis and Head, 1976), and in rat muscle a block of labelled sodium influx by decamethonium can be demonstrated but only at dosage of decamethonium in the mM range (Creese, Franklin and Mitchell, 1977).

* Palade and Barchi (1977) found that in rat diaphragm, 85 per cent of the resting membrane conductance was attributable to chloride.

Input Resistance in a Passive Cable

At least three kinds of channels are encountered in skeletal muscle. First there are the channels for passive cable properties. The ions responsible are chiefly K^+ and Cl^-, and the equilibrium potential for K^+ is near -100 mV (Adrian, 1956). Secondly there are channels for the action potential, chiefly for Na^+, with equilibrium potential near +50 mV. And thirdly there are channels induced by the transmitter and by end-plate agonist drugs, with equilibrium potential at 0 to -15 mV.

In frog muscle the choride ions account for 68% of the total membrane conductance (Hutter and Noble, 1960), while in goat intercostal muscle the proportion is as much as 85% (Bryant and Morales-Aguilera, 1971)*. Experiments on detubulated fibres have led to the conclusion that the chloride conductance resides mainly in the circumferential surface membrane whereas the potassium conductance is shared between the surface membrane and the transverse tubules (Eisenberg and Gage, 1969). The surface membrane also seems to contribute to the property of anomalous rectification such that outward current flow produces a fall in potassium conductance (Adrian and Freygang, 1962).

For passive spread of electrotonic potential along a long cable produced by a rectangular input I the steady state expression is

$$V = I R_{in} \exp(-x/\lambda) \qquad (1)$$

where the input resistance R_{in} is $\frac{1}{2}(r_m r_i)^{\frac{1}{2}}$ and the D.C. space

constant λ is $(r_m/r_i)^{\frac{1}{2}}$. This expression was used by Fatt and Katz (1951) and others, and a derivation is given by Hubbard et al (1969). The terms r_m, r_i are defined in the list of abbreviations (p 15) and V is the voltage signal recorded by an internal electrode at a distance x from another electrode which is used to inject current I. R_{in} is the value of V/I when x is zero and is obtained by extrapolation (eg Fig. 6.7); V/I is the effective input resistance measured at a distance x from the signal.

The terms r_m and r_i can be obtained from R_{in} and λ. If the fibre diameter is known the transverse membrane resistance R_m and longitudinal internal resistance R_i can be calculated. Hodgkin and Nakajima (1972) have listed the chief sources of error in these measurements. Smaller fibres have a relatively higher input resistance and low membrane conductance (low frequency measurements), which is consistent with the concept that the tubular system of the fibre contributes substantially to the conductance (Hodgkin and Nakajima, 1972).

The exponential decrement with distance along the fibre has been confirmed for mammalian muscle by Boyd and Martin (1959); Zolovick, Norman and Fedde (1970); Adrian and Marshall (1977), and Glavinovic (1979). The value of 0.9 mm for the space constant in guinea-pig muscle (Table 6.3) lies between that for cat and for rat muscle (Hubbard et al, 1969, Table 3.1). If the distance between the electrodes is taken as 80 μm (Methods) then the term x/λ is 0.1 and $\exp(-x/\lambda)$ is 0.90; and if V/I is used to estimate the input resistance the error is approximately 10 per cent. The term V/I is

the "effective resistance" of Manthey (1966).

The terms r_m and R_m are proportional to $(R_{in})^2$. Transmitter drugs reduce R_{in}. If R_{in} falls to 0.7, this implies that r_m and R_m are approximately halved. The relation $1/(R_{in})^2$ or I^2/V^2 is termed "input conductance" by Geduldig (1968). The space constant λ is also proportional to R_{in}, so the decrement produced by inter-electrode distance would be much affected by changes in R_{in}, and this is considered below.

Input Resistance During Synaptic Action

Stimulation of the nerve trunk releases transmitter which induces the opening of chemosensitive channels and produces an end-plate potential, this generates an action potential which travels along the muscle fibre. In frog muscle the action potential recorded near the end-plate has a "step" in the rise and a "hump" in the fall, and the spike has less "overshoot" than that recorded at some distance along the fibre. Fatt and Katz (1951) and del Castillo and Katz (1954b) interpreted the diminished overshoot as due to the shunting effect of the channels opened by the transmitter by which the input resistance (and the space constant λ) was reduced to 0.1 of the resting value and r_m and R_m were reduced to 0.01. The short-circuit effect of the epp on the action potential was also studied by Maeno (1966) and was interpreted by a model in which the transmitter opened

separate K^+ and Na^+ channels, which behaved in a different manner. The evidence that one set of channels is opened by the transmitter and that Na^+ and K^+ (and Ca^{++}) share the same end-plate channels is given by Dionne (1979).

The action potential recorded in the end-plate region in cat muscle shows an epp and spike in the presence of tubocurarine (Boyd and Martin, 1956) but in the absence of drugs there was no clear indication of "step" or "hump". The results of Muscholl (1957) also show no step or hump in rat muscle. Fig. 9 of Muscholl's paper (1957) shows action potentials at the end-plate and also 3 mm away, with some reduction in the overshoot in records at the end-plate.

Fig. 8.1A shows the action potential in the guinea-pig diaphragm in the region of the end-plate, with a small overshoot which was +12 mV in 20 fibres. Records of action potentials at a distance from the end-plate have not been made in the present study, but from the results in other mammalian muscles the action potential shown in Fig. 8.1A should be regarded as reduced as compared with that recorded outside the region of the end-plate. The further reduction in the action potential which occurred in the presence of carbachol when the resting potential had recovered, is considered below.

Relation Between Extent and Rate of Depolarization

The rate of depolarization varies greatly in different experiments. Table 9.1 shows the maximum rate, expressed as mV/min, collected from Tables 4.1, 4.2, 4.5, 5.4 and 5.5. The total depolarization produced by carbachol (80 μM) or decamethonium (10 μM) is also shown, and in general a rapid maximum rate of depolarization was associated with a greater amplitude of depolarization. The results in Table 9.1 are grouped and show this trend.

In Table 9.2 the half-time in sec has also been listed, being the time taken for the membrane potential to fall to half the total extent, measured from the first indication of a change in the trace. The relation between the response (mV) and distance is exponential in the case of electrotonic potentials (Hodgkin and Rushton, 1946), and in Fig. 9.1 the change in voltage (response) has been plotted against the half-time from grouped results with the former on a logarithmic scale.

The simplest explanation for this relation is that the fast traces represent recording with the electrode very close to the synaptic cleft, which would correspond to focal recording (del Castillo and Katz, 1956), while the slow records presumably represent results with the electrode not perfectly positioned. All recordings gave spontaneous miniature end-plate potentials (mepps) with a rapid rise (Fig. 2.7), but in only a few cases was it possible to

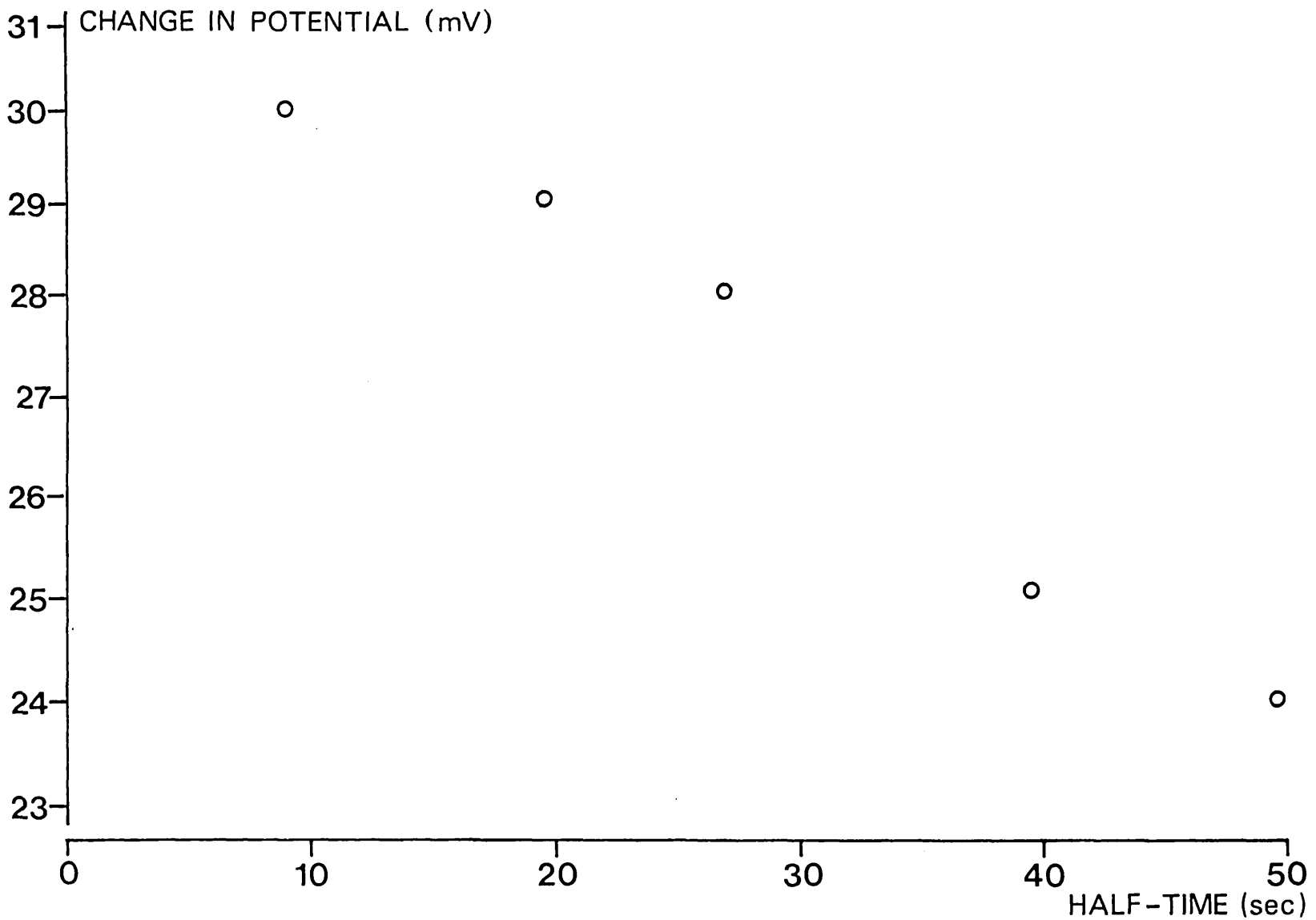
Table 9.1 Rate and Extent of Depolarization

Maximum Rate of Depolarization						Change in Potential	
Table 4.1	4.2	4.5	5.4	5.5	Group Median (mV/min)	Change (mV)	Group Median (mV)
	(mV/min)						
	15.8					24	
				17.6		17	
				24.0	24.5 (6)	24	24.0 (6)
		25.0				24	
	25.0					23	
25.9						24	
	27.8					31	
			28.6			24	
			30.0			26	
	31.6				31.6 (7)	26	26.0 (7)
		33.3				26	
				37.5		31	
		38.5				28	
			40.0			30	
	50.0					30	
		55.6				32	
60.0					60.8 (8)	31	28.5 (8)
				61.5		27	
				61.5		31	
			66.7			19	
			66.7			22	
68.2						26	
71.4						26	
			75.0		75.5 (5)	40	32.0 (5)
				100.0		32	
	113.2					43	
125.0						41	
			133.3			27	
160.0					146.6 (4)	37	33.5 (4)
		166.7				30	
(6)	(6)	(5)	(7)	(6)			

Table 9.2 Half-time and Extent of Depolarization

Half-time for Depolarization						Change in Potential		
Table					Group Median	Change	Group Median	log
4.1	4.2	4.5	5.4	5.5	(sec)	(mV)	(mV)	mV
	82.5					24		
	53.4					31		
		49.5			49.5 (5)	32	24 (5)	1.380
			45.0			24		
				43.5		24		
41.4						24		
		40.2				28		
		39.0			39.6 (4)	24	25 (4)	1.398
			39.0			26		
	37.5					23		
				34.5		17		
	28.5					26		
		27.0				26		
				27.0	27.0 (8)	31	28 (8)	1.447
	26.4					30		
25.5						31		
			24.0			30		
				24.0		31		
				21.0		27		
			20.1			40		
			19.5			22		
	19.5				19.5 (8)	43	29 (8)	1.462
16.5						26		
16.2						26		
				15.0		32		
15.0						41		
			12.6			19		
			9.0		9.0 (5)	27	30 (5)	1.477
9.0						37		
		5.4				30		
(6)	(6)	(5)	(7)	(6)				

Figure 9.1 The relation between change in membrane potential and half-time for depolarization. Data for this graph come from Table 9.2 and represent pooled results for both carbachol (80 μM) and decamethonium (10 μM). Change in potential is given in mV; half-time is given in sec. Large depolarizations are associated with a short half-time.


CHANGE IN POTENTIAL (mV)
31
30
29
28
27
26
25
24
23
0
10
20
30
40
50
HALF-TIME (sec)

obtain inverted mepps which would indicate focal recording. In the case of the end-plate potential produced by nerve stimulation the diminution in amplitude and in the rate of depolarization as the recording electrode is moved away from the optimal point has been described by Fatt and Katz (1951).

The results imply that the rate of depolarization for a well positioned electrode may be around 150 mV/min (Table 9.1) with a half-time of perhaps 5 sec (Table 9.2). On this interpretation a small displacement of the electrode would give a much slower rate of response, although the extent of the depolarization seems to be less affected. The electrical changes which are seen when the depolarizing drugs are applied are much slower than the electrotonic effects which were studied by Fatt and Katz (1951) and it is likely that the rate of response is slowed by lags due to ionic changes which include equilibration of chloride ions (Jenkinson and Terrar, 1973).

*Depolarization and input resistance:

$$\frac{\Delta V}{V-V_{rev}} = -\frac{g}{g+G}$$

and $$1-\frac{g}{g+G} = \frac{G}{g+G} = \frac{G}{G'} = \frac{R'_m}{R_m} = \left(\frac{R'_{in}}{R_{in}}\right)^2$$

Input Resistance During Depolarization Induced by Carbachol

The effect of agonist drugs on the end-plate may be studied by the voltage clamp method, and in a recent paper Head (1983) measured the current produced by acetylcholine and by carbachol in rat diaphragm at a steady resting potential of -80 mV. This method has not been used in the present study which was designed to investigate the secondary effects associated with depolarization including the spontaneous recovery process and the operation of the sodium pump. The direct measurement of agonist action on input resistance is subject to systematic errors, and fortunately other estimates can be made from the results.

If depolarization is produced then the conductance change has to be inferred from the results. The simplified circuit for the action of depolarizing drugs is shown in Fig. 9.2A which is derived from Ginsborg and Jenkinson (1976, p239), and from Ginsborg (1967, Fig. 1). The effect of the agonist drug is to add a shunt conductance g so that the depolarization is

$$\Delta V = V_o g/(g + G) \qquad (2)$$

where G is the input conductance in the absence of drug, and V_o is resting potential minus reversal potential $(V-V_{rev})$ which is termed the synaptic driving potential by Auerbach and Betz (1971). If G is not known, g/G may be found by multiplying $\Delta V/V_o$ by the factor $(1 - \Delta V/V_o)^{-1}$ (see Ginsborg and Jenkinson, 1976). In the present study the input resistance without drug was measured so that G could be obtained. The term g/(g+G) was found from $\Delta V/V_o$.

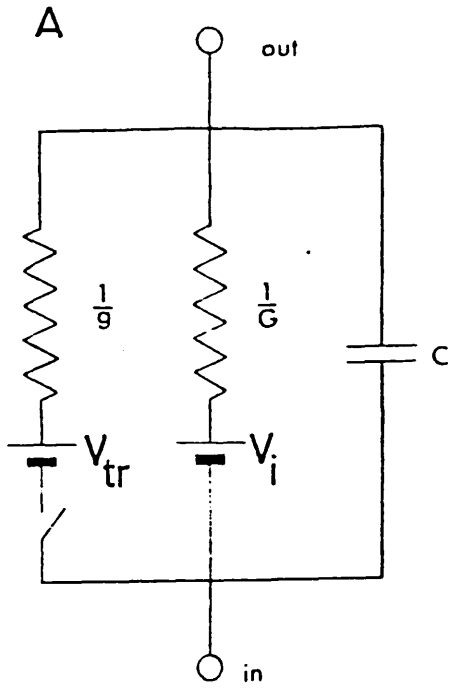
Figure 9.2. Equivalent circuits referred to in the text.

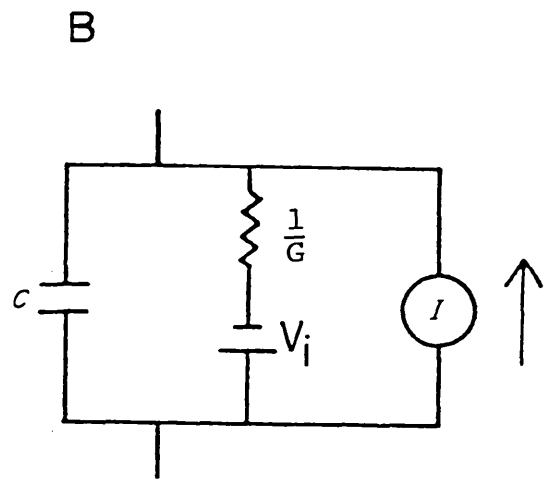
A. Slightly modified from Ginsborg and Jenkinson (1976; p239). Simple equivalent circuit for transmitter action at the neuromuscular junction. Non-chemosensitive membrane represented by a battery V_i, a conductance G and a capacitance C. The elements on the left indicate the additional ion channels opened by transmitter, and comprise another battery V_{tr} in series with a conductance g and a switch which closes to mimic transmitter action.

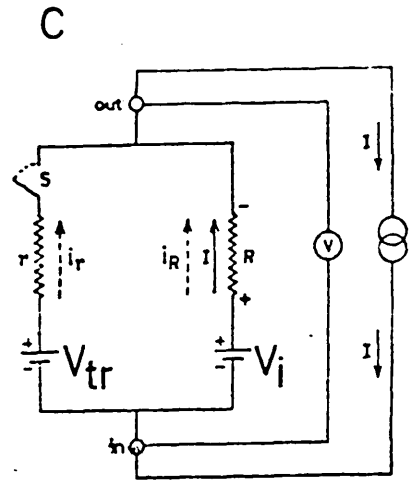
B. From Hubbard et al (1969;p48). Equivalent circuit for the non-chemosensitive part of the membrane, incorporating a non-neutral ionic pump. The flux of charge produced by such a pump would be equivalent to a circuit, and could act independently of the membrane potential. The membrane potential would be $V_i + I/G$, where I is the current produced by the 'constant-current generator'. If the pump were outwardly directed (eg more Na^+ extruded than K^+ brought in), the current would re-enter the membrane in an inwards direction resulting in hyperpolarization.

C. Slightly modified from Ginsborg (1967, p294). Simplified equivalent circuit for the constant current and voltage clamp methods which can be used in the determination of transmitter equilibrium potentials. The depolarization caused by transmitter should be abolished when the membrane potential is equal to V_{tr}; and if the membrane potential is made less negative than V_{tr}, the effect of the transmitter on the membrane potential should be reversed in direction.

D. Equivalent circuit incorporating the model for transmitter action (A) and also the model for the pump (B). See text for details.


A
out
$\frac{1}{g}$
$\frac{1}{G}$
C
V_{tr}
V_i
in


B
C
$\frac{1}{G}$
V_i
I


C
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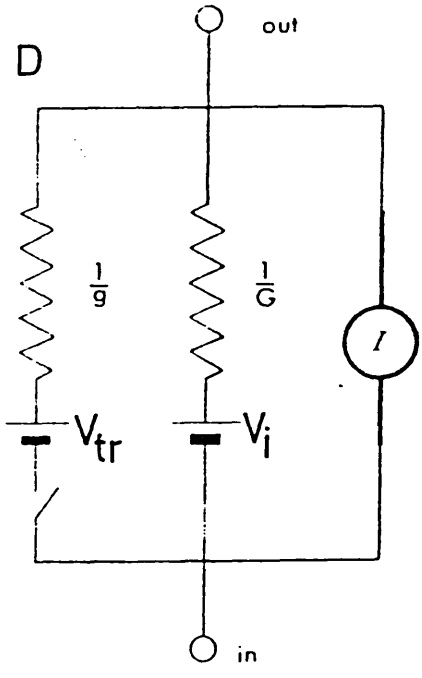
D
out
$\frac{1}{g}$
$\frac{1}{G}$
I
V_{tr}
V_i
in

If $g/(g + G)$ is f then the ratio g/G is $(f^{-1} - 1)^{-1}$, as used in Table 9.4. There are several uncertainties in this method for estimating the shunt conductance. The reversal potential in the presence of the agonist has to be known; this was found to be -17.5 mV and is discussed below. Non-focal positioning of the voltage recording electrode could produce an under-estimate of ΔV and hence of $g/(g + G)$, and chloride redistribution might produce lag and attenuation of the response (Jenkinson and Terrar, 1973; Feltz and Jaoul,1974). The use of the method implies that the value of G (and R, which is 1/G) does not appreciably change with depolarization, and that the voltage-current relation in the range which was used can be treated as linear.

When current is injected in normal fibres the voltage changes for small displacements are linearly related to the current (Boyd and Martin, 1959; Hubbard et al., 1969, p99). For large displacements non-linear characteristics may appear. Depolarization is accompanied by a rise in chloride conductance as expected from the constant field theory (Hodgkin and Horowicz, 1959; Hubbard et al, 1969, p41). Depolarization also produces a fall in potassium conductance; outward current is then accompanied by an increase in membrane resistance, and this has been termed 'anomalous rectification' (see Hubbard et al., 1969, p60, p41; Hodgkin and Horowicz, 1959). The voltage-current curves in frog muscle were studied by Hutter and Noble (1960), and have been summarised by Hutter and Warner (1972). The non-linear curves associated with potassium (in chloride-free solutions) are largely linearised

in the presence of chloride. The rectifying effect on the membrane is considered by Ginsborg (1967).

The effect of a depolarizing drug is to produce an additional conductance which is found to reduce the locally recorded rectification, (measured in depolarized fibres), and this is explicable if the chemo-sensitive channels allow potassium and other cations to move in both directions with much the same facility (del Castillo and Katz, 1955b). Curvature in current-voltage plots in the presence of agonist drugs may also be due to the effect of voltage on the number of end-plate channels which open (Mallart, Dreyer and Peper, 1976; Colquhoun et al., 1979). In Fig. 8.8 the plot of epp voltage against membrane potential in the presence of carbachol has a definite curvature if continued to positive values of membrane potential. Depolarization by carbachol is usually 30 mV (Table 6.2), and in this range the curvature is small. In practice the value of $\Delta V/V_o$ has been taken as an estimate of $g/(g + G)$, as used by Ginsborg (1967) and others, with the reservations listed above.

Depolarizing drugs open shunt channels and produce a fall in input resistance, and Zaimis and Head (1976) have included this method for the estimation of agonist action. The input resistance can be measured in the presence and in the absence of agonist and an estimate of the ratio R'/R can be made by the use of two electrodes. The method is not generally applicable because with large changes in r_m and R_m the decrement due to the inter-electrode distance

changes, and if V'/I' and V/I are the effective input resistances in the presence and absence of agonist respectively, then the ratio V'/I' to V/I, which can be experimentally determined, will be an over-estimate of the effect of the agonist on input resistance (see below). Now, in favourable cases the depolarization is followed by spontaneous repolarization in which the original resting potential is restored, and in these circumstances desensitization has occurred so the error in the measurement of input resistance is likely to be much smaller. The method has been used in the present study as a qualitative indication of a change in input resistance (see below).

If the shunt conductance is calculated from the ratio $\Delta V/(V-V_{rev})$ then the change in input resistance can be found, as on p.219. The membrane resistance in the presence and absence of agonist is R'_m/R_m which is $1 - g/(g+G)$, and R'_{in}/R_{in} is $(R'_m/R_m)^{\frac{1}{2}}$.

The measured value of ΔV is likely to be attenuated in the presence of chloride ions, and this factor could be considerable if the chloride conductance is 85 per cent of the total as in rat diaphragm (Palade and Barchi, 1977). The measured depolarization would be reduced by the chloride factor, and also by any opposing current due to an electrogenic pump (below). Calculations of shunt conductance g given in Table 9.4 (p.247) have been obtained from the use of equation (2), without corrections. At peak depolarization (Table 9.4) the term R'_{in}/R_{in} comes to 0.71 (with V_{rev} taken as -17.5 mV) and to 0.77 (with V_{rev} as - 3.6 mV). The value of R'/R measured by current injection is 0.31 (5, from Table 6.2), and after correction for changes in space constant the value comes to 0.38 (see p.226). The attenuation in the measured depolarization may account for these discrepancies.

Using the value of 0.38 obtained by current injection, the expected depolarization from equation (2) would be 50 mV (taking V_{rev} as -17.5 mV) or 62 mV (V_{rev} = -3.6 mV); that is, about twice the measured depolarization. Jenkinson and Terrar (1973) found that when chloride was replaced by isethionate, the depolarization of frog skeletal muscle was increased from 15 mV to 40 mV. It seems likely then, that chloride redistribution may limit depolarization in guinea-pig diaphragm to a similar degree.

The microelectrode for voltage recording was inserted first and can on occasions be accurately placed in the focal region as shown by inverted mepps on withdrawal (Fig. 2.7). The second electrode, for passing current, is necessarily inserted in such a way as not to disturb the first, and the inter-electrode distance is commonly "within 50 μm" (Manthey, 1966; Burke and Ginsborg, 1956); "less than 100 μm" (Auerbach and Betz, 1971); "5-10 μm" (Glavinovic, 1979). If this is less than 80 μm in the present study (see Methods) then the distance as a fraction of the space constant is on average less than 0.1 (Table 6.3). When an agonist is applied the space constant changes, and this introduces a systematic error in measurements of input resistance in the presence of agonist.

Possible Corrections for Direct Measure of Input Resistance

Two procedures have been used in an attempt to estimate the error, both intended for amphibian muscle. Fatt and Katz (1951) used eqn. (1) directly and estimated the change in R_{in} and hence in r_m (which is proportional to R_{in}^2), and in λ (which is proportional to R_{in}). Hence if R_{in} falls to 0.1, λ also becomes 0.1 of its former value, and r_m and R_m

Table 9.3 Effect of change in membrane resistance on apparent input resistance

r_m	$(r_m)^{\frac{1}{2}}$	$1/(r_m)^{\frac{1}{2}}$	x/λ	$\exp(-x/\lambda)$	V/I	V/I as fraction
1	1	1	0.1	0.905	0.905	1.0
0.9	0.949	1.05	0.105	0.900	0.854	0.944
0.8	0.894	1.12	0.112	0.894	0.799	0.883
0.7	0.837	1.19	0.119	0.888	0.743	0.821
0.6	0.775	1.29	0.129	0.879	0.681	0.752
0.5	0.707	1.41	0.141	0.868	0.614	0.678
0.4	0.632	1.58	0.158	0.854	0.540	0.530
0.3	0.548	1.82	0.182	0.833	0.456	0.504
0.2	0.447	2.24	0.224	0.799	0.357	0.394
0.15	0.387	2.58	0.258	0.773	0.299	0.330
0.1	0.316	3.16	0.316	0.738	0.237	0.257
0.05	0.224	4.47	0.447	0.640	0.143	0.158
0.01	0.100	10	1.0	0.368	0.037	0.041

$$V/I = (\tfrac{1}{2})\ (r_m r_i)^{\frac{1}{2}} \exp - x/(r_m/r_i)^{\frac{1}{2}} \qquad (1)$$

The term x/λ is taken as 0.1 initially. The term r_m is supposed to change over all of the fibres. The first column gives r_m. The fourth column gives x/λ. The term V/I gives the apparent input resistance, and the last column gives the measured term V/I as a fraction of the initial value (0.905).

are 0.01. This type of calculation is shown in Table 9.3 in which the effect of change in r_m on the apparent input resistance V/I has been calculated. The columns give r_m, x/λ, V/I the apparent input resistance, and V/I as a fraction of the initial value (0.905). For $x/\lambda = 0.1$ (before drug), the term V/I is modified as shown in the penultimate and final columns, and the effect of a change in λ can be assessed. In practice an apparent value of 0.31 for R'/R (Table 6.2), obtained by electrical stimulation, would imply a value of 0.38 for R_{in}'/R_{in} (Table 9.3, second column, putting 0.31 in last column).

The direct use of eqn. (1) in this form might be valid for a uniform change in the value of membrane resistance along the whole cable, such as that envisaged for slow fibres of the frog (Burke and Ginsborg, 1956) or perhaps for denervated muscle. In twitch fibres of amphibian muscle the effective chemosensitive area is over 200 μm (Gage and McBurney, 1973; Rang, 1974), or possibly 500 μm (Fatt and Katz, 1951), and the two electrodes can be placed in the chemosensitive area. The change in λ which would accompany the action of depolarizing drugs has been considered by Jenkinson and Terrar (1973), who did not attempt a quantitative estimate.

Rang (1974, p323) has calculated the effect of inter-electrode distance in a different manner, based on results by Hodgkin and Nakajima (1972, p117) and Harrington (1973). R_{in} was 0.32 MΩ for frog fibres of 80 μm, and λ was 1.9 mm, so r_m which is $2 R_{in} \lambda$, was 0.12 MΩ cm and r_m^{-1} was 8 μS cm^{-1}. Acetylcholine (bath applied) produced an increased conductance

g of 10^{-5} mho or 10 μS. The end-plate length was taken as 300 μm or 0.03 cm so the new value of r_m^{-1} comes to 330 μS cm^{-1}, which implies that the end-plate conductance was increased by a factor of 40. This would reduce λ by a factor of $40^{\frac{1}{2}}$ so that λ would now be 1.9/6.3 or 0.3 mm, and Rang (1974) used this calculation to argue that with increasing concentration of agonist the area controlled by a clamping electrode would be progressively reduced in frog muscle.

If this procedure is applied to guinea-pig muscle, R_{in} is 0.53 MΩ (Table 6.3), λ is 0.9 mm (Table 6.3) so $r_m (= 2R_{in}\lambda)$ is 0.095MΩ cm and r_m^{-1} is 10.5μS cm^{-1}. For the extra end-plate conductance with 80 μM carbachol, g/(g + G) is 0.504 (25, from Table 6.2) so g/G is 1.016 and if G is 1.89 μS, g is 1.92 μS. In rat diaphragm the end-plate length has been taken as 30 μm (Auerbach and Betz, 1971) and if this is applicable to guinea-pig muscle then r_m^{-1} is 640 μS cm^{-1} so that end-plate conductance has increased by a factor of 61. Hence λ would decrease by $61^{\frac{1}{2}}$ or 7.8times, so that x/λ would change from 0.1 to 0.78 and the term exp $(-x/\lambda)$ would change from 0.9 to 0.46.

However, the end-plate in mammalian muscle is short. If the voltage-recording electrode is focally positioned the current-passing electrode would be outside the end-plate and a pulse from the latter would traverse mainly an area outside the end-plate region, in which the concentration of receptor complexes is probably low (Miledi, 1962; Case et al., 1977), with membrane conductance intermediate between end-plate and non-synaptic regions. Hence this calculation of change in λ cannot be applied to mammalian muscle and no

convincing method of estimation is available.

A depolarization of 30 mV (Table 6.2) with R equal to 0.53 MΩ would require a current of 57 nA (if the current-voltage relation can be regarded as linear for a depolarization of this magnitude), and the current flows through the transmitter channels and outwards through the rest of the membrane (Ginsborg, 1967). The conductance effects can be calculated, but the change in input resistance measured by current pulses is over-estimated because the effects of inter-electrode distance are enhanced by an unknown factor. The systematic error in estimation of the membrane conductance by the use of two electrodes is very much reduced when the resting potential has returned to normal, and in this situation other methods are available which provide a check on the results (see below).

Input Resistance when Membrane Potential has Recovered

In Fig. 6.2 prolonged application of carbachol produced depolarization with subsequent complete recovery of membrane potential, while the effective resistance V/I remained low as compared with the initial value. Manthey (1966) carried out similar experiments in frog muscle with a concentration of carbachol which reduced the effective resistance by 90% of its control value, and this was followed by spontaneous repolarization with restoration of the effective resistance in most cases.

In Table 6.1 the effective resistance in the presence of agonist drug at a time when membrane potential had returned to the initial value (or was within 2 mV of the initial value) was 83% (range 69, 90% in 10 muscles). If the standard relation in eqn. (1) is applicable the length constant would also change and a correction factor would be needed. It can be argued that a consistent fall in the effective resistance must imply that a shunt conductance is present, though the precise correction factor for the exponential term $\exp - x/\lambda$ is not known. From Table 9.3 a fall in effective resistance to 0.82 when x/λ equals 0.1 indicates that $(r_m)^{\frac{1}{2}}$ and R_{in} are close to 0.84. In any case the correction due to the exponential term will be less than at peak depolarization.

In Fig. 4.1 the membrane potential recovers in the presence of the agonist drug, and if the effective resistance is not restored to the initial value then this implies that some end-plate channels continue to open, and it can be inferred that the restored membrane potential would be partly maintained by another factor which is likely to be the sodium pump. This interpretation is supported by the effects of withdrawal of the drug, which produced consistent hyperpolarization (Fig. 4.1). From Table 4.3 the hyperpolarization is 6.5 mV (combined results from decamethonium (10 μM) and carbachol (80 μM) following exposure from 16 to 32 min , median of 8 muscles). The input resistance was not measured in this series, but if the value in the absence of drug can be used (Table 6.3), the current which produced the hyperpolarization would be $(6.5 \times 10^{-3})/0.53 \times 10^{6})$ or 12 nA per end-plate.

For a situation in which the resting potential is restored to the initial value this polarizing current is presumably equal and opposite to that produced by the opening of channels in the presence of the drug. It is of interest to compare these results with those of Head (1983) who worked with rat diaphragm at 37°C. He found by voltage-clamp measurements that the initial current produced by carbachol (40 μM) was approximately 90 nA (Fig. 11, Head, 1983), and this rapidly fell by desensitization to around 40 nA and then declined more slowly. Recordings were not continued beyond 5 min.

The calculation of membrane conductance when the potential has been restored in the presence of agonist can be illustrated by supposing that the hyperpolarization of 6.5 mV on withdrawal of agonist (Table 4.3) is reversible. This might have been shown by restoring the agonist after 3 min at the end of the experiments in Fig. 4.1, when presumably the membrane would depolarize by 6.5 mV, from -83 mV (Table 4.3). On this assumption the fraction $g/(g + G)$ from eqn. (2) is 0.110 if V_{rev} is -17.5 mV, and 0.089 if V_{rev} is -3.6 mV (Table 9.4). Then, if G is 1.89 μS, g is 0.21 or 0.17 μS. The shunt conductance g has fallen from 1.92 μS by desensitization. The input resistance when recovery has occurred in the presence of agonist, in experiments shown in Fig. 6.1 and Fig. 6.2, would be R_{in}'/R_{in} which is 0.95 or 0.96 (Table 9.4), which can be compared with the value of 0.83 found by injection of current (Table 6.1). The input resistance in the desensitized muscles would be 0.96 x 0.53 MΩ or 0.51 MΩ, and r_m^{-1} is g/(end-plate length) or $0.17 \times 10^{-6}/0.003$ which is 57 μS cm^{-1} for V_{rev} = -3.6 mV, and 70 μS cm^{-1} for V_{rev} = -17.5 mV (Table 9.4).

The current produced by channel opening at a time when the resting potential has returned to the initial value can be calculated in another way. In rat diaphragm at 37°C with a clamped potential of -80 mV the single channel conductance of channels opened by carbachol is 17 pS and the current is 1.2 pA, obtained by noise analysis (Head, 1983). If these values are applicable to desensitized guinea-pig muscle with a current of 12 nA per end-plate, the number of channels open at any one time would be $(12 \times 10^{-9})/(1.2 \times 10^{-12})$ or 1.0×10^{4} per end-plate with conductance 0.17 μS.

Some other results obtained when the resting potential has returned to the initial value remain to be considered. The mepp in guinea-pig muscle is near 1 mV with focal recording (Fig. 2.7). When agonist drugs are applied the mepps disappear (Fig. 4.3) and similar findings have been reported by Roberts and Thesleff (1965), Galindo (1971), Evans (1974) and Creese and Mitchell (1981). The disappearance of mepps may be regarded as a manifestation of the general process of desensitization, and this factor would presumably reduce the mepp to the noise level.

Nerve stimulation, at a time when the resting potential has been restored in the presence of an agonist, can for a time still produce action potentials (Fig. 8.1B) and muscle contractions (Fig. 3.1, Fig.4.5). It is likely that the transmitter acts on the post-synaptic area of the muscle end-plate at saturating concentrations, perhaps 1 mM (Matthews-Bellinger and Salpeter, 1978), so that in this situation the transmitter must depolarize to the threshold level in spite of the desensitization produced by the agonist drug.

The "safety factor" (Paton and Waud, 1967) has not been assessed in guinea-pig muscle, but it is apparent that considerable interference in neuro-muscular transmission can exist in guinea-pig muscle before failure occurs.

At the end-plate the action potential is diminished in comparison with that recorded at a distance from the end-plate (Fatt and Katz, 1951). There is a <u>further</u> reduction at the end-plate in the presence of carbachol (Fig. 8.1B). A similar effect, in which the spike does not reach the zero level of potential, has been found in frog muscle treated with carbachol. This was shown by Nastuk and Gissen (1965), who did not attempt an explanation. The rising phase of the action potential is dependent on the inward sodium current, and the falling phase is attributed to sodium inactivation and to the delayed outward rectifying potassium current (Adrian, Chandler and Hodgkin, 1970). When the membrane potential has returned to normal there remains an increase in membrane conductance (Fig. 6.2) which would produce some further addition to the shunting effect during synaptic transmission and any increase in potassium conductance would decrease the overshoot (Hubbard et al, 1969). Recently Almers, Roberts and Ruff, (1983) have used the "loose-patch" voltage-clamp method to record membrane currents following step depolarization, and they have been able to measure separately the rapid inward and delayed outward currents. In human intercostal muscle the delayed outward current was small relative to that described for frog, so that this factor may be less

significant in mammalian muscle. The reduction of the action potential is considered again in connection with discussion of the reversal potential: if V_{rev} is -17.5 mV in muscles which have repolarized in the presence of carbachol, then the spike in Fig. 8.1B still becomes more positive than V_{rev}, although it does not cross the zero line.

Neuromuscular transmission is only temporarily restored and long-continued application of agonist leads to a secondary neuro-muscular block (Fig. 3.1, Fig. 3.3) with decline of epps (Fig. 8.3).

Labelled Decamethonium in Guinea-pig Muscle

In Fig. 7.2 the uptake of decamethonium in the junctional region extends laterally, with a Gaussian distribution which has a standard deviation of at least 1 mm. This is similar to rat muscle (Case et al., 1977). These authors have argued that the spread which is found in whole diaphragm cannot be attributed to a distribution within individual muscle fibres. Decamethonium becomes rapidly bound in the fibre and lateral diffusion was found to be negligible: and in any case autoradiography of single fibres has shown uptake for only a few hundred microns beyond the end-plate, compatible

with some extra-junctional receptors as shown electrically by Miledi (1962). The spread of radioactivity seen in whole diaphragm was attributed by Case et al. (1977) to the scatter of end-plates in a thick muscle, and a similar explanation would seem likely in the guinea-pig (Fig. 7.2).

The ratio of peak to end (Fig. 7.2) is much smaller in guinea-pig than in rat muscle, and is largely attributable to the large size of fibres in guinea-pig diaphragm (see Chapter 7), so that the number of end-plates per mg of muscle is considerably smaller in guinea-pig diaphragm.

In Table 7.1 the uptake in the central slices of muscle has been summed, and this is necessary if the scatter of end-plates extends over several mm. The continued uptake of labelled decamethonium (Fig. 7.3) gives some support to the view that end-plate channels continue to open with long-continued application of the drug, and the results can also be used, with some assumptions, to give an estimate of the current through the drug-induced channels. This depends on the finding that in rat muscle the uptake of labelled decamethonium and labelled sodium were related. When the rate of uptake was divided by the concentration the values for decamethonium and for sodium were similar (Fig.5, Creese et al., 1977). The units for (uptake rate)/concentration are p-mole mg^{-1} $time^{-1}$)/(p-mole l^{-1}), which is l. mg^{-1} $time^{-1}$ and is a rate of clearance (μl. $mg^{-1}hr^{-1}$ in Table 7.1).

If this can be extended to guinea-pig muscle the slope in Fig. 7.3 may be used to estimate the rate of entry of sodium. In Table 7.1 the rate of clearance is 0.320 μl. mg^{-1}

hr^{-1}, which is 88.9 pl. $mg^{-1}sec^{-1}$. The sodium content of the external solution is 0.145 p-mole pl^{-1} and there are 130 fibres mg^{-1}, so the rate of uptake of sodium is (88.9 x 0.145)/130 or 0.099 p-mole sec^{-1} end-$plate^{-1}$. If the faraday is taken as 10^5 coulombs $equiv^{-1}$ the sodium current comes to 10 nA end-$plate^{-1}$.

This estimate is probably not inconsistent with the value of 12 nA end-$plate^{-1}$ obtained by electrical measurements. The isotope and electrical methods are independent, and a serious discrepancy would have indicated some flaw in the interpretation. The greatest source of error is likely to be the figure adopted for the number of end-plates per mg muscle, which was obtained from the square of the representative fibre radius.

Reversal Potential and the Sodium Pump

Measurement of the reversal potential in skeletal muscle presents difficulties because micro-electrodes small enough for internal use may not pass sufficient current for adequate depolarization, and secondly because depolarization beyond the threshold may excite an action potential (this difficulty can be avoided by the use of tetrodotoxin). In addition nerve stimulation is liable to produce a number of artefacts which have been described by Fatt and Katz (1951), and some of these effects have been attributed to extracellular recordings from other fibres.

Studies on normal guinea-pig diaphragm at 37°C (without drugs)are summarised in Table 8.1 and the change in mepp on depolarization is shown in Fig. 8.5 and Fig. 8.6. The muscles were depolarized until the mepp changed sign, so that the reversal potential was found by interpolation. The plot of amplitude of the mepp against membrane potential was curved as expected (see Colquhoun et al., 1979, and later Discussion) and the curve was fitted by a method similar to that used by Mallart et al. (1976). The potential at which the mepp was zero was estimated as -3.6 mV (median of 6, limits -0.7, -10.9 mV), and these results are similar to those found in rat muscle by Glavinovic (1979), in mouse muscle by Linder and Quastel (1978), and in frog muscle (Katz and Miledi, 1977; Colquhoun et al., 1979).

Reversal potentials were also measured in muscles exposed to carbachol (80 μM), in which the membrane potential had recovered to the former value (as Fig. 4.1, Fig. 4.4). No mepps were present (Fig. 4.3) and the method was modified by recording the epp produced by nerve stimulation. The muscles were desensitized and small epps were obtained which did not usually reach the threshold for action potentials, so that neuromuscular block had been produced (eg Fig. 3.1, Fig. 3.3, Table 3.3). An example is shown in Fig. 8.7 and Fig. 8.8, with a summary in Table 8.2. The estimates of V_{rev} varied from -12.8 to -23.3 mV, with median -17.5 mV (6). These values are clearly very different from those of control muscles (Table 8.1, median -3.6 mV), and the two series do not overlap. The results in Table 8.2 (epps from desensitized

muscle) were obtained in a somewhat different manner from that used for controls (mepps, Table 8.1) and this weakens the conclusions which may be drawn. The epp might have been compared in both series if tubocurarine had been used in the controls. However,it is difficult to attribute the difference in results to the methods which were used. These pilot experiments require confirmation, but for the purpose of this Discussion the results in Table 8.2 are assumed to be valid, so that the reversal potential in guinea-pig muscle at 37°C in the presence of carbachol (80 μM), at a time when the resting potential has recovered, is considerably less positive than that in control muscles untreated by carbachol.

In normal muscle, the membrane voltage at which the end-plate potential change is reversed is accepted as a measure of the equilibrium potential (Hubbard et al., 1969, p209). In amphibian muscle there were claims that desensitization changed the equilibrium potential, but Katz and Miledi (1977) have shown conclusively that frog muscles desensitized with acetylcholine have the same reversal potential as normal controls.

If a change in reversal potential does occur in guinea-pig muscle exposed to the prolonged action of carbachol then this might be attributed to an alteration in ionic content or possibly to a change in the Na/K conductance ratio, although the concept that Na^+ and K^+ traverse separate channels at the end-plate has not been sustained (Dionne, 1979). A change in internal ionic composition probably occurs but this does not seem likely to change the equilibrium potential to the required extent. An early attempt to calculate the equilibrium potential was in terms of the "constant field"

equation, a version of which is shown below (at 37°C),

$$V = -61 \log_{10} \frac{K_o + b\ Na_o}{K_i + b\ Na_i} \qquad (3)$$

(see Rang, 1974, p290) where b is the permeability ratio P_{Na}/P_K which changes in the presence of agonist compounds, and V is in mV. Changes in ion content in the end-plate region following depolarization have not been adequately documented in any species but may be extensive (Zaimis and Head, 1976). In denervated guinea-pig the loss of internal potassium following decamethonium (1 μM) in vitro is as much as 30 mM, with subsequent restoration in the presence of the drug (Creese et al., 1983). A gain of sodium is also likely. Such changes in ionic content could produce some shift in the reversal potential, but the effect is probably small because the ratio P_K/P_{Na} is close to unity, probably about 1.2 in frog muscle treated with acetylcholine (Lewis and Stevens, 1979) and it is difficult to see how even large changes in cation concentration could give more than a few mV change in equilibrium potential. The derivation of the "constant field" equation requires independent movement of cations through end-plate channels, and in practice other models of ion permeation have had to be developed to explain the effects of ion concentrations on the equilibrium potential (Rang, 1974; Lewis and Stevens, 1979).

The sodium pump has been implicated as one factor in the spontaneous recovery of resting potential in the presence of depolarizing drugs (Creese and Mitchell, 1981; Creese et al., 1983) and the present results in guinea-pig muscle

give support to the concept. A simple circuit (in the absence of agonist drug) is shown in Fig. 9.2B. Here a non-neutral ionic pump acts as a constant current generator. If the pump were outwardly directed the current would re-enter through the conductance G and cause hyperpolarization i_p/G were i_p is the pump current, and any increase in membrane conductance would tend to reduce the hyperpolarization (Hubbard et al., 1969). Experiments such as that in Fig. 5.2 could give an estimate of pump current which might approach the maximum value. Here a muscle has been depolarized by agonist in potassium-free solution and the membrane potential has become steady. Replacement of potassium 4 mM (in the presence of agonist) produced a marked restoration of membrane potential. In Table 5.2 and Table 5.3 there are records of 8 experiments of this type (3 with decamethonium, 5 with carbachol) and the median restoration in voltage is 23 mV. If the input resistance in the desensitized muscle can be taken as 0.48 MΩ, then the pump current would be $(23 \times 10^{-3})/(0.48 \times 10^{6})$ or 48 nA (the estimate would be larger if the input resistance is smaller). This may be compared with values in canine Purkinje fibres where currents of up to 30 nA have been described following sodium loading and potassium activ ation (Gadsby, 1980, Fig. 4).

In Fig. 9.2C the circuit is shown for injection of current in the presence of agonist drug (pump absent), taken from Ginsborg (1967), and the arrangement can be used to determine the reversal potential. If external current is used to depolarize the end-plate to a pre-determined value V, then the driving force for current through the shunt is $(V - V_{tr})$.

If mepc's were then recorded (with a small additional conductance g produced by a quantum of transmitter) the shunt current would be $(V - V_{tr})\ g$. This would be zero when V equals V_{tr}, so that the reversal potential would equal the transmitter equilibrium potential (eg. Hubbard et al., 1969, p64; Ginsborg, 1967).

Fig. 9.2D shows a constant current generator plus a shunt. Such a circuit may be used to model the effect of the pump in diminishing the depolarization produced by an agonist (see below). The current generator would affect membrane potential, but would not in this form affect the measurement of the reversal potential. A pump associated with a variable resistor, which would then act as an additional battery, might however affect the voltage required for zero shunt current.

If indeed the reversal potential is more negative in the presence of carbachol, then this might partly explain the apparent lack of overshoot in the action potential in Fig. 8.1B. In experiments like that shown in Fig. 8.1A (no carbachol) the action potential goes to +12 mV (Chapter 8); the reversal potential is -3.6 mV (Table 8.1) and the overshoot is 15.6 mV more positive than V_{rev}. In Fig. 8.1B (with carbachol) the action potential goes to -9 mV but the V_{rev} is now -17.5 mV (Table 8.2), so there is still an overshoot of 8.5 mV more positive that V_{rev}, although the action potential is diminished as considered earlier.

Spontaneous Recovery from Depolarizing Drugs, Potassium-free Solution and Ouabain

The spontaneous recovery from depolarizing drugs (Fig. 4.1, Fig. 6.1) may be considered firstly as a process of desensitization, which has been interpreted in rat muscle in terms of diminished frequency of channel opening (Head, 1983). The results presented here indicate that another factor is involved, and there is evidence for a separate recovery process in guinea-pig muscle which is sensitive to external potassium and to ouabain and which has the characteristics of the electrogenic sodium-potassium pump. The suggestion that the sodium pump may counteract part of the depolarizing effect of agonists has been made before (Mooij, Evers and Ras, 1976; Mooij, 1976; Creese, Franklin and Mitchell, 1976; Bolton , 1973).

During depolarization by agonists there is loss of K^+, gain of Cl^- (Feltz and Jaoul, 1974), gain of Ca^{++} (Evans, 1974) and probably gain of Na^+. In denervated guinea-pig muscle there is complete restoration of potassium in the recovery period (Creese et al, 1983), and this would be compatible with the restoration of membrane potential. In rapid recovery the possibility of depletion of external potassium has been considered in some tissues (Geduldig , 1968; Gadsby and Cranefield, 1979; Gadsby, 1980) and an increase in conductance may occur (Geduldig , 1968; Fig. 2). In practice some degree of hyperpolarization accompanied recovery from carbachol 80 μM (Fig. 4.1, Fig. 4.4, Table 4.2, Table 4.5).

There are several problems in estimating electrogenic activity by pump inhibition, including (a) a decreased

membrane potential due to gradual loss of internal potassium, (b) the possible accumulation of potassium on the outside of the membrane itself in an unstirred layer (Adrian and Slayman, 1966; Glynn and Karlish, 1975; Gadsby, Niedergerke and Ogden, 1977), (c) changes in the permeability of the membrane to sodium and potassium secondary to the depolarization itself, and (d) opposition to depolarization by changes in chloride gradient, secondary to altered movement of positive ions (Jenkinson and Terrar, 1973).

There is an additional complication created when decreasing the external potassium concentration. Such a reduction has a direct and immediate effect on the electrochemical potential which, from the Goldman equation, will be in the direction of hyperpolarization. The effect of removal of potassium has been reported in this study as an increase in polarization of 15 mV from a resting value of -80 mV (Table 5.1, n = 10). Hence, any decrease in electrogenic pumping in the resting state is concealed by the membrane potential varying in a similar manner to a potassium electrode (Hodgkin and Horowicz, 1959). Bearing in mind this effect on the resting membrane potential, then the consequence of removal of external potassium during exposure to a depolarizing drug would depend on (a) whether or not the pump is electrogenic in such a situation, (b) the relationship between the change in external potassium concentration and the half-optimum external potassium concentration for pump activity, (c) the permeability of the membrane to potassium, and (d) the pre-existing potassium gradient.

The results showed that potassium-free solution in the absence of agonist produced marked hyperpolarization (Fig. 5.1), and hyperpolarization was also produced with K-free solution when recovery of resting potential had occurred (Fig. 5.3, Fig. 5.4). Spontaneous recovery was minimal in the absence of potassium (Fig. 5.2), although some degree of recovery was usually found (Table 5.2). Brown et al (1972) found that K-free solution did not completely prevent ganglionic after-hyperpolarization, and attributed this in part to an appreciable pericellular concentration maintained by a potassium leak from cells. This factor may have some relevance here.

During the process of spontaneous recovery, removal of potassium produced not hyperpolarization but either depolarization or a reduced rate of repolarization (Fig. 5.3 and Fig. 5.4). The hyperpolarizing effect of K-free solution due to a change in the potassium equilibrium potential should be more pronounced in a depolarized muscle. The striking effects in Fig. 5.3 and Fig. 5.4 are consistent with the concept of the action of the sodium pump, and give no support for increased potassium permeability as an alternative explanation of the spontaneous recovery process.

It was found that the reversal potential was -3.6 mV (6) in normal muscles and -17.5 mV (6) in muscles exposed to carbachol, and this high negative value in the presence of agonist might be attributed to the action of the sodium pump. If this were so then the prediction can be made that in solutions in which the sodium pump is inhibited, the depolarization produced by carbachol should be enhanced

(eqn. 2). This was indeed found in two sets of experiments. In normal solution the depolarization was 30 mV (25 results, Table 6.2), while in the presence of ouabain the value was 37 mV (6, Table 5.6), and in K-free solution the depolarization was 42 mV (from an initial value of -86.5 mV: 6 results, Table 5.3). Bolton (1973) has found similar results in smooth muscle.

The depolarization produced by carbachol (80 μM) in the presence and absence of ouabain can be compared to see whether the differences are explicable in terms of an altered reversal potential. In Table 6.2, with no ouabain, and V_{rev} taken as -17.5 mV, the term $\Delta V/(V - V_{rev})$, which is $g/(g + G)$, comes to 0.504 (25). In Table 5.6 (with ouabain), the term $\Delta V/(V - V_{rev})$ comes to 0.501 (6), so that the calculated conductance comes to a similar figure in both series if the reversal potential remains at the normal value when both ouabain and carbachol are present (Fig. 5.5).

The depolarizing current in the absence of the pump can be estimated from the results with ouabain. The assumption is made that Fig. 9.2A may be taken as the equivalent circuit and that the resistance R of the non-junctional membrane may be considered constant. Then the current is $\Delta V/R$, where ΔV is 37 mV and R is 0.53 MΩ (Table 6.3), so the current is 70 nA in experiments shown in Fig. 5.5 and Table 5.6. In the absence of ouabain, ΔV is 30 mV (Table 6.2) and R is 0.53 MΩ so the current is 57 nA, and if a pump is present the equivalent circuit is now Fig. 9.2D. The shunt current passes outward through R, while the pump current passes inwards through R , so the

net depolarizing current is reduced by 13 nA. If the pump current passes inward through both R and the shunt, then the total pump current could be greater than 13 nA, perhaps double if r and R (and g and G) are approximately equal at peak depolarization (see Table 6.2). The figure of 13 nA for the pump current is thus a minimal estimate.

In Fig. 5.5 there is no recovery in the presence of ouabain. This particular result is not inconsistent with an ouabain-sensitive pump, but can hardly be cited in support of the concept since there is a depolarizing action on control muscles (Fig. 5.6A). The use of ouabain in guinea-pig diaphragm is chiefly of value in experiments such as that of Fig. 5.5 in which ouabain modified the extent of the peak depolarization, at a time when ouabain had only been acting for one or two minutes.

The Use of Guinea-pig Diaphragm

Guinea-pig muscle produces a depolarization block with end-plate agonists, and in this it resembles human and cat muscle (see Zaimis and Head, 1976). In addition the fibres are large so that internal microelectrodes can be maintained for long periods. This has meant that repolarization can be demonstrated in the presence of agonist without repeated insertion of electrodes, and in favourable cases two electrodes can be retained until the resting potential has fully recovered.

Prolonged recording has produced evidence that some channels continue to open in the presence of depolarizing drugs. The end-plate current at a time when resting potential has recovered has been estimated electrically by the effects of withdrawal of drug, and also isotopically by the use of labelled decamethonium from which sodium influx can be inferred, and the results are in fair agreement (12 nA and 10 nA end-plate^{-1}).

At this stage the muscle is desensitized and the reversal potential can be explored in this preparation without the need for methods to prevent contraction. Measurement of reversal potential in the presence of carbachol led to an estimate of -17.5 mV, and this high value is unexpected.

The preparation of guinea-pig muscle can be used to show that an electrogenic pump is switched on at an early stage by the action of agonist drugs (presumably by sodium entry). The pump current can approach 50 nA when activated.

Guinea-pig diaphragm has proved a useful preparation for the study of agonist drugs in mammalian muscle at body temperature. Table 9.4 provides a summary of the values used in this preparation for calculation of the changes produced by carbachol (80 μM) at peak depolarization and at a time when recovery of resting potential has occurred.

Table 9.4 Summary of effect of carbachol (80 μM)

	V_{rev} (mV)	R_{in} (MΩ)	G (μS)	g/(g+G)	g/G	g (μS)	R'_{in}/R_{in}	Shunt Current (nA)	r_m (MΩ cm)	r_m^{-1} (μS cm^{-1})
Normal	-3.6	0.53	1.89	-----	-----	----	----	--	0.095	10.5
Peak Depolarization	-17.5	——	----	0.500	1.016	1.92	$(0.50)^{\frac{1}{2}}=0.71$	57	——	640.0
	-3.6	——	——	0.414	0.706	1.33	$(0.59)^{\frac{1}{2}}=0.77$		——	443.3
Spontaneous Recovery	-17.5	——	----	0.099	0.110	0.21	$(0.90)^{\frac{1}{2}}=0.95$	12	——	70.0
	-3.6	——	——	0.082	0.089	0.17	$(0.92)^{\frac{1}{2}}=0.96$		——	57.0

or -3.6 mV

V_{rev} has been taken as -17.5 mV at peak depolarization and at recovery (Table 8.2).

g/(g+G) was obtained from $\Delta V(V-V_{rev})$: see eqn. (2).

If g/(g+G) is f, g/G is $(f^{-1}-1)^{-1}$.

R'/R is taken as G/G' or 1-g/(g+G), and R'_{in}/R_{in} is $(R'_m/R_m)^{\frac{1}{2}}$.

r_m^{-1} in μS cm^{-1} is obtained from $(2\,R_{in}\lambda)^{-1}$ without drug, and as g/(end-plate length) in the presence of drug, with end-plate length taken as 30 μm or 0.003 cm, as in rat muscle.

The normal value for r_m in guinea-pig is similar to that for cat muscle (Boyd & Martin, 1959) and for frog muscle (Fatt & Katz, 1951).

Values for normal muscle are from Table 8.1 and Table 6.3.

Values at peak depolarization are from Table 6.2 and calculations on pp 227 & 244.

Values after recovery are from Table 4.3 and calculations on pp 229 & 230.

APPENDIX

Curve Fitting for Estimation of Reversal Potential

Mallart, Dreyer and Peper (1976) measured synaptic current in voltage clamped frog muscle following iontophoretic release of acetylcholine. They considered the synaptic current, I, to be proportional to the driving potential $(V-V_{rev})$ and also to an exponential function of the membrane potential, so

$$I = (V-V_{rev})\ C\ \exp(-AV) \qquad (4)$$

where A and C are constants.

In the experiments described in Chapter 8 the change in potential, ΔV, was recorded as mepps or epps, and eqn. (4) needs to be modified.

From the electrical model for transmitter action (Fig. 9.2A), eqn. (2) is obtained, which can be written as

$$(V-V_{rev})/\Delta V = (g+G)/g = 1+G/g$$

so

$$G/g = (V-V_{rev})/\Delta V-1 \text{ and } g/G = \Delta V/(V-V_{rev}-\Delta V)$$

The shunt current, I, is $g\ \Delta V$ or $G(\Delta V)^2/(V-V_{rev}-\Delta V)$ and I is also given by eqn. (4). Hence

$$G(\Delta V)^2/(V-V_{rev}-\Delta V)(V-V_{rev}) = C \exp(-AV)$$

and $\ln(\Delta V)^2/(V-V_{rev}-\Delta V)(V-V_{rev})$ plotted against V should be linear.

In practice, various values of V_{rev} were tried until the regression of the logarithmic term on V gave a minimum sum of squares. A desk calculator (Commodore S61) was used. The results of curve fitting are seen in Fig. 8.6 and Fig. 8.8. The initial calculations were made by Professor Creese.

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(with Additions)

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