STUDIES ON NEUROMUSCULAR TRANSMISSION AND THE EFFECTS OF LACK OF POTASSIUM

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submitted by

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Abstract

An investigation has been made of the effect of low extracellular potassium concentration on the functioning of the rat diaphragm phrenic nerve preparation, at 24°C.

Potassium free bathing solution causes an augmentation of the mechanical response of the muscle under conditions of indirect tetanic stimulation. A cyclic stimulation procedure is described consisting of alternate 1 Hz and 50 Hz stimulation. Using this procedure the augmentation of the tetanic response is particularly evident both in amplitude and maintenance of tension.

Investigations have also been made using intracellular recording techniques. Potassium free solution has been shown to cause an increase in the amplitude of the curare blocked end-plate potential greater than the increase which its action on the resting potential would predict. Removal of potassium has little action on the miniature end-plate potential amplitude recorded from the junction under resting conditions.

Experiments using paired stimuli have demonstrated a differential, amplitude enhancement of the second end-plate potential at intervals between 5 msec and 600 msec. The effect of potassium free solution was shown to be different from that of increased calcium concentration, and to reverse the depression of amplitude caused by this under these conditions.

By the use of short trains of stimuli under conditions of low potassium, an increase in the immediately available store of transmitter has been inferred. This increase was augmented in some cases by the addition of choline to the bathing solution.

The action of both d-Tubocurarine and Hemicholinium-3 on the mechanical response of the preparation has been shown to be similar, using the cyclic stimulation procedure, and opposite to that of potassium free solution.

An hypothesis is suggested linking the augmentations observed to an effect on the presynaptic store of transmitter in the nerve terminal caused by an increase of transmitter synthesis under potassium free conditions.

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INTRODUCTION

Chapter 1

Early Theories about Neuromuscular Transmission

The origin of speculation as to the manner in which nerve influences muscle and causes contraction may be traced back to the earliest writings on the subject of physiology. These we owe to Erasistratus who can be considered the first known Physiologist. Working in Alexandria in about 300 B.C. he observed that a muscle is entered by three vessels, a vein, an artery and a nerve. These vessels were seen to branch repeatedly and it was therefore logically assumed that they continued to divide beyond the limit of vision and so formed the tissue of the muscle. The nerves themselves he thought to be hollow tubes filled with a pneuma or spirit, and his theory suggested that the muscle was inflated by this fluid which flowed down the nerve and caused the muscle to contract by distension.

The work of the Alexandrian school is known through Galen, a Greek practising medicine in Rome in the second century A.D. He reviewed the earlier work and tried to refute some of the errors. He asserted that both arteries and veins contain blood, but aparently considered the old ideas about nervous fluids satisfactory. He believed that the blood containing natural spirits travelled to the heart where vital spirits were produced and then to the base of the brain where they were changed to animal spirits. It was these that flowed down the nerves and caused the distension of the muscles. Although based on no experimental evidence, the writings of Galen were authoritative and were accepted without question until well into the seventeenth century.

Thus, the first ideas about neuromuscular transmission were to remain for 1500 years. Even the great anatomists, Vesalius and Leonardo da Vinci, show in their drawings that they believed the tendon of a muscle to be a continuation of the nerve. The publication of De Magnette in 1600 by William Gilbert reflected the changing attitude towards investigations. His work is symptomatic of a new era of planned experiment and the overthrow of the ancient doctrines. After Harvey, in 1628, had shown Galen capable of error in his theories on circulation, investigation began into other accepted ideas in physiology.

The first investigations into the validity of the animal spirit hypothesis were made by Borelli. He was one member of a group of nine experimental scientists working in Florence. The work of this small group was based entirely on experiment and had far reaching effects on the rest of European science when their proceedings, published in 1667, became known. Borelli had cut the muscles of a struggling animal under water and seen that no gas emerged. He therefore concluded that the nervous medium must be a liquid. His concept of contraction was that of an explosive fermentation within the muscle, causing it to swell but still initiated by the nervous liquid.

At the same time as Borelli was experimenting on muscle, the philosopher Rene Descartes formulated his own theory as to the actions of the nervous system. He maintained that the pineal gland was the seat of the soul, and as such directed the nervous energy which he still considered to be like a "wind or very subtle flame", into the muscle, which then swelled up like a balloon. Similar ideas were held by William Croone, one of the founders of the Royal Society. His view was of the muscle as a series of small bladders in which the nervous

liquid mixed with the muscle juice and caused it to swell, "like a bladder blown up".

It was not until very late in the seventeenth century that any experiments were performed which cast doubt on the accepted theories. Francis Glisson demonstrated by immersing an arm in water, that the volume decreased on contraction. Swammerdam, in Holland, obtained a much more convincing result. He placed an isolated frog nerve-muscle preparation in a container filled with water, with a capilliary tube attached. The muscle was stimulated via the nerve and thus he demonstrated that there was no change in muscle volume on contraction.

In the eighteenth century a great deal of evidence accumulated against the animal spirit theory in general. The Swiss physiologist, Albrecht Von Haller, a pupil of Boerhaave, maintained that muscles could contract independently of nervous action. Muscles were not continuous with arteries and veins, and since their nerves were few it seemed unlikely that so many muscle fibres could arise from them. He emphasised that the muscles were not small bladders as suggested by Croone, but still spoke of a nervous liquid stimulating the muscle and forcing the elementary particles of it together. As he had shown that nerves do not swell when ligated, nor leak when cut, the liquid, he maintained, must be not the usual sort.

Toward the end of the eighteenth century there did degin to arise the possibility of electricity being a biological agent. However, the entire unsuitability of tissues as conductors or insulators caused this fact to escape Von Haller. It was not until after his death at the end of the eighteenth century, that Galvani began to experiment with frogs legs and gave rise to the possibility of animal electricity.

The experiments were attacked by Volta, also working on electrical effects and a controversy started that was to lead to the development of the bi-metallic battery by Volta, and eventually the observation of the action potential by Du Bois Reymond.

Galvani had attached frog's legs by brass hooks to an iron ballustrade and thought that the contraction observed was due to conduction of animal electricity through the circuit. Volta maintained that the brass iron junction generated sufficient electricity to stimulate the muscles. However, Galvani later showed that when the cut end of a frog's spine was placed in contact with a muscle, contraction occurred. Thus, the effect occurred in the absence of any metals.

This fact was developed by the Italian, Matteucci. His experiments demonstrated that a current could be measured between the cut end of a muscle and its undamaged part. This injury current could be measured using a very crude galvanometer. The experiments were repeated in Berlin by Du Bois Reymond who managed to measure a drop in the injury current during tetanic stimulation. He called this the negative variation and went on to show the same thing in nerve. He thus discovered the action potential that Matteucci had suspected but failed to find, due to his less sensitive galvanometer.

With the development of the microscope a better idea of the structure of muscle became known. Bowman in 1840 showed that nerves are attached to muscles at specified places or end-plates, and it was only in these areas that muscle contraction began. It is therefore from this date that the true history of neuromuscular transmission begins, for before this time the nerve and muscle had been considered a single unit.

In 1862 Kuhne a pupil of Bruche published a paper on the end-organ of motor nerves. He noted the histological difference between nerve and muscle and suggested that the action currents of the nerve flowed over to the muscle and caused it to contract.

Du Bois Reymond noted a delay at the junction and even at this early stage considered the possibility of a chemical influence. This he thought to be ammonia and lactic acid, shown to be contained in muscle by Leibig in 1849. However, he went on to postulate an electrical theory of transmission and went as far as drawing a diagram of the possible field set up from the nerve to the muscle showing the way in which an end-plate might act electrically.

This theory was developed by Du Bois Reymond's pupil Bernstein, and as a result an electrical hypothesis of nerve and muscle action came fully into being. Bernstein showed that the nerve and muscle membranes were polarised, having negative ions on the inside and positive on the outside. He further stated that the action potential was self propagated and due to a conducted depolarisation. This was based on the assumption that the membrane is selectively permeable to potassium ions. His explanation of the injury current was that it was due to a break in the membrane and a leakage of ions.

It was noted by Kühne in 1888 that the nerve could only excite the muscle when connected to it at the end-plate. He explained this by postulating an increased effectiveness of the action potential produced by the end-plate at that region of muscle fibre.

Concurrently with the development of the electrical hypothesis of nervous action came evidence that chemical agents would also directly affect the nerve-muscle system. The first of these shown to have a

definite effect was the South American arrow poison, curare. This was first brought to Europe by Sir Walter Raleigh from Guyana in 1595. It had been experimented with by various people including Brodie and Von Humboldt, but the first person to make a careful systematic study of its action was Claude Bernard. (Claude Bernard 1878, Foster 1899). He started working with the drug in 1845 and made a short communication to the Académie de Sciences and to the Société de Biologie in 1850. He simply stated that the drug was not absorbed through the alimentary canal but abolished reflex actions if injected directly into the blood stream. From then until 1856 he published nothing more about curare. Only then was he forced to publish his work due to a publication by Kölliker stating the same general results as he had already obtained. These were, that the main effects of curare were on the motor nerve, thus injected curare prevented contraction of muscle by block of the motor nerve near to the muscle, leaving the muscle and all the sensory nerves untouched.

Towards the end of the century a second drug, nicotine, was also found to affect neuromuscular transmission (Heidenhain 1883, Langley 1905, 1908, Edmunds and Roth 1908). It caused the stimulation of skeletal muscle and this contraction was in turn prevented by the action of curare. The site of action, therefore, could only be the muscle membrane. The nerve muscle junction was thus established as a drug-receptor system and became a pharmacological entity.

Elliot (1905) suggested on the basis of the very close similarity between the normal action of the sympathetic nerves on smooth muscle and the action of the drug adrenaline that the nerve ends might release an adrenaline-like substance, which would act on the cells and modify their activity.

Langley applied the same ideas to skeletal muscle. He used curare and nicotine in a series of experiments which demonstrated that the receptor substance was directly beneath the motor nerve terminations. (Langley 1905, 1907, 1909, 1914). It followed from these experiments that the nerve too must excite the muscle through the agency of this same receptor substance.

Further proof of the hypothesis was provided by Otto Loewi. In a long series of superb experiments (1921, 1926), he showed that the nerve could have an action at a site removed from its own immediate vicinity. This 'action at a distance' was demonstrated by the slowing of a frog heart when perfused with effluent from a heart, the vagus nerve of which was being stimulated.

To the chemical mediator thus shown to be acting, he gave the name 'vagus stoffe', much later this was shown to be acetylcholine. (Feldberg and Krayer 1933). In other experiments when using atropinised hearts, Loewi found that the actions of the heart could be augmented due to some accelerator substance liberated when stimulating the accelerator nerve.

Further proof of what was now being termed chemical transmission was obtained when an adrenaline-like substance was obtained, from sympathetic nerve stimulation, which also stimulated the heart. (Cannon & Bacq 1931, Cannon & Rosenblueth 1933).

Reid Hunt and Taveau (1906) had described the properties of acetylcholine as early as 1906 and shown it to be 100,000 times more effective than choline on the cardio-vascular system. Dale (1914) classified the pharmacological actions of the choline esters and divided the powerful effect of acetylcholine into 'muscarine-like' or 'nicotine-like', depending on the cells involved. The diverse

actions suggested to Dale that it might have some function similar to, but opposite in effect to that of adrenaline. At that time however there was no evidence for the existence of acetylcholine in the body. It was fifteen years later that Dale and Dudley (1929) isolated acetylcholine in reasonable amounts from ox spleen, and proved its physiological existence.

In a long series of experiments Dale and his co-workers extended the hypothesis of chemical transmission to the neuro-muscular junction and eventually Dale, Feldberg & Vogt (1936) showed that acetylcholine was present in the eserinised perfusate of stimulated skeletal muscle. Thus it was shown that the transmitter which had been postulated by Langley as early as 1909 was probably acetylcholine. These experiments were followed by others in which acetylcholine was injected into the artery close to the point of entry into the muscle. This technique of 'close arterial injection' was used to mimic as near as possible the arrival of acetylcholine from the nerve. Injection of acetylcholine $^{(1-5ug)}$ in this way gave rise to small twitches resembling the nerve produced twitch but slower in time course. However, allowing for the problem of the slower access to the muscle by the drug there were striking similarities of action. (Brown, Dale & Feldberg 1936). Later this twitch was in fact shown to be a short tetanus.

The effects of anticholinesterase agents were also shown to be consistent with the theory that these allowed accumulation of acetylcholine in the normal course of activity. (Bacq & Brown 1937).

Dale and his colleagues believed that they had assembled sufficient evidence to establish acetylcholine as the transmitter

at the neuromuscular junction.

There were however still rumblings of the old electrical hypothesis. Lapicque (1936), after considering the strength duration parameters of nerve and muscle concluded that the end-plate caused the nerve to be so uniquely matched to the muscle that the action potential would flow across. He concluded that substances such as curare modified the muscle membrane stimulus parameters and prevented it from responding. Other investigators, particularly Eccles, were unwilling to accept this primitive idea of transmission and suggested that the effects of acetylcholine were purely pharmacological and unrelated to the physiological processes normally occurring, which were electrical in nature. This hypothesis was not to last in the face of overwhelming evidence in favour of chemical transmission and the last defence of the electrical theory, still essentially the same as that put forward by Du Bois Reymond in 1877, was made by Eccles (1946).

At the time, the mechanism by which the chemical transmission caused muscle action potentials was unknown. Some light was brought to the subject by Göpfert & Shaffer (1938). They found that a local potential could be recorded from curarised muscle at the nerve-muscle junction. After nerve stimulation a rapid negative deflection and slower return to the initial potential could be recorded with external electrodes. This came to be known as the end-plate potential (e.p.p.) and was extensively investigated physiologically and pharmacologically. Successive reduction was found with increasing doses of curare, and a prolongation, with drugs interfering with acetylcholine breakdown such as eserine. This work indicated that the e.p.p. was due to the release of acetylcholine and if of sufficient size could elicit a muscle action potential. (Eccles, Katz & Kuffler 1941, 1942). (Eccles & MacFarlane 1949).

Final proof of the chemical theory for neuromuscular transmission could only come with final disproof of the electrical one. After the introduction of the micro-pipette and intracellular recording by Ling and Gerrard (1949) it became possible to make an accurate estimate of the charge transference across the membrane during the action of the e.p.p.

In a paper which now marks the start of modern knowledge of the mechanism and of the neuromuscular junction, Fatt & Katz (1951) analysed the events using intracellular electrodes. They showed that stimulation of the nerve produced a change at the muscle end-plate which could not be mimicked by passing current into the muscle fibre itself. The change was a short circuit of the end-plate membrane which caused a depolarisation of the muscle fibre and the propagated action potential. By analysis of the cable properties of the nerve and muscle they proved that the nerve could not possibly excite the muscle directly. Thus the difficulty Kühne had forseen in 1888 eventually led to the final downfall of the electrical hypothesis and the acceptance of a chemical transmission system.

Present day work is concerned with the clearer elucidation of the detail of the chemical transmission processes themselves for that is the current theory based as it is on clear experimental evidence and acceptable to us, as were the theories of our forebears acceptable to them.

Chapter II

Current Concepts of Neuromuscular Transmission

The work drawn upon to substantiate the present theory of neuromuscular function comes from a variety of different preparations. The most usual tissues for the study of neuromuscular phenomena are the frog sartorius muscle and the rat diaphragm phrenic nerve preparation. Other preparations include human intercostal muscle, crustacean claw muscle, cat tenuissimus, guinea pig seratus, and also a number of nerve-nerve synapses such as in superior cervical ganglia and the squid giant synapse. Each preparation has its own merit and as the results obtained seem remarkably comparable there appears to be little against this practice. However there are species differences and care must be exercised when generalising theories resulting from work carried out with one particular junction.

In 1952 due to the increasing use of intracellular recording and the development of better electronic circuitry, a new and subsequently important phenomena became known. Fatt & Katz (1952) whilst recording from the junctional area in frog muscle noticed some small spontaneously occurring noise-like signals. The similarity of these in all respects except amplitude to the generated e.p.p. led them to be called miniature end-plate potentials (m.e.p.p.). The same potentials were also shown to exist both in rat and cat. (Liley 1956, Boyd & Martin 1956). Subsequent investigation has shown them to posess all the characteristics of small packets of acetylcholine released spontaneously from the nerve terminal. If these effects were due to molecules of acetylcholine affecting reception sites, the effect of curare should

be to block statistically, one molecule at a time. The fact that the process is a slow decrease in the amplitude on application of curare suggests a multi-molecular make up. The small packets were shown to be identical in size with those that would make up the evoked e.p.p. if this were quantal in nature. (Castillo & Katz (1954) for frog, Liley (1956a) for rat). The idea was put forward that the occurrence of the e.p.p. was due to a transient increase by the nerve spike of what is normally a slow spontaneous release of quantal acetylcholine. This view led to a great deal of investigation into the properties of the e.p.p. and the study of similar potentials at other synaptic junctions.

At the same time as improvements in technique led to the discovery of the m.e.p.p., improvements in electron microscopy led to a better understanding of the structure of the myo-neural junction. It was characterised as having a synaptic cleft dividing the post-synaptic receptor structure from the presynaptic nerve ending. The nerve ending was seen to have abundant numbers of mitochondria and numerous vesicles of approximately 500 Å diameter. (Robertis & Bennett 1955, Robertson 1956, Birks, Huxley & Katz 1960). The occurrence of these vesicles was found to be a characteristic of all synapses that were considered to be chemically transmitting. The obvious conclusion therefore was to equate the vesicles with a quantal store of acetylcholine in the nerve terminal. (Robertis & Bennett 1955, Castillo & Katz 1955, 1956). The results of nerve degeneration experiments (Birks, Katz & Miledi 1960, Miledi & Slater 1968) appeared to show that m.e.p.p.s and vesicles disappeared at the same time. In addition, Hubbard & Kwanbunbumpen (1968) have shown alteration in vesicle numbers and distribution within the nerve terminal after changes in the extracellular potassium concentration which is known

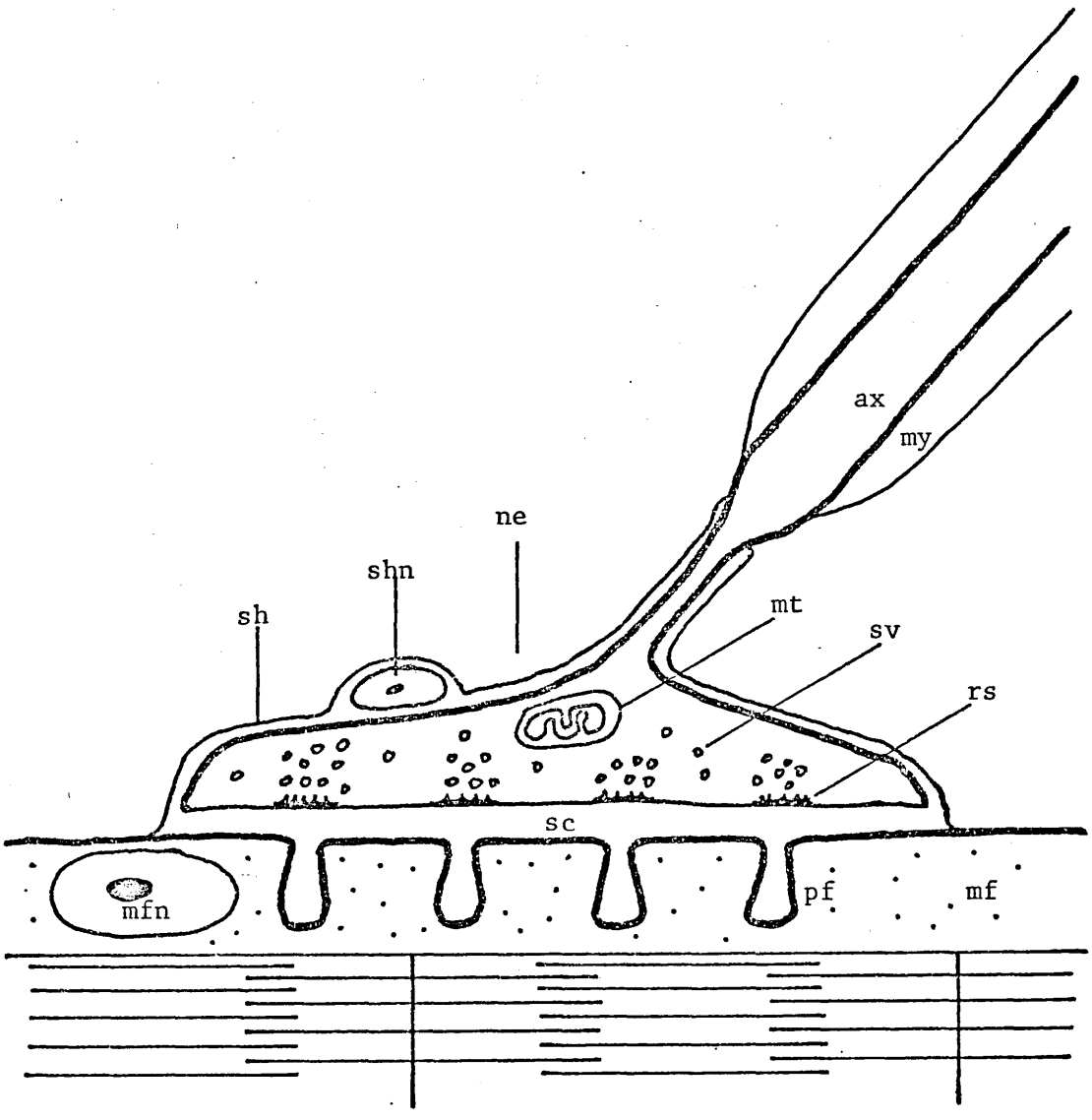


Fig. 2.1 Schematic diagram of the ultra-structure of a typical neuromuscular junction.

ax. axon, my. myelin sheath, ne. nerve ending, sh. schwann cell, shn. schwann cell nucleus, mt. mitochondrion, sv. synaptic vesicle, sc. synaptic cleft, pf. post-junctional fold of muscle membrane, mf. muscle fibre, mfn. muscle fibre nucleus.

to affect m.e.p.p. discharge frequency.

Fig. 2.1 is a diagramatic illustration of the structure of the neuromuscular junction as seen with the aid of the electron microscope. It illustrates the structural points described in this and the following chapter.

The normal e.p.p. is typically a depolarisation of 25-30 mV with a threshold for activation of the muscle fibre of about 10-15 mV. For efficient study of the properties of the e.p.p. with intracellular micro-electrodes it is desirable that the muscle should remain in a relaxed state. Any movement of the muscle will cause damage to the impaled fibre and changes in the electrical 'base line' of the record. A normal contraction of the muscle fibre when internal recording is in progress immediately breaks the micro-pipette tip, releases potassium and ruins the junction concerned. The ways of avoiding this problem are, (a) reducing the amount of transmitter liberated, (b) reducing the effect of the transmitter, and (c) preventing the muscle fibre from responding.

Dealing with possibility (c) first, this usually involves damage to some part of the system. It is possible to stretch the muscle and pin it so that it moves less when stimulated. This unfortunately causes a deformation of the end-plate region inevitably changing the characteristics of the junction. Another method is to cut the muscle fibres, this lowers the resting potential and prevents the action potential from propagating along the muscle fibre and initiating contraction. (Barstad, 1962).

It is possible to treat the muscle with hyperosmotic solutions and damage the contractile mechanism or uncouple it. Soaking in 600 mM glycerol solutions then returning to normal

osmolarity achieves this. (Gage & Eisenberg 1967, Dulhunty & Gage 1973). Some work has been done on frog myoneural junction using this method when the m.e.p.p. and the e.p.p./action potential complex can be recorded without muscle movement. It is not known yet what damage this drastic treatment does to the overall storing and releasing system for acetylcholine.

The second possibility, (b), involves the use of pharmacological agents which interfere with the receptor mechanism for acetylcholine. The classical drug for this purpose is curare which has been known since the time of Claude Bernard. Careful use of curare depresses the e.p.p. just sufficiently to prevent excitation. This leaves a smaller sub-threshold e.p.p. the properties of which may be investigated. The main disadvantage is that due to the nature of the blocking action the me.p.p.s are obviously depressed to extinction, making quantal content determination extremely difficult to any great accuracy. The advantage of this method is that since the work of Dale, Feldberg & Vogt (1936) curare has been thought to have a purely postsynaptic action and therefore no action on the releasing process. Recently however evidence has accumulated, (Lilleheil & Naess 1961, Hubbard & Wilson 1973), that there may be some presynaptic element of curare action. This appears to only be a problem during high rates of stimulation and may not affect experiments where the release rate of transmitter is low.

Reducing the amount of transmitter is the first possibility mentioned, (a). This is easily acomplished by changes made to the calcium and magnesium concentrations in the bathing fluid. Investigations of the effects of changing these ions have led to a much greater understanding of the physico-chemical nature of the releasing process itself.

By raising the magnesium and lowering the calcium, the quantal release is depressed and the size of the e.p.p. is seen to fluctuate. The fluctuations are of a discrete step-wise nature and can be shown to be identical in size with the m.e.p.p. (Boyd & Martin 1956b, Castillo & Katz 1954).

A random response such as occurs with the m.e.p.p. at the neuromuscular junction, if it comes from a large population of releasable units each having only a small probability of release, should obey the statistical predictions made from Poisson's theorem. If the mean number of quanta released is represented by m, then the probability, P, of observing any given number of quanta, x, is given by:

$$P_x = \frac{m^x}{x!} e^{-m} \qquad \ldots\ldots (1)$$

For small e.p.p. (Martin 1955) m is given by:

$$m = \frac{\text{mean response}}{\text{mean spontaneous response}} \qquad \ldots\ldots (2)$$

If a sufficiently large number of responses are recorded then agreement should be found between the observed numbers of quanta making up the e.p.p. 0,1,2,3 etc. and the number predicted from the formula.

This has been checked for frog (Castillo & Katz 1954), for cat (Boyd & Martin 1956b) and for rat (Liley 1956b), and a good agreement has been found in all cases.

A second prediction using the failure rate of responses and the overall number of stimulations can also be used to predict the value of m. This involves no measurement of amplitude and for a

large number of events is an accurate and satisfactory method.

$$m = \log \frac{\text{No. Impulses}}{\text{No. Failures}} \qquad \ldots\ldots (3)$$

Values for m calculated in these two ways are found to agree closely.

Thus, the hypothesis that the m.e.p.p. represents the quanta of transmitter output which makes up the evoked e.p.p. and that its size remains unchanged during release appears to be correct.

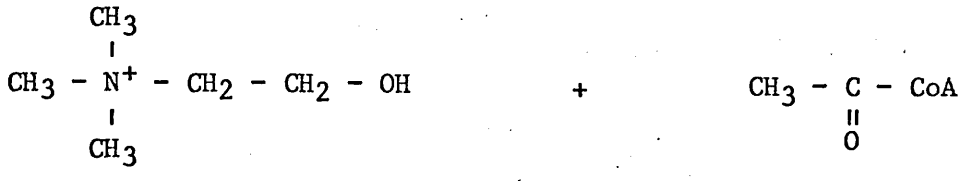
A statistical analysis such as the above is obviously dependent on the basic assumptions of the Poisson Theorem. One of these is that the amount available for release is large in relation to that liberated at any time. It is pertinent then to consider the storage of acetylcholine in the nerve terminal and its relation to the release mechanism.

Chapter III

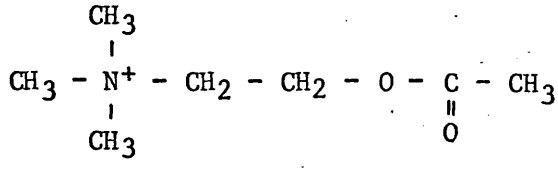
Physiology of the Neuromuscular Junction

Synthesis and Storage of Acetylcholine

Acetylcholine is synthesised from a combination of choline and acetate utilising acetyl-CoA as the acetate carrier and the enzyme choline-o-acetyl transferase (choline acetylase) to catalyze the reaction. The chemical structures of choline and acetylcholine are:



Choline + Acetyl CoA



Acetylcholine

The amount of choline located in the nerve terminal is small (Potter 1970) and sufficient for only 8-9% of the total acetylcholine store. Most of the choline therefore must come in from the extracellular fluid (Collier & MacIntosh 1969) transported in by a mechanism which may be inhibited by the drug Hemicholinium-3. (Chang & Rand 1960, Seakens & Stoll 1965, Chang & Lee 1970, Potter 1970). It appears that following an impulse at lease 50% of the choline from the liberated transmitter is reabsorbed back into the nerve terminal (Potter 1970),

and that this system for obtaining choline is effective enough to keep pace with release rates of 20 Hz for 5 minutes with no change in output. (Collier & Katz 1974). The muscle also has a similar system and so probably mops up the remaining choline. (Adamic 1970, Chang & Lee 1970).

The total amount of acetylcholine in nerve terminals can be considered to be divided up into a number of compartments. The evidence for this comes from a variety of biochemical and electrophysiological procedures rather than from morphological divisions.

The acetylcholine may be obtained from the nerve terminal in a number of ways. Stimulation via the nerve is the physiological method and produces no damage. Raised extracellular K+ has a graded effect and is similar in effect to the nerve action potential. Assay of the released transmitter may be chemical, pharmacological or electrophysiological using the e.p.p. or m.e.p.p. Less physiological is the process of grinding up of the nerve terminals and assaying either the vesicular fraction or the whole acetylcholine.

From these experiments the stores or compartments of acetylcholine storage have been postulated. It must be emphasised that as yet there is no real morphological correlation for this classification of stores but on the basis of acetylcholine release data it forms a useful hypothesis from which investigations may proceed.

To obtain any valid data about the stores of acetylcholine it is necessary to interfere with the synthesis of acetylcholine during the release procedures. This is usually achieved by the use of one of the hemicholinium group of drugs. Hemicholinium No. 3 (-dimethyl ethanolamine 4, 4' biacetophenonone) (HC3) (Scheuler 1955) is known to

block actylcholine synthesis in intact neurones but not in homogenates. The drug therefore would appear to interfere with the choline uptake mechanism rather than with the choline acetylase enzyme. (MacIntosh, Birks & Sastry 1956, Scheuler 1960, MacIntosh 1961). At the mammalian nerve-muscle synapse it was found that small doses (2×10^{-6} gm/ml) caused a run down of e.p.p. and m.e.p.p. amplitudes during prolonged stimulation. (Elmqvist et al 1963, 1964, Elmqvist & Quastel 1965). Higher doses (4×10^{-4} gm/ml) have a post-synaptic curare like effect. (Martin & Orkand 1961a, b, Brooks & Thies 1961).

In a junction not treated with HC3, at physiological rates of stimulation, the amount of stored acetylcholine remains constant. Thus, under normal working conditions synthesis can keep pace with release. In the presence of HC3 the acetylcholine appearing is not replaced, indicating that supression of choline uptake effectively interferes with synthesis (Potter 1970). However, when cessation of release indicated exhaustion, assay of the nerve endings showed that approximately 20% of the initial store size was still remaining, i.e. was not releasable. This fact gave rise to the idea of the two compartments, releasable and stationary acetylcholine. As all the acetylcholine can be shown by radioisotope labelling to be resynthesised during prolonged incubation (Potter 1970) the two stores must be in communication.

The total releasable store may be estimated by summing all the e.p.p.s and m.e.p.p.s after blocking of acetylcholine synthesis. Such measurements seem independent of the frequency at which they are obtained and so tend to suggest a complete block of acetylcholine synthesis. Elmqvist and Quastel have obtained values of 270,000 $\pm$ 70,000 initial quanta at a typical mammalian neuromuscular junction. (Elmqvist et al 1964, Elmqvist & Quastel 1965).

The number of vesicles estimated from electron microscope studies is 3×10^{-5} for frog junctions (Birks, Huxley & Katz 1960) which corresponds well with a value of 2×10^{-5} quanta in similar junctions. (Chen & Lee 1970, Longenecker et al 1970). Electron micrographs fixed at the time of release show changes in vesicle population (Chen & Lee 1970, Clark et al 1970, Clark, Hurlbut and Mauro 1972, Quilliam & Tamarind 1974). It may therefore be assumed circumstantially that the vesicles form part of the releasable store existing in a quantal form.

The number of quanta released during an impulse has been determined for various junctions and is about 100 - 300 (Martin 1955, Liley 1956b, Boyd & Martin 1956, Takeuchi & Takeuchi 1960, Elmqvist and Quastel 1965). This amount is small in comparison to the overall transmitter store. There is evidence to suggest however that not all the store is available for release at one time. Collier (1969) and Potter (1970) found that newly synthesised transmitter was released preferentially. Secondly at high rates of stimulation the quantal content of the first few e.p.p.s falls until a plateau level is reached which remains constant. A similar line of reasoning comes from observations made on pairs of impulses (Liley & North 1953, Lundberg & Quilish 1953a,b, Brooks & Thies 1962, Thies 1965, Elmqvist & Quastel 1965). The second e.p.p. of a pair is reduced in amplitude for periods of up to 10 seconds after the first, suggesting that depletion by the first e.p.p. causes a relatively long-lasting effect on the store, which affects the release of the quanta responsible for the second e.p.p.

Birks & MacIntosh (1961) found a similar effect in sympathetic ganglia and could not attribute it to either acetylcholine synthesis or frequency of stimulation. They suggested that a smaller store in

series with the main store, and filled from it, would have the same characteristics. These results also gave rise to the concept that a nerve impulse releases a constant fraction of the transmitter available for release. This is considered to be related to the probability of release of the quanta from the available store.

Values for the available or readily releasable store are given at about 300 - 1000 quantal units. (Elmqvist & Quastel 1965). A possible morphological correlate for this is the layer of vesicles found in E.M. sections close to or associated with the membrane.

In nerve terminals that have been treated with eserine and assayed for acetylcholine it is found that the level of acetylcholine has approximately doubled compared to that in normal endings. (Potter 1970). This extra acetylcholine exists in the cytoplasm probably as an overflow from synthesis and normally would be hydrolysed by the cholinesterase present and therefore does not contribute to release. With eserine present the output under prolonged stimulation is better maintained suggesting that the extra cytoplasmic acetylcholine can contribute in some manner to the releasable fraction. (Collier & Katz 1971).

For the purpose of investigating short term effects on transmitter release the most important compartment is the releasable fraction and its subdivisions. In the case of a single e.p.p. it is assumed that a constant small fraction, slightly varying with statistical changes in quantal content, is released each time. This will have little effect on the size of the immediately available store that will be replaced at the same time. Ca++ and Mg++ ions affect the number of quanta released and clearly have an effect on the fractional release and on the probability of a single quanta being

released. In the case of a train of e.p.p.s it has been found that the first few fall linearly in amplitude but are then maintained at a smaller size, until after prolonged stimulation they are seen to be smaller than the first amplitude plateau reached, due to a slower decline (Lundberg & Quilish 1953, Liley & North 1953). The plateau level is considered to be a reflection of mobilisation of the transmitter into the available store. The initial fall is then due to a depletion of this store down to the size that the replenishment rate can keep constant. The rate of the initial fall is a guide to the fractional release of the store with each e.p.p.

So far it has been assumed that the release occurs from the vesicles. This could occur in three ways: (1) the vesicles could be released into the synaptic cleft and discharge their contents by lysis. This is unlikely as no vesicles or debris have been observed in the cleft under the electron microscope, (2) the vesicles could discharge into the cytoplasm and the transmitter then difuse out. Recently Tauc et al (1974) have put forward evidence in favour of this theory. After injecting acetylcholine esterase in to the nerve ending, transmission was abolished. Vesicular acetylcholine should be protected from this procedure by the vesicle membrane. From this evidence the conclusion drawn is that acetylcholine must be free in the cytoplasm and open to attack by the injected enzyme.

(3) The vesicles could fuse with the nerve membrane at some point and then release their contents either directly or through a low resistance pore made in the membrane by fusion. Evidence has accumulated in favour of this from electron microscopic studies using horseradish peroxidase. Molecules such as this and ferritin or thorotrast are electron dense. After placing the substance in the

external solution close to the nerve ending and stimulating, it was found that the molecules were taken up into the vesicles within the nerve ending. Using this method a complete cycle of activity of vesicle structure has been worked out (Heuser & Reese 1973).

Release of Acetylcholine

The initial stimulus for the release of acetylcholine (whether it may come from the vesicles or from the cytoplasm) is the arrival of the nerve impulse at the terminal. The electrical change in the nerve ending resulting from this event is thought to cause a change in the permeability characteristics of the membrane to calcium and possibly other ions.

Evidence for the importance of calcium in the release process comes from a variety of sources. del Castillo and Katz (1954) found that removal of calcium had little effect on spontaneous m.e.p.p.s but reduced the quantal content of evoked e.p.p.s. Even when using the calcium chelating agent EGTA the m.e.p.p.s are not completely abolished. This discrepancy led to two mechanisms of release being postulated, a calcium dependent evoked release and a calcium independent spontaneous release.

More recently evidence has accumulated that the intracellular level of calcium is more important in release (Hubbard 1970, Baker 1972). This leads to the theory that spontaneous release it due to activation by the small intracellular calcium concentration and that evoked release is due to an increase in this during activation of calcium channels by the nerve impulse.

Calcium is found to bind extensively to proteins within the cell. Studies on this are usually carried out using the protein

aequorin. This is a calcium sensitive substance and emits light in response to small changes in ionised calcium at low calcium levels. Using this protein the ionised calcium in squid axon is found to be approximately 0.3 μM compared to about 400 μM total calcium. Thus such a low value will produce a high electro-chemical gradient directed into the cell and a small amount entering will cause a transient large change in the intracellular active calcium.

The mitochondria appear to provide a buffer system for this increase in calcium in the short term but the excess calcium must be removed for any long term process to remain stable. This is effected by means of calcium pumping sites in the membrane. Calcium pumping sites are known to exist in red cell membranes and in some excitable cell membranes including gut. The requirements appear to be sodium and/or potassium gradients and ATP. In squid nerve axon changes in the sodium gradient appears to affect intracellular calcium levels (Baker 1974). Replacement of sodium by lithium or choline leads to a reduction in calcium efflux.

Using the aequorin technique various factors can be shown to affect the intracellular calcium:-

a) a rise in external calcium

b) a fall in external sodium

c) a rise in internal sodium

d) inhibition of mitochondrial calcium accumulation.

All these conditions also affect the neuro-secretory processes. In the squid giant synapse the pre and post synaptic structures are large enough to allow the insertion of micro-electrodes. Katz and Miledi (1967) using this preparation were able to control the depolarisation of the terminal and measure the results. It was found that at a certain potential value (+130 mV) internal depolarisation

causes no release of transmitter. The calcium equilibrium potential of squid axon is calculated by the Nernst equation to be 130 mV. Thus, when the inflow gradient potential was balanced by the opposing electric potential no flow of calcium ions occurred. The similarity of these two values strongly suggests that a relation between depolarisation and calcium influx must exist.

Other work, (Hagiwara and Saito 1959, Katz and Miledi 1967a,b,c,d, 1968, 1969a,b,) shows the influx to be independent of sodium or potassium ions. Thus with tetrodotoxin (TTX) and tetraethylammonium (TEA) present, release still occurs in response to artificial depolarisation.

The effects of voltage changes on the calcium permeability have been studied by Baker, Hodgkin & Ridgeway (1971). They have shown that calcium entry occurs in two phases, an early phase comparable to the increase in sodium permeability and a late phase which occurs at about the same time as potassium permeability increase.

The two phases were studied with a variety of agents affecting the sodium and potassium permeabilities. The results suggest that the early phase of entry occurs directly through the sodium channel. The late phase however, appears to be quite independent of agents which affect potassium permeability and is probably a completely separate channel. The factors determining the release therefore are the voltage change which will affect the number of channels active, the length of time which they remain open and the calcium gradient.

Analysis of the effects of calcium on evoked potentials brings to light two features of the release process. At low

calcium concentration the relationship between the quantal content and external calcium is very non-linear and at high concentrations the quantal content tends to a constant value. (Dodge & Rahamimoff 1967, Jenkinson 1957). It appears that at low calcium concentrations there is a fourth power relationship between calcium and release and this gives way to a linear relation at higher external calcium. (Crawford 1973). At high external calcium concentrations the process appears to saturate. This might be caused by entry of calcium saturating, release sites saturating, or transmitter release may be limiting. It is difficult to distinguish between any of these possibilities at present.

Further evidence for the role of calcium in transmitter release has been obtained from investigations using tetanic stimulation. Following a short rapid train of pulses the evoked e.p.p. and the m.e.p.p. frequency are both potentiated. Investigations have led to the current theory that the potentiation is due to a transient rise in the intracellular active calcium, (Weinreich 1971), the intracellular concentration remains higher than normal causing subsequent pulses to release more transmitter until the internal calcium returns to normal.

The evidence therefore appears to be in favour of the depolarisation affecting calcium selective voltage dependent channels in the nerve membrane. Following the opening of a number of these channels calcium enters due to the electro-chemical gradient imposed on it by the distribution between inside and outside and so causes the release of the transmitter.

The mechanism whereby calcium causes the release of transmitter is still unknown. The charge on the calcium ion may neutralise the negative charge on the vesicle so facilitating

fusion with the membrane. A further possibility is that the calcium changes the viscosity of the axoplasm. Calcium is known to reversibly liquify extruded axoplasm from squid axons. Thus, the vesicles could conceivably approach the membrane more easily under the influence of calcium on the inside.

Postsynaptic Mechanisms

With a normally innervated muscle the sensitivity to transmitter is confined to the region immediately opposite to the presynaptic terminal as far as the response to released transmitter is concerned. There are other areas of low sensitivity termed extrinsic receptors but these probably differ in their properties to the normal intrinsic receptors concerned with the normal function (Feltz & Mallart 1971a,b,).

The receptor is probably four proteolipid molecules arranged to form a pore through the membrane and surrounded by acetylcholine esterase molecules. D-Tubo-Curarine was found to bind to a protein fraction of the muscle (Robertis 1973). The more specific ∝-bungarotoxin (∝Bgt) was found to bind with molecules of molecular weight 320,000. (Miledi, Molinoff & Potter 1971, Miledi & Potter 1971) from electroplax tissue. Similar results were obtained by Berg et al (1972) with mammalian muscle. Further evidence for a protein nature came from experiments showing inactivation by sulphydryl blocking durgs, heavy metals and substances forming covalent bonds with amino acids. (Albuquerque et al 1968, Barnard, Wieckowski & Chiu 1971, Castillo, Iscobar & Gijon 1971). Iontophoretic application of mercury can be shown to produce a depolarisation presumably by changing the tertiary structure of the receptor. (Castillo, Iscobar & Gijon 1971).

α-Bgt has been used for determining the number of acetylcholine receptors. Studies on rat diaphragm (Fambrough & Hartzell 1972) suggest 4×10^7 sites per end-plate. Studies on frog indicate 10^9 sites (Miledi & Potter 1971).

The actual structure of the binding site cannot be accurately determined until a better knowledge of the proteins comprising it is obtained. It is considered however that there are two parts of the receptor corresponding to the two sections of the acetylcholine molecule.

The main binding area is an anionic quaternary ammonium group receptor. It is at this point that substances such as curare are thought to act. Nitrogen free isoesters do not bind to the site indicating a specificity for the charged quaternary ammonium group.

A weaker cationic section binds the carbonyl group presumably by hydrogen bonding and a small charge attraction.

The existence of both muscarinic and nicotinic sites suggest that there may be a third section to the binding site which confers this specificity.

The action of substances such as curare is probably to bind to the anionic sites and simply to block the pore mechanically by the large bulk of the molecule.

The receptor protein is considered to undergo a conformational change producing a change in the sodium and potassium conductances.

(Eccles, Katz & Kuffler 1941, Kuffler 1942, Fatt & Katz 1951, Castillo & Katz 1954, Takeuchi & Takeuchi 1960).

Katz & Miledi (1972) have shown a noise increase at the time of transmitter action to be due to the effects of single molecules of acetylcholine reacting with receptor sites. The size of these events is about 0.3μV. Analysis gives a charge transfer of about 5×10^4 univalent ions.

Voltage clamp studies of the electrical changes show that the end-plate current (Takeuchi & Takeuchi 1959) rises to a peak and then declines exponentially. It was originally considered that the decline in end-plate current (e.p.c.) is due to a fall of acetylcholine concentration in the synaptic cleft. It is now thought the e.p.c. decline is related to the receptor action or to conductance change.

The time course of the e.p.c. is greatly influenced by the level of membrane polarisation. Gage & Armstrong (1968) found a two fold difference in e.p.c. time course when comparing m.e.p.p. currents at +50 and -50 mV. An immediate explanation was that the lengthening was due to a change in the sodium and potassium channels due to the voltage change. This view did not hold as e.p.c.s have a simple exponential decline at all voltages. (Kordas 1969, Magleby and Stevens 1972). Also the duration of the e.p.c. should not be prolonged at potential differences more negative than the potassium equilibrium potential. (Magleby & Stevens 1972). The most likely explanation at the moment is that the decay is due to events at the receptor level. (Kordas 1972).

Disappearance of acetylcholine from the synaptic cleft is more rapid than can be accounted for by diffusion alone. Most of the acetylcholine is destroyed by hydrolysis by the enzyme acetylcholine esterase (Nachmansohn 1939, Marnay & Nachmansohn 1938).

Changes occurring in the rate of hydrolysis can be seen as changes in the time course of the e.p.c. This does not reflect an effect directly on the e.p.c. as this is determined by receptor kinetics. Prostigmine however increases the duration of the e.p.c. probably by causing repetitions of the basic event due to a prolongation of the acetylcholine action.

The presence of acetylcholinesterase is vital to the functioning of the neuromuscular junction as any alternative method of removal of acetylcholine would be too slow for the mode of action of this synapse. This may be illustrated by the fact that after an 80% block of acetylcholinesterase molecules, nervous transmission during tetanic stimulation rapidly begins to fail (Barnard & Wieckowski 1970).

After hydrolysis the acetate normally disappears from the synaptic cleft by diffusion and the choline by either diffusion or a process of active reabsorbtion. Some evidence has accumulated to support the removal of choline by active reabsorbtion in some nervous as well as non-nervous tissues.

Active uptake has been shown to occur in incubated mouse cerebral cortex slices (Schuberth et al 1966). Hodgkin & Martin (1965) have demonstrated a carrier system for choline in squid axon, and Marchbanks (1968) has shown active uptake into synaptosomes prepared from guinea pig cerebral cortex. Absorbtion has also been shown to occur against a concentration gradient in red blood cells (Martin 1967, 1968a,b), Kidney (Sung & Johnson 1965) and rat diaphragm (Chang & Lee 1970).

The choline which finds its way back into the nerve terminal by either diffusion or reabsorbtion is then resynthesised into new transmitter which then becomes available for release.

Chapter IV

Background to the present investigations

Experiments reported here demonstrate augmentation of tetanic responses of a nerve-muscle preparation in potassium free bathing medium. The problem is, where in the chain of events between nerve stimulation and muscle contraction does this change actually take place? The action could be on the muscle contraction; on the resting potential of the nerve or muscle, thus causing a change in some property indirectly; or through a direct effect of low potassium on the nerve terminal and possibly modifying transmitter output.

The actual process of mobilisation of transmitter at the nerve end from synthesis through storage to release remains obscure. Despite its physiological importance, little work has been published relating directly to the problem. Agents which have been shown to have some effect are, hyperpolarisation, depolarisation and the effect of extracellular potassium (Hubbard & Willis 1962, 1968, Parsons, Hoffman & Feign 1965), i.e. all effects on the presynaptic resting potential and possibly the spike amplitude. The action of calcium is at the final stage of the process and is also under the control of the membrane polarisation.

There is a body of evidence (Hagiwara & Tasaki 1958, Takeuchi & Takeuchi 1961, Katz & Miledi 1967a,b,c,d) that suggests the amplitude of the presynaptic depolarisation affects the amount of transmitter released. There is also evidence from extracellular recording in frog muscle that the presynaptic action potential amplitude has little effect on transmitter release, the amount released remaining relatively constant during small changes of

potential. (Braun & Schmidt 1966). It has also been shown that depolarisation of the presynaptic nerve terminal in rat can cause a reduction in both the presynaptic nerve spike and the e.p.p. (Hubbard & Willis 1968). The reduction in e.p.p. amplitude was shown to be due to a reduction in the number of quanta released. Unfortunately, due to the small size of the nerve terminal it is impossible with conventional micro-electrodes to record intracellularly the nerve resting potential or spike potential.

The squid however has a giant synapse with a presynaptic nerve fibre large enough to penetrate and results from this work indicate that the amount of transmitter release is indeed sensitive to the depolarisation produced. (Takeuchi & Takeuchi 1962, Katz & Miledi 1968, 1969a,b). The depolarisation affects the calcium influx which governs release, and therefore, providing transmitter availability is not a limiting factor, the level of depolarisation will affect release to some extent through this factor.

Liley (1956a) originally reported that hyperpolarisation reduced m.e.p.p. rate, this has since not been confirmed (Landau 1969). However, as the resting rate is so low compared with possible rates of release or rate of release during the active phase, this is not a serious objection. Krnjevic & Miledi (1959) in a paper dealing with presynaptic failure of propagation in rat motor nerve terminals suggested that the major cause was anoxia. However, they showed the block of transmission was relieved by anodic polarisation, suggesting that the anoxia could have caused reduction of nerve terminal polarisation by inhibition of exchange pumping. Increase of extracellular potassium could therefore also be a contributing factor. During muscle action the concentration of potassium around the presynaptic nerve terminals must increase and so cause depolarisation.

If this led to a reduction of presynaptic spike amplitude and a smaller amount of transmitter liberated, it could contribute to other factors in causing failure of propagation. Similarly, the increase in extracellular potassium could result in depolarisation of the fine nerve branches and lead to block of transmission of impulses, so contributing to the block caused by anoxia as suggested by Krnjevic and Miledi (1958, 1959).

A survey of the relevant literature reveals little work having been published on neuromuscular transmission using solutions containing low extracellular potassium. The Goldman-Hodgkin-Katz equation, relating the resting potential to the permeabilities of active membranes and derived from the Goldman constant field theory, (Hodgkin & Katz 1949), predicts that no great increase in potential will result from the removal of the 5 mM of potassium ions in normal Krebs solution, the effect of the other ions being dominant. In addition, it is known that potassium permeability is affected in some muscles when deprived of potassium on the outside of the membrane. (Adrian 1956, 1958). The resting potential could therefore be expected to rise but then reach a limiting value.

Creese has demonstrated a significant increase in outward movement of potassium under conditions of stimulation. (Creese et al 1958). It has also been shown that anoxia can cause a rapid loss of potassium from the muscle. (Creese 1954). It could therefore be expected that the internal potassium of the nerve terminal will be lower during times of muscle activity and especially so under conditions of tetanic activation. During these periods, the supply of oxygen to the fine nerve terminals will be reduced due to constriction of the blood vessels while the muscle is contracted. Removal of

extracellular potassium could be beneficial in that it would tend to oppose the lower resting potential produced by changes in the potassium ion ratio between inside and outside. Under conditions of high activity therefore, removal of potassium from the extracellular fluid may enable the nerve terminal to retain more normal excitability.

Consideration of the above points led to an initial hypothesis that an increase in the potassium concentration around the presynaptic nerve terminals should cause some reduction in efficiency of the nerve-muscle preparation. Removal of potassium, under conditions of activity, should therefore have some effect, if this postulated increase in extracellular potassium were adversely affecting the responses of the preparation.

A trial experiment was performed to determine whether by using potassium free Krebs solution the degree of neuromuscular failure during tetanic stimulation could be reduced. It was observed, that following reduction of the potassium in the extracellular fluid, the amplitude and sustaining power of the tetanus was increased. As removal of the potassium should decrease the active state of the muscle (Goffart & Ritchie 1952), the amplitude of the response might be expected to be diminished. Removal of potassium therefore was having an effect which appeared to be directly beneficial to the functioning of the preparation, and of such a magnitude as to overcome the reduction in active state expected.

In this thesis the results of investigations into the causes of this effect are set out and an attempt has been made to offer some explanation.

SECTION II

MATERIALS & METHODS

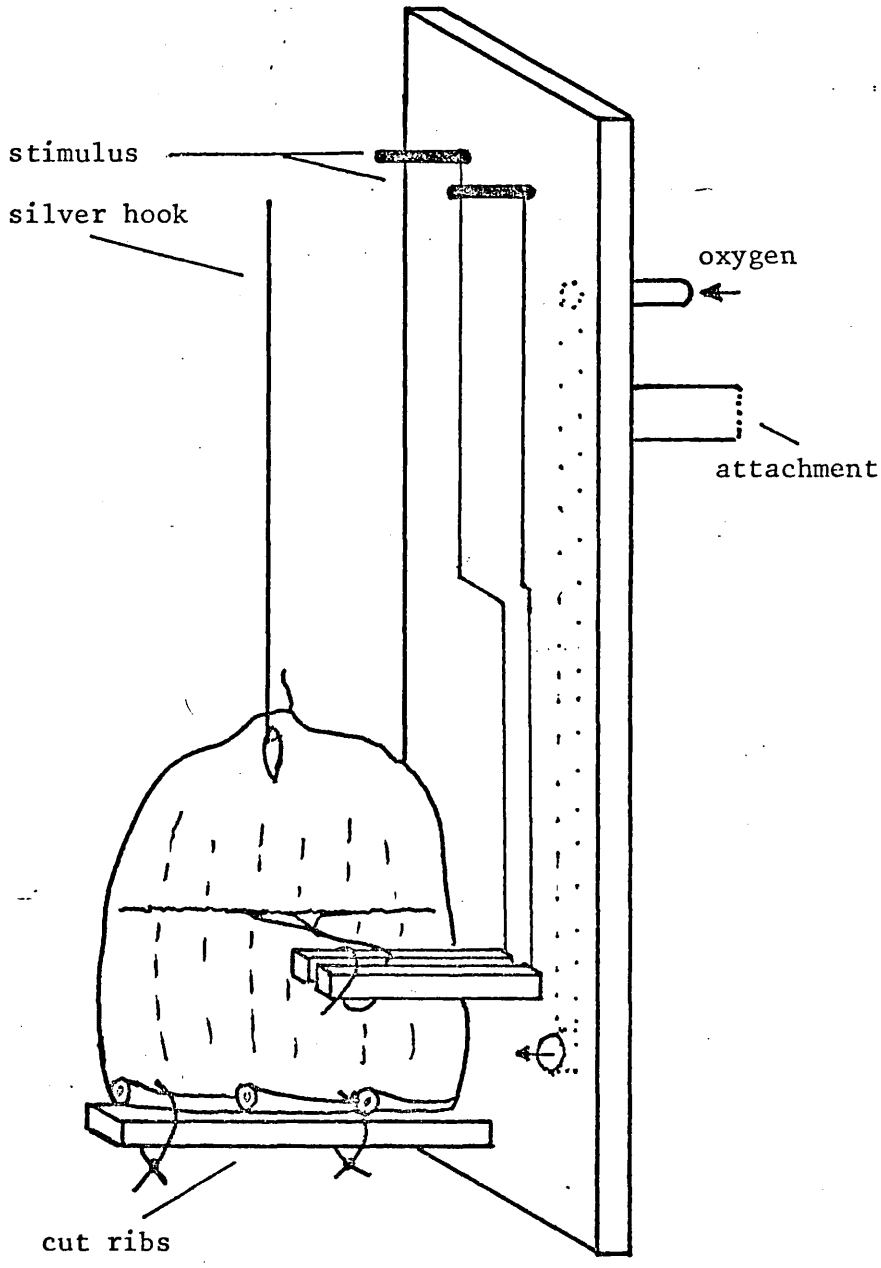


Fig. 5.1 Holder used for attaching diaphragm muscle and stimulating phrenic nerve in mechanical response experiments.

Chapter V

Methods 1 - General Methods

All of the results reported in this thesis were obtained from the rat diaphragm phrenic nerve preparation. Albino rats of Wistar origin weighing 200-300 grams were killed by a blow to the head and the left hemidiaphragm removed with approximately 2 cms of phrenic nerve attached. The preparation was placed in a Petri dish containing preoxygenated modified Krebs solution (for composition see section on Krebs solution later in this chapter) equilibrated with 95% O_2, 5% CO_2, and trimmed to size. The whole time from killing the animal to placing the preparation in Krebs solution was kept under 4-5 minutes.

The investigative procedures used are divided under two main headings: experiments using the mechanical response of the muscle and experiments using electrophysiological methods of investigation.

Mechanical response recordings

The method used was similar to that described by Bulbring (1946). The preparation was mounted on an electrode assembly that acted also as a support for the diaphragm muscle. Fig. 5.1. The nerve was passed between two stimulating electrodes made of silver wire and the loose end passed lightly around the electrodes to prevent movement. The whole assembly was placed in a 50 ml isolated organ bath and attached to a spring loaded heart lever arranged to write on a smoked paper attached to a Kymograph drum. In experiments where isometric recording of tension was used, the lever was replaced by a transducer (see section iv) and the smoked paper by a "Devices" M4 pen recorder.

The preparation was stimulated indirectly through the phrenic nerve with impulses of 0.05 ms duration and of sufficient amplitude to ensure supramaximal stimulation. This was usually of the order 8-12 volts indicated on the stimulator. The actual voltage applied to the nerve was considerably less, due to the shunting action of the solution. With such short pulses, there is however little likelihood of muscle stimulation, even at high voltages. The experiments were carried out at room temperature (24°C), this having been shown to produce more consistent results from the preparation than at normal body temperature. (Krnjevic & Miledi 1958).

Drugs or ionic solutions were added directly to the bath to produce the required concentration. Solution changes were accomplished by running out the contents and running in new solution from the bottom.

Electrical activity investigations

This procedure is similar to that described by Fatt and Katz (1951). The basic method for removal of the diaphragm was the same. When trimmed to size the diaphragm was about ½" wide, (the muscle fibres running longitudinally), this was placed in a specially designed perspex bath. The bath contained a chamber for the muscle with a side arm for the nerve. The muscle chamber was set with synthetic rubber compound (Siloset) around the edges to enable pins to be inserted through the diaphragm for attachment. The chamber contained about 5 ml of fluid. This was circulated through the bath from a reservoir where oxygenation took place and then allowed to drip into a beaker. The side chamber had silver wire electrodes for nerve stimulation, the nerve having been drawn under the electrodes to keep it in place. A third hole in the bath

contained a push-in chlorided silver wire electrode.

The tissue was illuminated from a light source and mirror arranged to pass light through the base of the bath and observed by means of a Wild binocular microscope using a magnification of between 6 and 25 x.

Glass micro-pipettes were used for intracellular recording. These were drawn to a fine tip and filled with 3M.KCl. These were inserted into a perspex holder attached to a micro-manipulator. Connection was made by agar/saline and a silver/silver chloride electrode. The whole assembly was contained within a small Faraday cage (2' x 2' x 2') and mounted on a thick aluminium base.

The recording electrode was connected by a short wire to a high impedance probe unit (see section iv), and this to a Tektronix 502A oscilloscope. A calibrator unit and backing off device were connected in series with the indifferent electrode to enable D.C. voltages to be applied for the purpose of balancing electrode potentials and equalising resting potentials. This was necessary when using a high sensitivity setting on the oscilloscope. An electrometer was connected in parallel to the oscilloscope to continuously display resting potentials on a meter. Fig. 5.2.

The output of the oscilloscope was fed to an audio monitor, a Telequipment storage oscilloscope and a Racal 3002 tape recorder. Data were recorded on the tape recorder (frequency response 10 KHz) and subsequently photographed from the oscilloscope screen during play-back. End-plate potentials recorded on film were measured by projection onto graph paper by means of a photographic enlarger. All measurements being made from the centre of the base line noise.

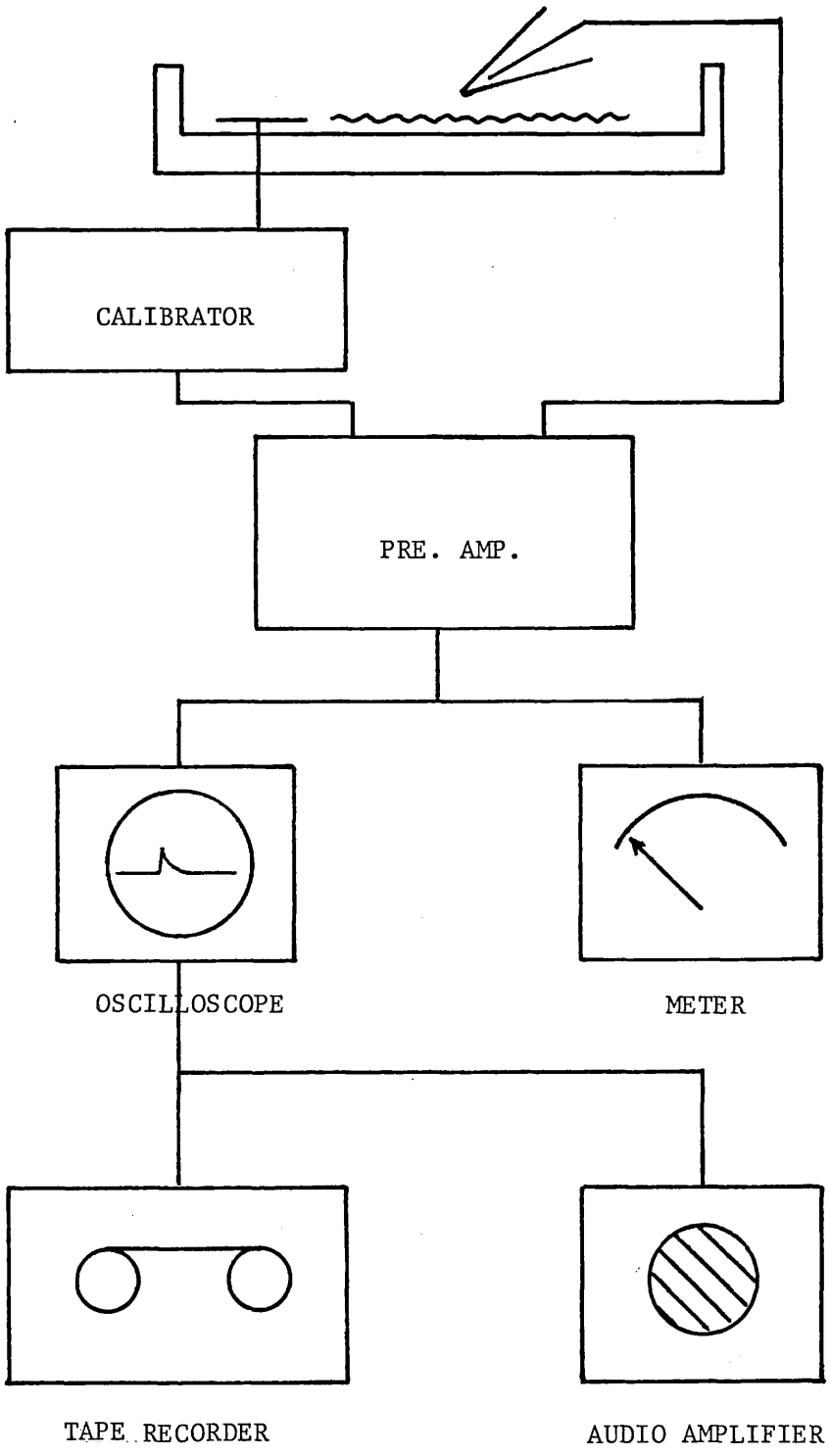


Fig. 5.2 Block diagram of connections between electronic aparatus used for intracellular recording.

Stimuli to the nerve were produced from a Grass SD5 stimulator or from the duration and output circuit shown in Fig. 6.3. driven from a Devices Digitimer coupled with a small digital timing unit made from 74N series Transistor Transistor Logic (T.T.L.) integrated circuits.

Procedure

Electrodes were pulled from 2 mm glass tubes, the pulling aparatus being a vertically mounted machine using gravity to effect the pull when the glass was hot enough. The end of the glass was attached to a 410 grm. weight which slid freely up and down. The tip was drawn out to about 1 cm before breaking at a tip diameter of about 0.5 μM, the length being controlled by the temperature of the heating element. This procedure produced fine flexible electrodes which stood up to considerable bending if muscle fibres moved.

The electrodes were selected for initial sharpness by viewing with a phase contrast miscroscope of magnification 720 x with a water immersion objective lens. During use, electrodes were selected on a basis of the highest resistance concomitant with acceptable noise characteristics.

Junctions were localised by the process of moving the electrode, viewed by the microscope, by means of a micromanipulator until the maximum amplitude of e.p.p. was obtained. E.p.p.s with slow rise times or which looked unually small in amplitude were discounted. Where it was required to replace an electrode back into a specified end-plate the field was mapped noting blood vessels, nerve junctions and other landmarks and the electrode replaced as close as possible to the original site. Trial runs of this procedure

appeared to produce repeatable results and as the e.p.p. in the region of the end-plate is so different from the electrical changes occurring in the surrounding tissue, this procedure was considered acceptable.

The results deal mainly with amplitude changes of the e.p.p. and as the errors in the measuring were no larger than the statistical variations of the e.p.p. the method used was judged to be satisfactory.

Krebs solution

Composition:

The composition of the normal Krebs solution was as follows:-

Na^+ 150mM, K^+ 5mM, Ca^{++} 2mM, Mg^{++} 1mM,

Cl^- 148mM, $^-HCO_3$ 12mM, $^-H2PO_4$ 1mM, Glucose 11mM.

The potassium level used in the control solution in all of these investigations was 5mM, and all references to normal Krebs solution presume this level of potassium.

When the concentrations of calcium and magnesium were changed, the amount of sodium chloride was varied to prevent changes in osmotic pressure.

Potassium free Krebs solution

Measurements were made of the levels of potassium in the Krebs solution made up containing no potassium. All such solutions contained an extra 5mM sodium chloride to maintain equivalent osmolarity.

The method used to determine the residual potassium was to compare samples of the solution with standard solutions containing known amounts of potassium, by flame photometry.

The concentration of potassium in samples of Krebs solution, made up as potassium free, was found to be 0.015mM.

Samples were also taken from the fluid after it had passed through the bath during the measurements of resting potentials and e.p.p.s. The muscle of the preparation was at rest during this time

and thus, any extra potassium would have come from leakage from the muscle or nervous activity. The concentration of potassium in these samples was found to have risen to 0.05mM.

Equipment

Transducer	Ether dynamometer UFI ± 4oz.
High Impedance Probe	EPIL HIP 151C
	D.C. - 2.5 KHz
	10MΩ input impedance

Materials

Electrode glass	Jencons, H15/10
Synthetic rubber compound	Siloset, Radiospares

Chemicals

Water	Glass distilled
Sodium Chloride	
Potassium chloride	
Calcium chloride	
Magnesium sulphate	
Magnesium chloride	All analar grade reagents
Sodium bicarbonate	
Sodium hydrogen phosphate	
Glucose	
Choline chloride	
d-Tubocurarine	Boroughs Wellcome 'tubarine'
Hemicholinium-3	Aldrich Chemical Company
Ouabain	British Drug Houses
Digoxin	Boroughs Wellcome
Nicotine	British Drug Houses
Adrenaline	Antigen Ltd. (adrenaline injection)
Acetylcholine	British Drug Houses

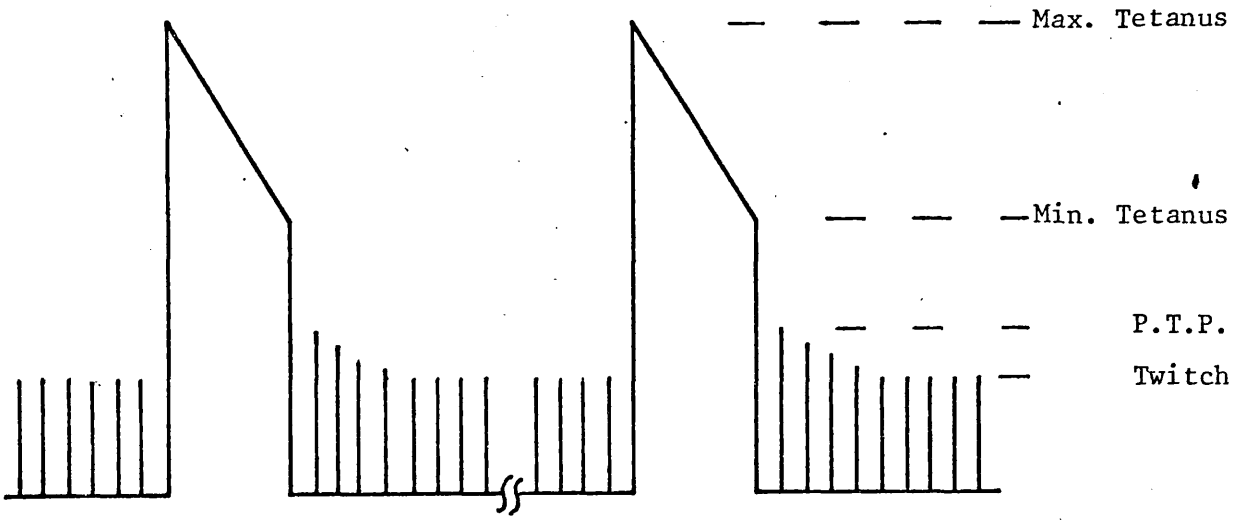


Fig. 6.1 Diagramatic chart record of the isometric diaphragm contractions obtained when using the cyclic stimulation method. Contraction is upward. Stimuli were given at 1 Hz for 55 seconds followed by 50 Hz for 5 seconds. The time scale is expanded compared with that usually used for the experiments. The important features of the record are labelled.

Methods 2 - Cyclic Stimulation Technique

The initial results obtained from mechanical response investigations into the effect of potassium removal from the bathing solution of the rat phrenic nerve diaphragm preparation indicated that the main response to the change was seen during tetanic stimulation. The results so obtained indicated that an effect was demonstrable but gave little further information. An improved method was therefore devised to record the results in a reproducible and semiquantitative manner.

The preparation was set up as previously described, but stimulated with a different pulse pattern. Supramaximal pulses were applied to the phrenic nerve at a frequency of 1 Hz. At one minute intervals a short tetanic stimulus was given by feeding a 5 sec. duration 50 Hz. pulse train into the stimulator external output socket. Thus a mixing of the two frequencys occurred and on cessation of the tetanus, the preparation was once more exposed to pulses at 1 Hz.

It was hoped that by using more tetani at shorter intervals a better indication of the general time course and that of any transient effects could be obtained. The preparation was found to settle to this pattern of alternating twitch and tetanus within 10 minutes, after which no further fatigue occurred providing the conditions were not changed.

The tension was recorded by means of a strain gauge isometric transducer and after amplification the output from the bridge was displayed on a Devices pen recorder. The features of the record obtained are shown in Fig. 6.1.

Using this system of stimulation, the preparation was found to be extremely sensitive to all agents commonly known to affect neuromuscular transmission. During the tetanic portion of the cycle, the safety factor for transmission across the synapse is probably lower due to a reduction of transmitter output, this could allow agents to exert their effects more easily and at lower concentrations.

The results may be analysed for a number of parameters known to be measures of neuromuscular function: Twitch height, tetanus height, tetanus droop (decline in tetanus height from onset to termination of tetanus), twitch/tetanus ratio, post-tetanic potentiation (PTP) or post-tetanic depression (PTD). These can be related to either the number of pulses or to time, whichever is more relevant.

As experience was gained in using the method, it was found that very small changes in oxygen tension and also ionic strengths caused changes in the record. The bathing solution constituents were therefore kept constant by making up large volumes of solution sufficient to complete an experiment. The solution was passed through the bath at a rate of 10 ml per minute. The solution entered the bath from a reservoir attached to the base and was sucked off from the top continuously. This allowed changes in the solution with minimal disturbance to the preparation. The oxygenation was carried out both in the reservoir and in the bath.

During preliminary trials it was evident from the records that the timing of the tetanus in relation to the single stimulation was important. It was found that because the two frequencys were unrelated, the first tetanic pulse could occur at any time between two twitch pulses giving rise to anomalous changes in tetanic amplitude. It was

decided that the logical time for the beginning of the tetanic train should be at the time of the next twitch pulse. Thus the first e.p.p. of the tetanus would be equivalent to a normal twitch e.p.p. and subsequent e.p.p.s would decline from this level rather than from an unpredictable starting value.

As no commercial instrument was available that would adequately carry out this function a cyclic stimulator was designed and produced. The circuit diagram for this is shown in Fig. 6.2 and Fig. 6.3.

The action of the circuit is to produce two pulse frequencys which are independently variable. The lower frequency is the unit interval and is also used as a timer and gate for the higher frequency which is the tetanic stimulation frequency. Thus it is possible to produce a short train which is an exact number of unit pulses in length.

The output from the unit interval generator is fed to the output and also into a four bistable divider circuit, set, by means of internal logic feedback, to divide by 10. The output from this is then divided by a further three bistable circuit, set to divide by 8. Selected outputs from the bistables are gated together and used to set or reset two bistable latches. When set one of these starts the higher frequency generator and the other disables the unit output.

The circuit counts in unit intervals up to a maximum of 80. The counters reset after a certain interval depending on the setting of the selection switches. Following the reset pulse the train frequency is produced until the unit counter output stops it after a selected interval. The counter then goes on until

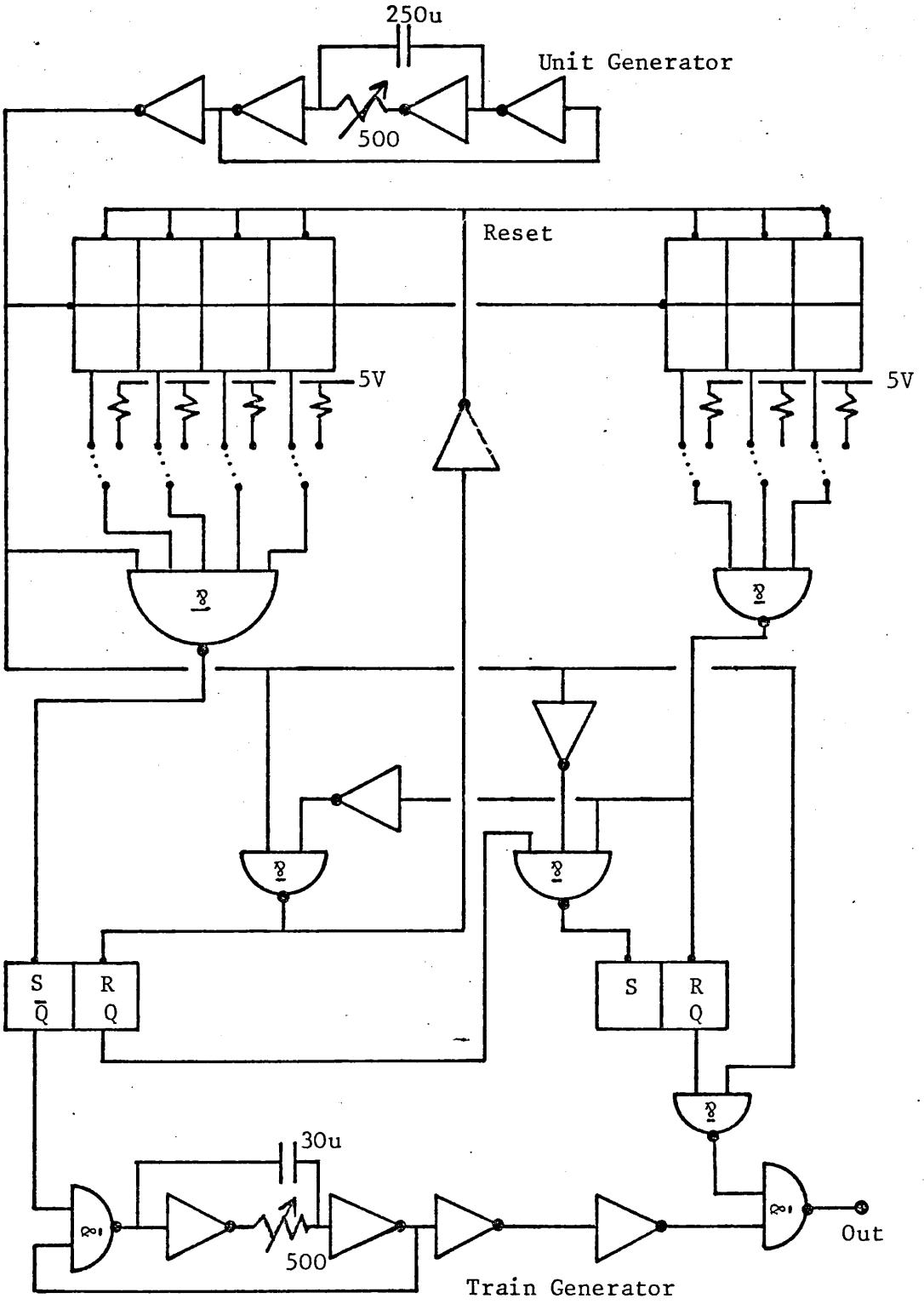


Fig. 6.2 Logic circuit diagram of pulse timing unit. The device is constructed from standard 74N series TTL logic gates. Both frequencies, train length and train interval are independently variable. For further details see text.

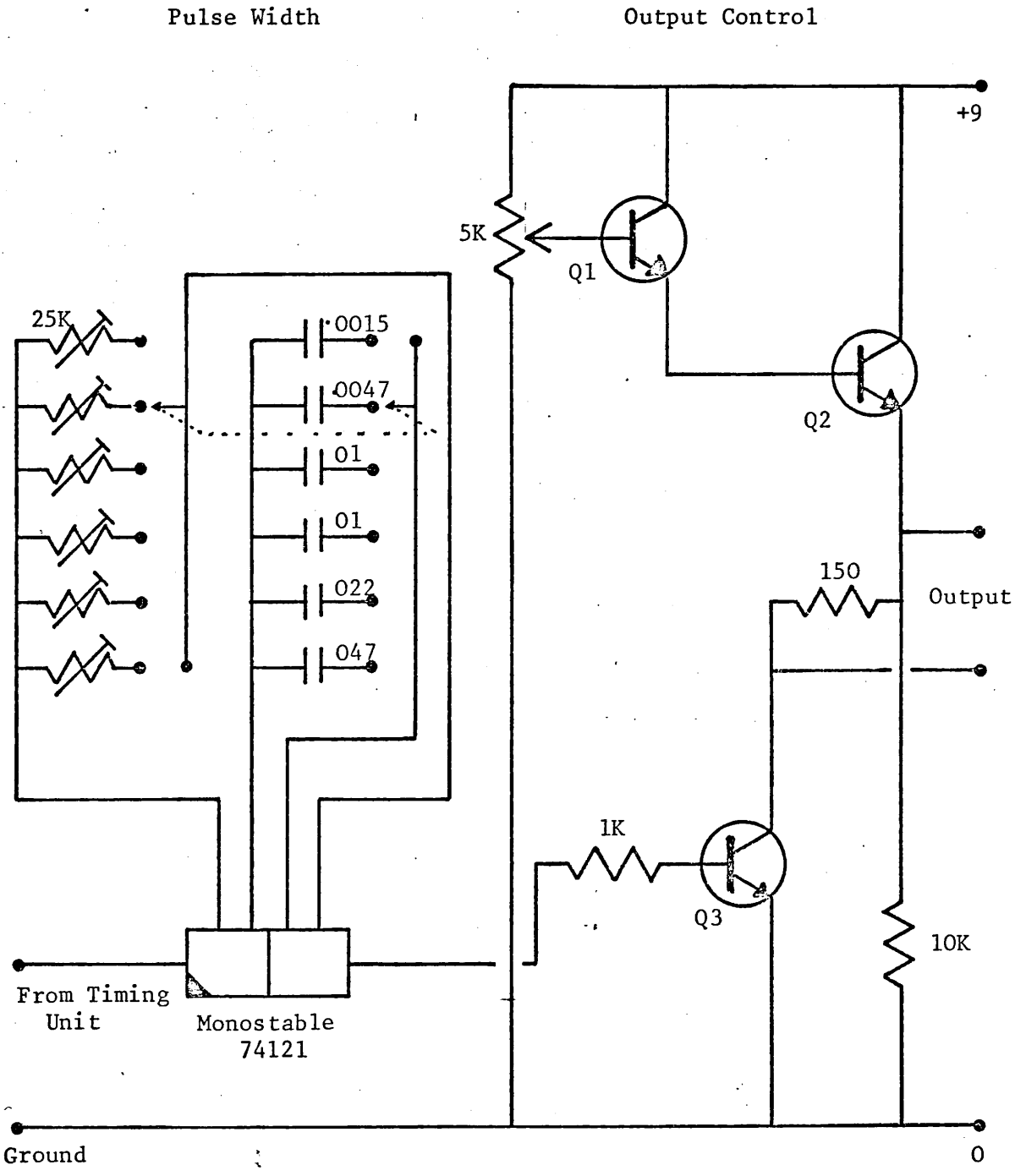


Fig. 6.3 Pulse width and output section of cyclic stimulator circuit. Pulse width section fed from 5V power supply for timing logic.

Q1, Q3 2N3053; Q2 2n3055.

Capacitance values in uF.

the next reset pulse when the cycle repeats.

The duration of the train may be any number of unit intervals up to 10 and the cycle period can be selected in units of 10 basic pulses up to 8.

The output of this circuit is fed to a single monostable package with switched resistor-capacitor combinations for pulse duration selection. The value is adjusted by varying the resistance of the R.C. circuit. Fig. 6.3.

The output voltage is controlled by an emitter follower circuit which sets the voltage and provides a low output impedance for driving preparations immersed in solution. The voltage set by this part of the circuit (Q_1, Q_2) is switched by a third transistor (Q_3) in the emitter circuit using the pulse from the monostable output.

This instrument greatly improved the quality of the results obtained. Experiments carried out with preparations which had come to equilibrium, showed that in control experiments, there was no change in any of the parameters measured, for periods in excess of one hour. More important however, was the fact that the maximum tetanic amplitude did not appear to vary, as was previously found to be the case, since beating of the two frequencys was avoided. Results obtained in normal Krebs solution typical of control responses are shown in Fig. 6.4.

It has already been stated that small changes in O_2 tension have a distinct affect on the preparation. This was much more noticeable than with the standard recording methods such as described for the initial experiments. In view of this it is

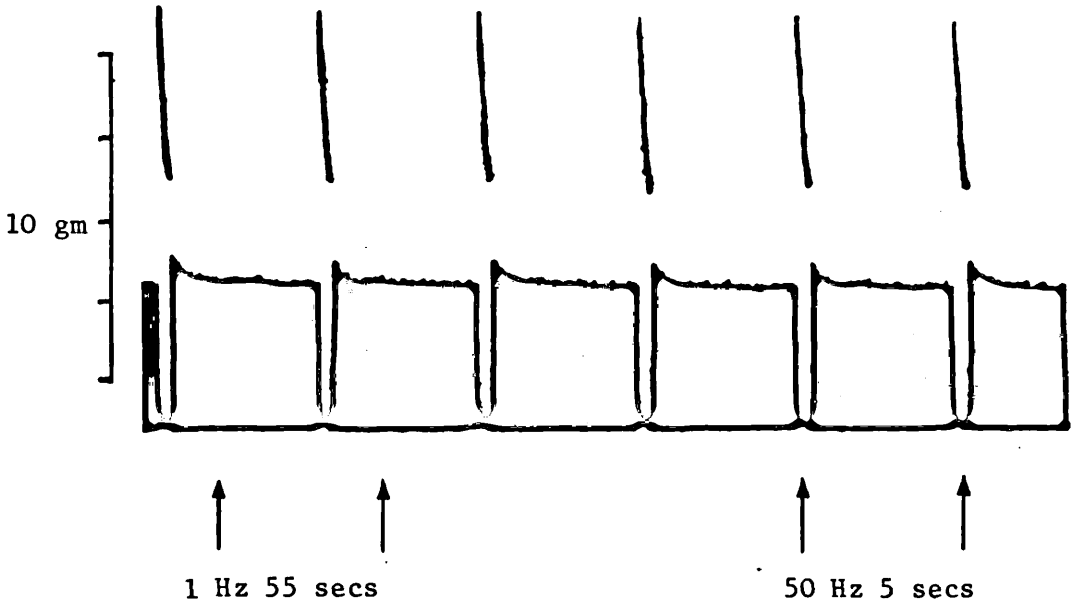


Fig. 6.4. Control response of rat diaphragm phrenic nerve preparation immersed in normal Krebs solution. Stimuli were given each second for 55 seconds producing the lower marks on the trace. This was followed by 5 seconds of 50 Hz tetanic stimulation producing the upper line of marks and the gap in the twitch response part of the record. The rapid upward and downward deflections of the pen at the start and finish of the tetanic period have made no mark on the chart. Post-tetanic potentiation of the twitch amplitude following the tetanic stimulation can also be seen at the start of the twitch stimulus period.

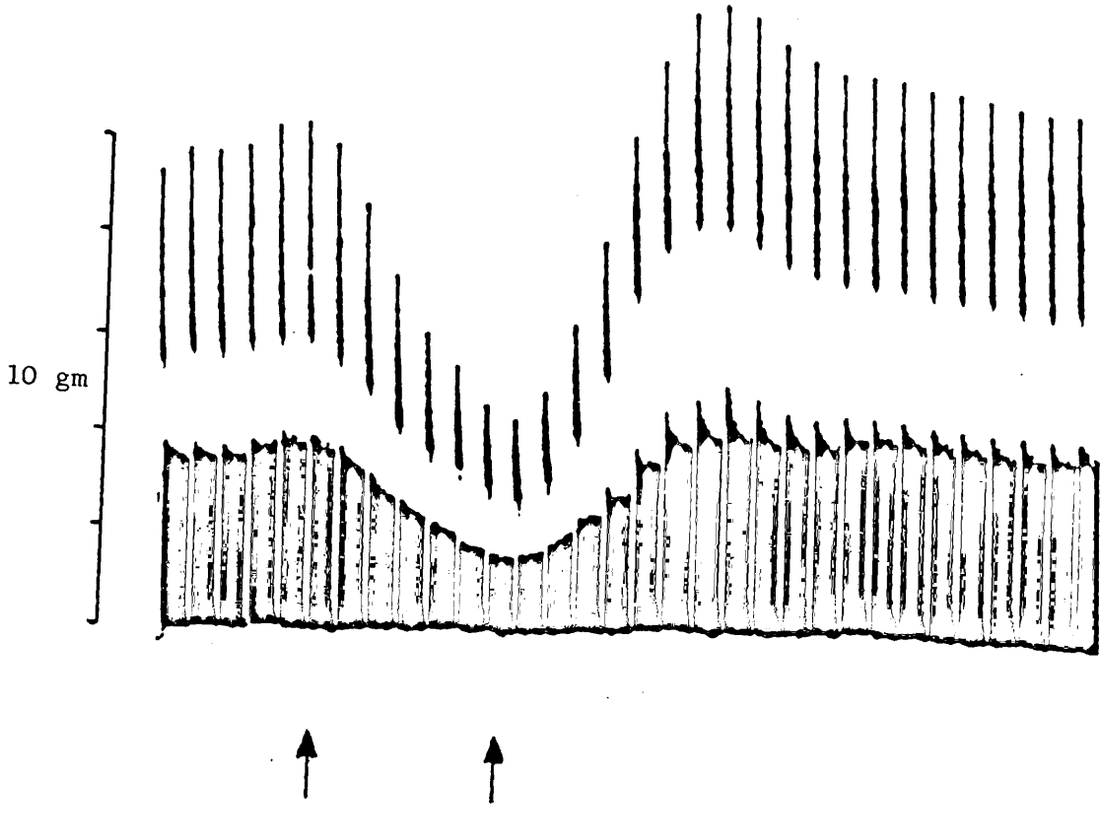


Fig. 6.5. The effect of anoxia on the mechanical response of the rat diaphragm phrenic nerve preparation. Stimulation was 1 Hz for 55 seconds and 50 Hz for 5 seconds repeated. Calibration shows isometric tension developed. The oxygen supply was interrupted for the period between the two arrows.

possible that the preparation as used was more starved of O_2 than is usually the case. Slight variations in rate of gas inflow to the bath had little effect on the preparation as judged from the record after the system had equilibrated. However, a preparation was required which was extremely responsive to conditions of either block or enhancement. Susceptibility to O_2, providing the preparation showed no erratic changes of response during control stimulation was therefore considered an asset. The effect of complete anoxia was determined to ascertain the changes which might occur and a typical result is shown in Fig. 6.5. This also demonstrates some interesting features of the method.

There is an immediate fall in both twitch tension and maximum tetanic tension. Strangely, although the maximum amplitude is less, the maintenance of the tension throughout the tetanus is improved. The effect was completely reversible when the preparation was only deprived of O_2 for a short time. An interesting feature of the recovery is facilitation of the response for a period following reoxygenation. The maximum tetanic amplitude is increased and a marked post-tetanic potentiation is present, both of which eventually return to normal values. By comparison of the responses obtained with those produced by agents, the action of which is well understood, it is considered that this method can provide a great deal of information about the mode of action of less well understood agents, by examination of a characteristic response pattern.

The main advantages of the method are that information may be obtained about a number of characteristics at once and that it appears to cause a greater sensitivity of response overall.

SECTION III
RESULTS

Chapter VII

Investigations using the Mechanical Responses of the Muscle

In all excitable tissues, there is an exchange of a small amount of potassium within the cell for sodium from outside the cell during activity. This loss of potassium is normally restored by exchange pumping of sodium for potassium during periods when activity is less. Thus, during contraction of skeletal muscle there will be a small but significant rise of potassium in the extracellular fluid surrounding the tissue. During periods of prolonged tetanic stimulation of the muscle, via its motor nerve, the transmission eventually begins to fail and the contraction declines in strength. A possible explanation for this decline is, therefore, build up of extracellular potassium surrounding the fine nerve terminals and the neuromuscular junctions. This build up, if of sufficient concentration, could exert a depolarising effect on the fine nerve terminals and adversely affect their functioning.

The above is only one of a number of possibilities put forward to explain failure of transmission during tetanic stimulation. (Krnjevic & Miledi 1958, 1959). Other possibilities are O_2 lack, transmitter or precursor exhaustion, increased muscle fibre threshold or build up of metabolites, any of which may adversely affect the junctional region. These authors however, were satisfied that the causes of the observed fatigue were localised within the fine nerve filaments or in the neuromuscular junctions themselves, and not in the main nerve trunk or the muscle.

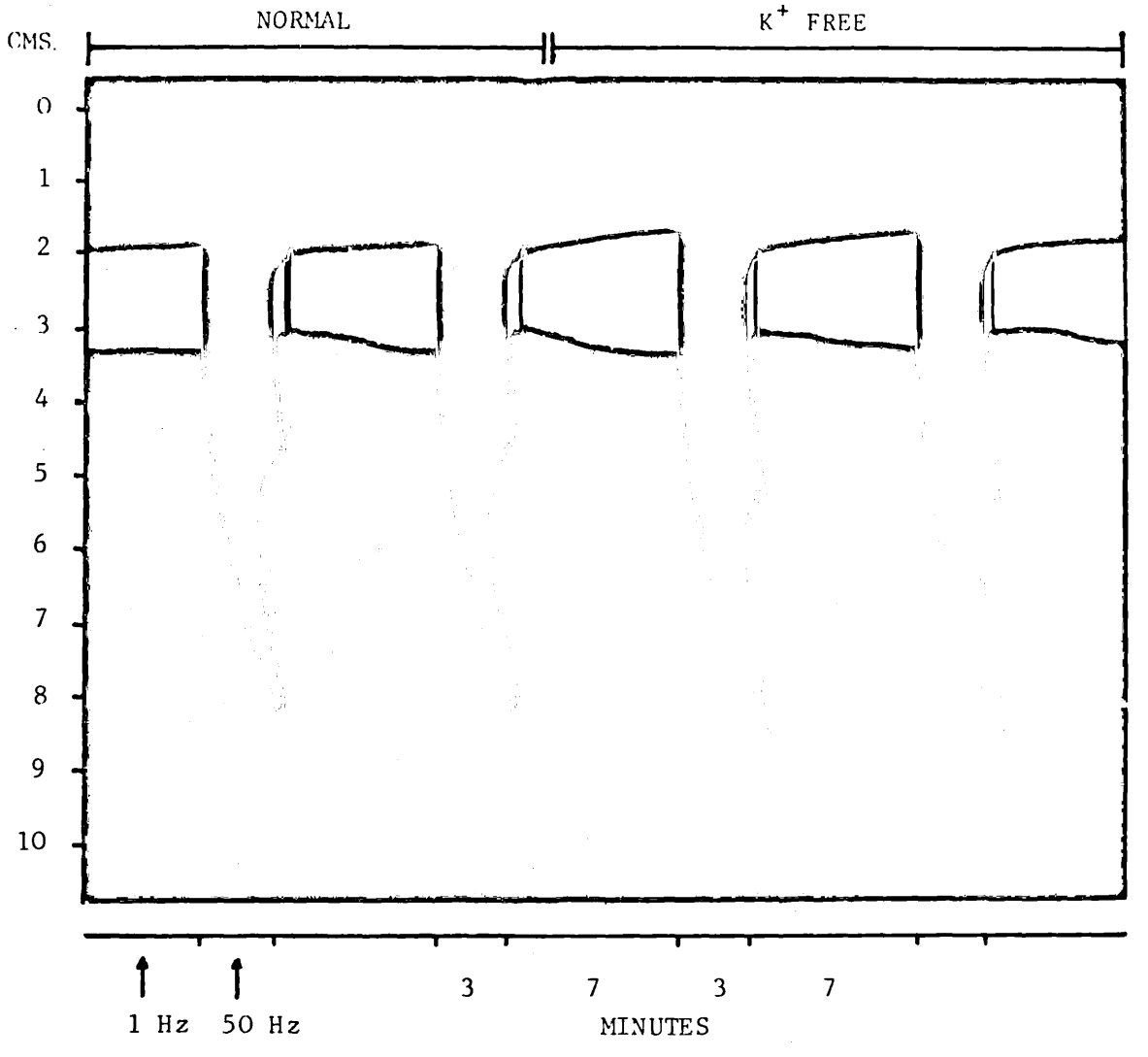


Fig. 7.1. Smoked drum recording of isotonic contraction of indirectly stimulated diaphragm in normal and potassium free solution. Contractions are downward. (5 cm ≡ 4 g). Length scale in cm. Stimulation indicated by arrows was 1 Hz for 7 min and 50 Hz for 3 min.

To investigate the possibility of potassium involvement, an attempt was made to lower the extracellular potassium concentration by removing this ion from the bathing Krebs solution. If the increase in potassium concentration was affecting the preparation during a tetanus, then by lowering the concentration it would be expected that a delay in "fall off" of tetanic amplitude would be seen until the potassium concentration in the vicinity of end-plates increased again.

A standard rat diaphragm phrenic nerve preparation was set up to record the tetanic contraction and the experiment performed. The results may be seen in Fig. 7.1.

The record is a smoked drum recording of isotonic downward contraction of the diaphragm muscle stimulated by the phrenic nerve. 1 Hz twitch contractions were elicited for a period of 7 minutes. Where indicated, the rate was changed to 50 Hz and maintained at this for 3 minutes. The preparation was then allowed to recover at 1 Hz for 7 minutes. The cycle of events was continued in normal Krebs solution until a repeatable effect was obtained. The bathing solution was then changed to one containing no potassium and the cycle was continued.

Inspection of the record reveals little change in the twitch response following change of solution. However, the maximum tetanic amplitude has been increased, as also has the sustaining power of the tetanic contraction. This may be seen from the smaller change of amplitude during the tetanus in potassium free solution compared with that in normal solution.

Fig. 7.2. is an analysis of responses of this type obtained from two diaphragms and indicates the magnitude of the changes observed. in twelve diaphragms examined, all showed a consistent increase in maximum tetanic amplitude. Of these, eight demonstrated a significant increase

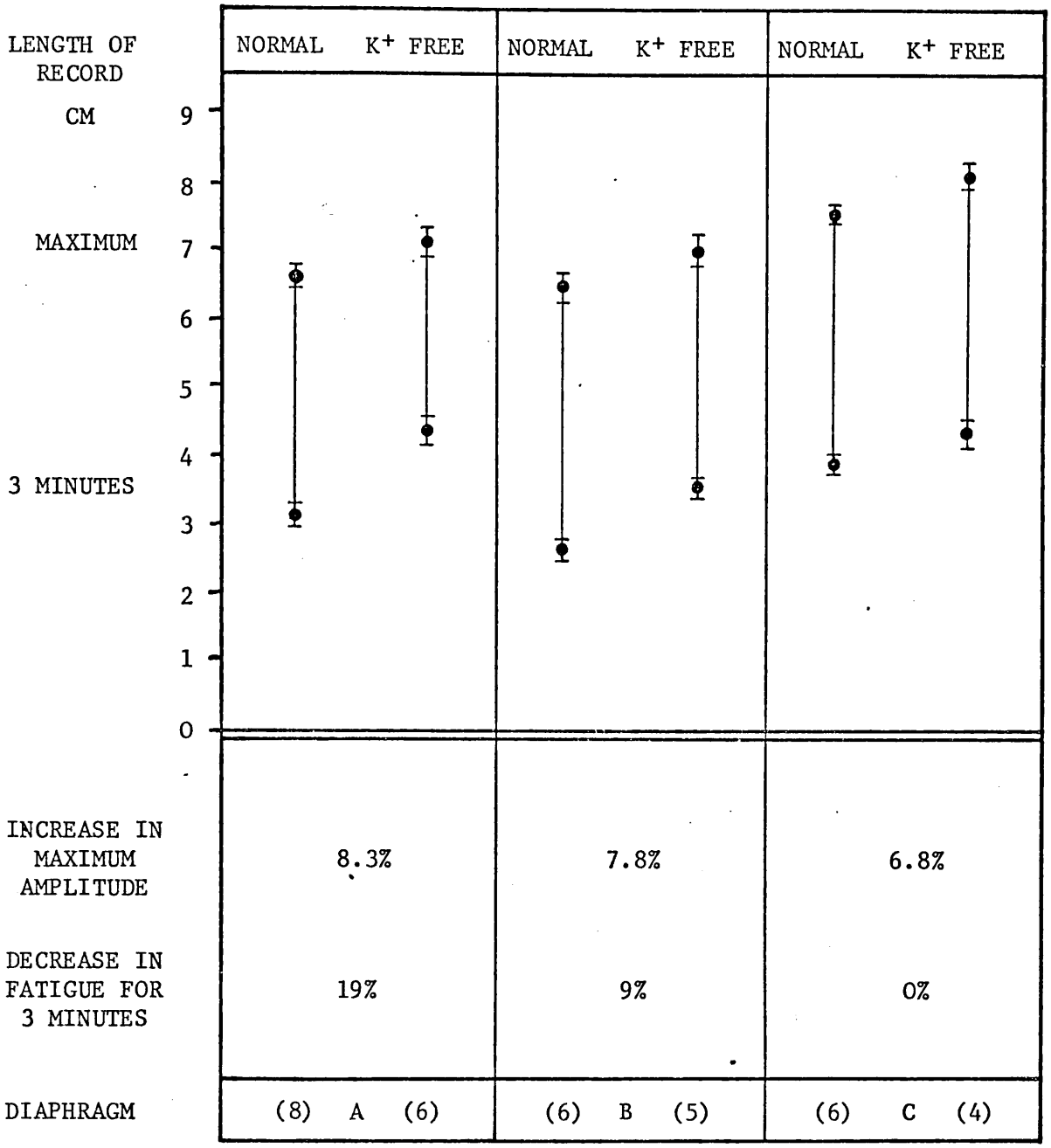


Fig. 7.2. Tetanic response of three indirectly stimulated diaphragm preparations in normal and potassium free Krebs solution. Points are mean ± S.D. of maximum response, and response after 3 minutes. Figures in brackets refer to number of determinations in each solution used to calculate means. Percentage changes are also shown for increase in amplitude, and decrease of fatigue after solution changes.

Changes in tetanic response in potassium free solution

Diaphragm	Increase in maximum tetanic amplitude %	Decrease in fatigue %
A	8.3	19.3
B	7.8	9.3
C	6.8	0.0 *
D	13.2	6.5
E	8.1	11.0
F	13.0	0.0 *
G	7.8	5.1
H	11.0	3.8 *
I	6.9	8.4
J	9.3	4.3 *
K	12.6	6.0
L	7.6	8.5

Table 7.1. Changes in maximum amplitude, and fatigue during 3 minute tetanic stimulation, produced by a change from normal Krebs solution to potassium free bathing solution. All changes significant at less than 0.01, except those marked *. (Student's 't' test, correlated data).

in sustaining power, and four did not. The mean change in tetanic amplitude obtained was 9.4%. All of the amplitude changes were significant with $p < 0.01$ (correlated t test).

The increase in sustaining power expressed as a percentage change from the control value in normal solution varied between no change and 19%. Results are summarised in Table 7.1.

The effect on sustaining power was variable in magnitude between diaphragms and not consistent from trial to trial in any one diaphragm. It was most obvious after the first solution change and thereafter tended to reduce in size. However, when the sustaining power of the tetanus at the end of a series of solution changes was compared with that of the tetanus at the start of the experiment, it was found always to have increased. The sustaining power in normal solution at the end of the experiment was always better than that at the beginning, tending to reduce the differences between normal and potassium free responses. As the experiments were often continued for periods of 3-5 hours, a cumulative long-term effect of potassium free exposure on sustaining power is suggested.

The effect of removal of potassium ions from the medium, on the mechanical response of the muscle, might be expected to be a decrease in isotonic tetanus amplitude. Goffart and Ritchie (1952) have shown potassium ions to increase the active state of the muscle, and hence complete removal could be expected to cause a decrease in the contractile properties. The result obtained therefore is an enhancement despite a predictable decrease in active state of the muscle.

To exclude any direct effects on the muscle, the experiment was repeated using direct muscle stimulation in the presence of

d-tubocurarine such that neuromuscular transmission was fully blocked. No enhancement was obtained. The experiments however were not completely satisfactory owing to the practical difficulties of using stimuli of long duration and large amplitude necessary to stimulate the muscle directly.

With indirect stimulation the tetanic enhancement was essentially reversible on returning to solutions containing normal potassium, although after prolonged exposure to potassium free solutions the effect appeared to be less reversible. To further investigate these somewhat qualitative results and also to determine more accurately the time course of the effect, an improved method was devised as a development of the basic experimental procedure.

The procedure used was cyclic stimulation of the phrenic nerve for a set period at 1 Hz followed by a short tetanic train of stimuli at 50 Hz, as described in Methods II. In the initial experiments these periods were 7 minutes and 3 minutes, respectively. By changing the periods to 55 sec and 5 sec, i.e. 10 complete cycles in the time taken for one previously, a marked improvement in the repeatability and quantitative nature of the results was obtained.

The lengths of the two periods were determined mainly by trial and error until it was considered that a satisfactory response was obtainable without undue fatigue occurring after prolonged stimulation. In normal Krebs solution, none of the characteristics of the record showed any change after many hours of stimulation using this cyclic technique, provided adequate oxygen and glucose were supplied. For further details of the method, reference should be made to Methods II.

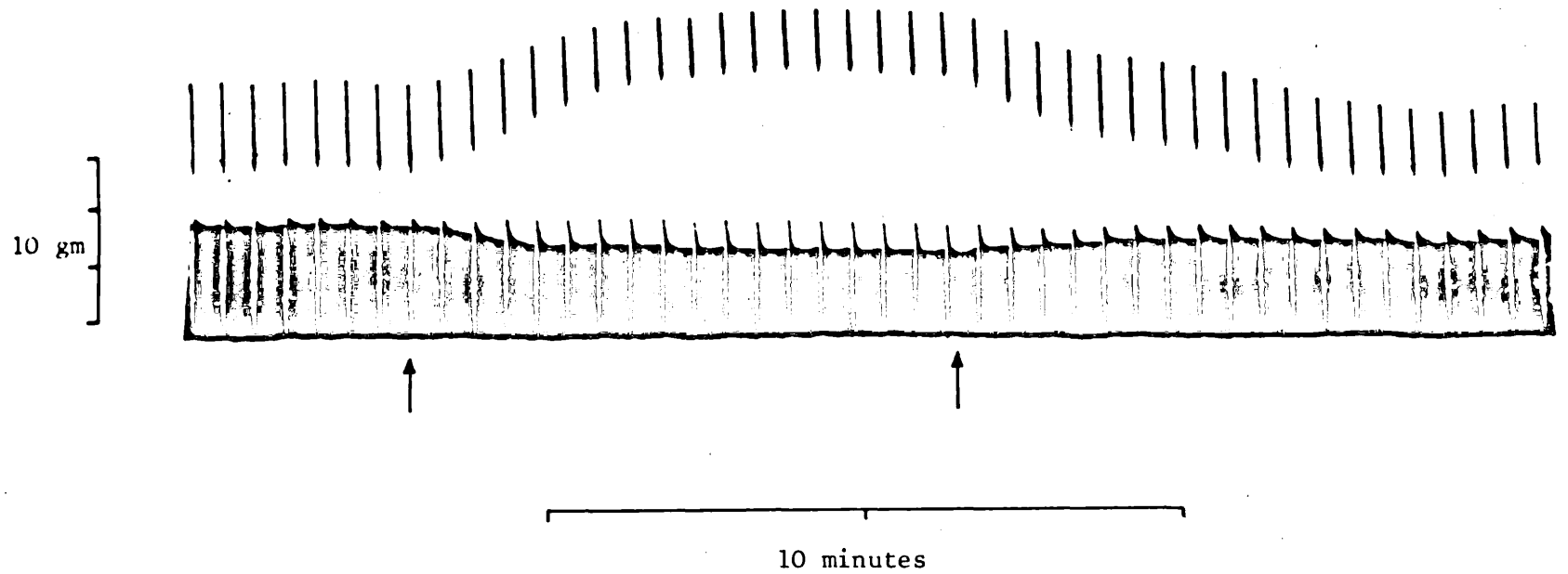


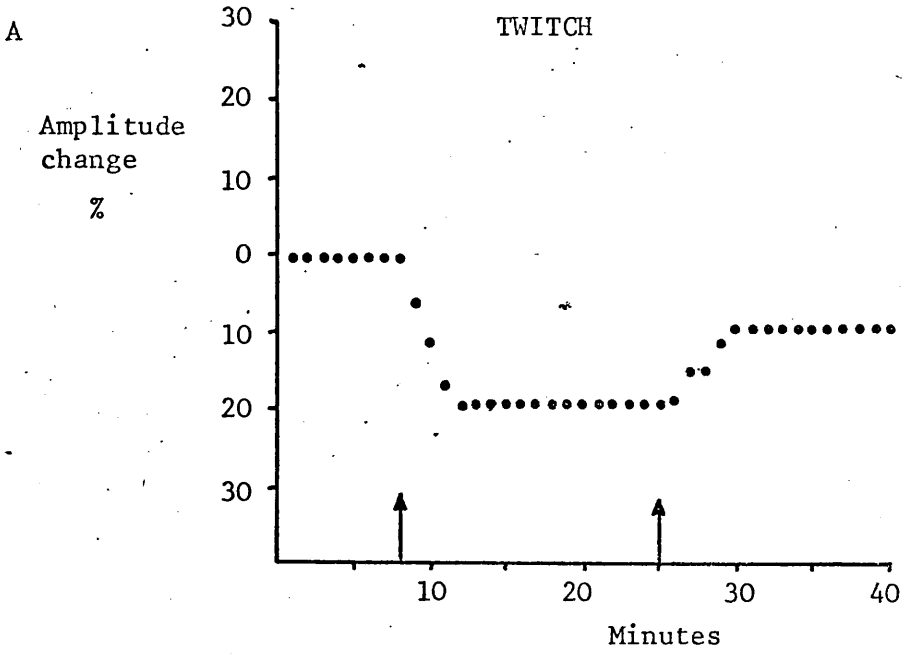
Fig. 7.3. Record of isometric twitch and tetanic response produced by the rat diaphragm stimulated through its phrenic nerve. Stimulation was 1 Hz for 55 seconds and 50 Hz for 5 seconds with the cycle repeated. At the point shown by the first arrow the solution was changed to potassium free. Potassium re-admitted at the point shown by the second arrow.

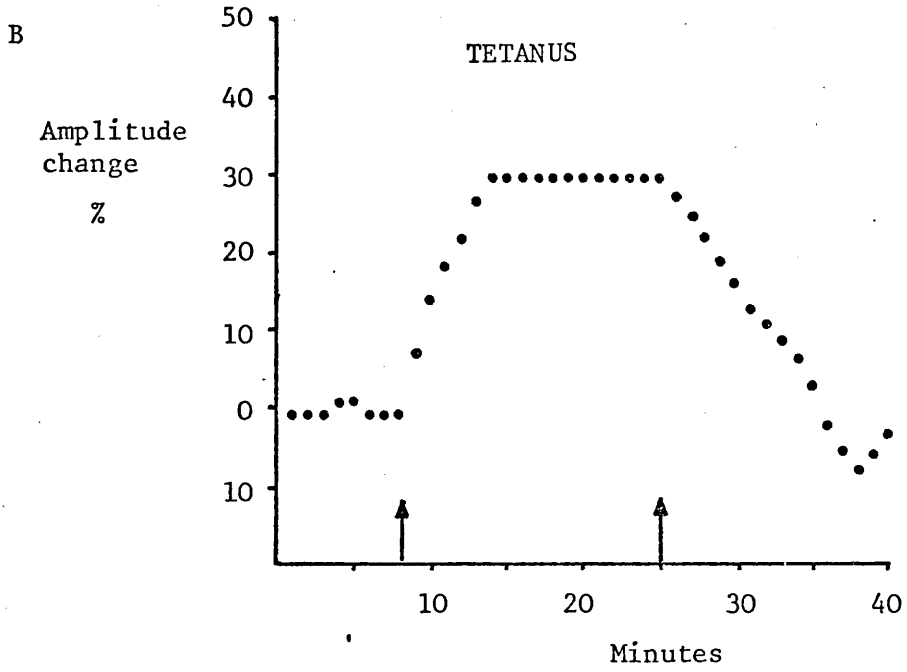
The change in the record obtained with substitution of potassium free Krebs solution for normal Krebs solution is illustrated by the chart record shown in Fig. 7.3. At the points indicated on the record the solution in the bath was rapidly and completely changed with no halt in the stimulation or paper drive.

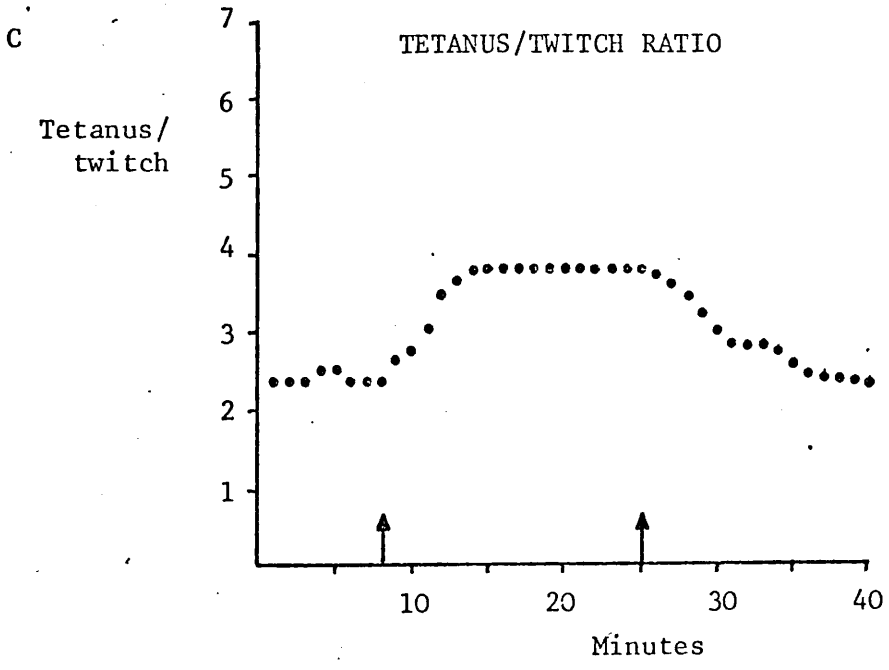
The lower markings on the trace are the results of the 55 twitch stimuli per cycle with contraction in an upward direction from the base line. Due to the characteristics of the pen recorder, the sweep up to the highest point (the start of the tetanus) does not show on the paper, but the slow decline causes a mark to appear. Likewise, the rapid return to the base line after cessation of the tetanic stimulation also produces no marking. Thus the upper line of marks represents the holding ability of the tetanus. The drop in height of the trace from the start to the end of tetanic stimulation will be defined as 'droop'. The length of the line represents the drop in tetanic tension, thus a shorter line indicates a more sustained muscle contraction, and an increase in length represents a more rapid decline of the tension under tetanic conditions.

A number of changes in the characteristics of the trace may be identified after a change from normal to potassium free Krebs bathing solution. These changes may be represented graphically as in Fig. 7.4.

Fig. 7.4a. shows the change in twitch amplitude obtained during exposure to potassium free solution and is consistent with the earlier remark about the effect of potassium on the active state of the muscle, thus on exposure to potassium free solution, the twitch amplitude is seen to be smaller than in the normal state. This effect did not become manifest in the earlier experiments to the degree obtained with the present method.


A
TWITCH
Amplitude change %
30
20
10
0
10
20
30
10
20
30
40
Minutes


B
TETANUS
Amplitude change %
50
40
30
20
10
0
10
10
20
30
40
Minutes


C
TETANUS/TWITCH RATIO
Tetanus/ twitch
7
6
5
4
3
2
1
10
20
30
40
Minutes

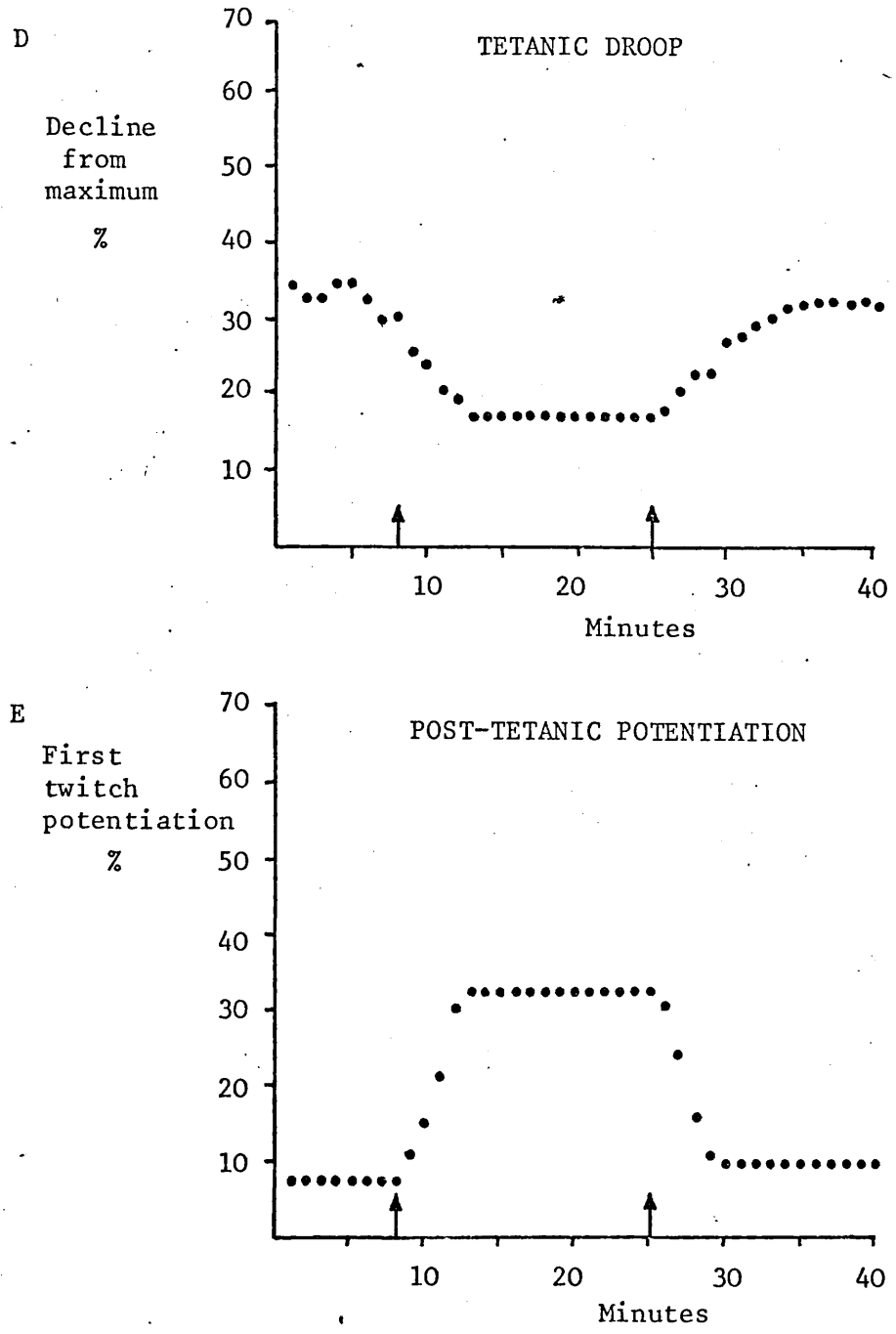


Fig. 7.4. Changes in mechanical response characteristics of the preparation when exposed to potassium free bathing solution. Solution was changed to potassium free at the point indicated by the first arrow in each case, and to normal Krebs solution at the second arrow. Changes are expressed as percentages and relate to the record shown in Fig. 7.3. For further explanation see text.

Fig. 7.4b. represents the improvement in tetanic tension obtained, and as in the earlier experiments, a marked increase is seen to be obtained throughout the period of exposure to potassium free solution. In the smoked drum experiments it was difficult to determine the onset of the potassium free effect. The solution was changed and then after waiting an indeterminate period for equilibration, a tetanic effect was elicited. It can be seen from the figure that the effect may be followed from onset to maximum tetanic amplitude, at which point the tension remains at a constant amplitude until potassium is re-admitted, thus a much greater degree of accuracy and repeatability is obtained.

The twitch/tetanus ratio shown in Fig. 7.4c. gives an indication of the increase in tetanic tension over twitch tension and thus takes into account the fall in the twitch tension due to decline of active state occurring after removal of potassium.

Fig. 7.4d. represents the change in tetanic 'droop'; as stated previously this is the change in tetanic amplitude over the period of the 5 sec. tetanus. This is the parameter that was difficult to investigate in the first experiments. Using the current method it may be now seen to be consistently decreased during the period of lowered potassium. It is expressed as a percentage ratio of the change in amplitude over the 5 secs to the total tetanic amplitude initially produced.

Fig. 7.4e. shows the change in post-tetanic potentiation (P.T.P.) or depression (P.T.D.). This is the change in twitch amplitude following a tetanic train and depending on duration of the tetanuc may be either an augmentation or depression of twitch amplitude. Long tetani usually produce a depression probably

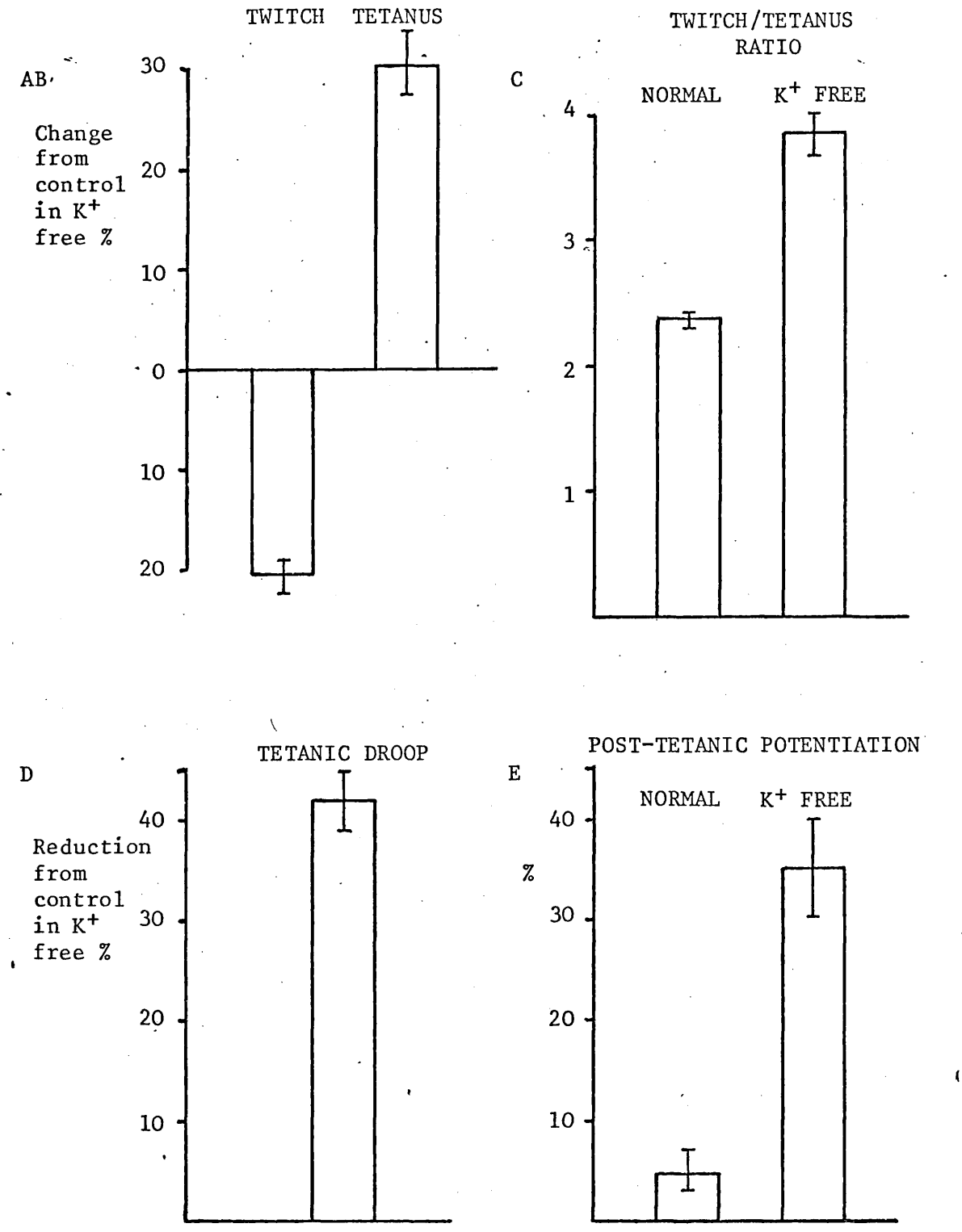


Fig. 7.5. Summary of changes in the characteristics of the mechanical response of the preparation as in Fig. 7.3. and Fig. 7.4. AB. Change in twitch amplitude and maximum tetanic amplitude as a percentage change from control. C. Twitch/tetanus ratio in normal and potassium free solutions. D. Reduction in tetanic droop expressed as a percentage reduction from control. E. Post-tetanic potentiation in both solutions. Data from 10 experiments. Values are mean $\pm$ SE. All changes significant p less than 0.001 (t test).

linked with transmitter exhaustion. Short tetani usually produces an augmentation. (Hughes 1958). Some of the early theories about the nature of P.T.P. link the effect to hyperpolarisation or changes in potassium concentration. (Lloyd 1949, Lilley & North 1953, Gage & Hubbard 1966). Recently however calcium entry has been shown to be directly linked to the phenomenon of P.T.P. (Weinreich 1971, Rahamimoff 1968, Hubbard et al 1971). The P.T.P. observed in these experiments may possibly be an artifact due to a transient removal of the active state depression following potassium release from the muscle during tetanic stimulation. However, the fact that following prolonged exposure to potassium free solutions, it was observed that the P.T.P. remained even after twitch size had returned to normal seems to argue in favour of a true P.T.P. produced in some way by exposure to potassium free solutions.

The procedure was repeated many times both during the development and testing of the cyclic stimulation apparatus and later when experiments were made just to test the effect of potassium free solution. In every case the results were entirely repeatable, both between trials on the same preparation or on separate preparations. The results are summarised in Fig. 7.5.

The net result of these experiments is to suggest that the effects are maybe due to a change in transmission phenomena from the nerve to the muscle associated with an increase in transmitter output causing an enhancement of tension rather than a physico-mechanical effect on contraction.

Chapter VIII

Preliminary Electrophysiological Investigations

Measurements of refractory period

Increased potassium in the extracellular fluid surrounding the fine nerve branches during muscle contraction could affect the refractory period of these structures. At high stimulus rates during a tetanus, the nerve might only let a certain percentage of the impulses through to the end-plate. Krnjevic and Miledi (1959) suggested that the cause of transmission failure during tetanic stimulation could be intermittent conduction block in the intramuscular portion of the nerve. The cause of the block was considered to be oxygen lack in this part of the fibre. The ultimate effect would therefore be reduction of ionic pumping followed by depolarisation. At a stimulation frequency of 50 Hz, block of conduction by an increase of refractory period would require this to be extended to 20 msec or approximately ten times normal. A large change such as this should be easily observable.

Krnjevic and Miledi found that when muscle contraction was prevented, block was less frequently produced by short periods of stimulation. In the present experiments, an enhancement of tetanic sustaining power was demonstrated during short periods of stimulation with the cyclic procedure under potassium free conditions. It is unlikely that during the short tetanus period in these experiments, oxygen lack was occurring to any great extent. The removal of potassium is therefore unlikely to be an antagonism of such an effect. A further explanation was therefore sought, and the effect of low extracellular potassium on the refractory period of the paralysed

preparation was investigated.

Measurements were made of the refractory period of the system by recording e.p.p.s with micro-electrodes, from the curare blocked muscle end-plate. The shortest time interval between two stimuli given to the nerve in succession, which still produced e.p.p.s was taken as a measurement of the refractory period. Measurements made at a total of seventy two end-plates in both normal and potassium free bathing solutions showed surprisingly little variation. Although a range of refractory periods was obtained from 1.7 to 3.8 msec with a mean of 2.3 msec, the difference between measurements made in the two solutions at any one end-plate was not significant. Similarly, even after prolonged tetanic stimulation, the refractory period between test pulses rarely exceeded 6.0 msec. With O_2 lack however, even to a very mild degree, the refractory period rapidly approached the significant 20 msec and nerve block occurred.

The oxygen supply to the nerve terminals is reduced during periods of tetanic contraction, both in vivo and in vitro. Prolonged contraction will stop blood flow in muscle in vivo and may reduce access of the dissolved oxygen from the bathing solution in vitro. Removal of potassium appeared to have little effect on the lengthening of the refractory period by oxygen lack, furthermore the effect of oxygen deprivation would be similar in both solutions. The effect of potassium lack therefore does not appear to be an antagonism of the effect of oxygen lack.

The measurements were made under conditions of curare blocked end-plates. The loss of potassium from the muscle, caused by activity, was therefore prevented in these experiments. Thus,

testing for changes in refractory period from normal to possibly shorter than normal by removal of potassium, is different from the experimental situation where the refractory period has possibly been increased by excess potassium and reduced to more normal levels by removal of potassium. These conditions cannot be exactly reproduced in a test situation as it is impossible to record from a functioning muscle fibre without causing damage. As no examples of change in refractory period were obtained which could be related to change in potassium levels, refractory period variation was therefore excluded as a major contributing factor.

Resting potentials in potassium free solution

The normal functioning of nerve or muscle cells is intimately related to the magnitude of the potential difference across the cell membrane known as the resting potential. This potential is determined mainly by the distribution of potassium between the inside and outside of the cell. Considering potassium only; an electrochemical gradient exists with an equilibrium potential given by the Nernst equation:

$$E = \frac{RT}{F} \log_e \frac{K_o}{K_i}$$

where

E = equilibrium potential

R = universal gas constant

T = absolute temperature

F = Faraday's Constant

K_o = external potassium concentration

K_i = internal potassium concentration

In practise, although the resting potential is close to the potassium equilibrium potential and governed by changes in it, the actual value is always less negative. This is caused by a small

permeability of the membrane to other ions, especially sodium. A more accurate calculation of the resting potential takes these permeabilities into account. Thus, the value of the resting potential may be obtained from the formula:

$$E = \frac{RT}{F} \log_e \frac{K_o + a\,Na_o}{K_i + a\,Na_i}$$

Na_o = external concentration of sodium

Na_i = internal concentration of sodium

a = ratio of sodium and potassium permeability

The constant a is the ratio of the permeability of sodium to that of potassium. The calculation therefore takes into account the leakage of sodium into the muscle fibre down both its electrical and chemical concentration gradient. For frog muscle, the sodium permeability appears to be about 1 - 1.5% of the potassium permeability. A value for a would thus be 0.01 - 0.015. Using this figure, the measured resting potential agrees well with the value predicted from the concentration of sodium and potassium. (Hodgkin & Horowicz 1959).

Removal of potassium from the bathing solution will result in some degree of hyperpolarisation. The increase in the potassium equilibrium potential calculated from the Nernst equation will be quite large. The increase in resting potential will however be limited by the effect of the sodium permeability and also by the effect of low external potassium concentration on the potassium permeability. This is known to reduce when the potassium on the outside of the membrane is low. (Hodgkin & Horowicz 1959, Adrian 1960, Adrian & Freygang 1962). When the external potassium concentration is low, the effect of sodium ions will be dominant and

thus the equation for the resting potential predicts that a limiting value will be reached governed by the resting permeability to sodium and the external sodium concentration.

The resting potential of the rat diaphragm muscle fibres was monitored during the experimental determination of the refractory period of the preparation. It was found that the fibres hyperpolarised by about 25 - 30 mV when exposed to solution devoid of potassium ions. Many of the properties of the muscle and nerve are linked with the resting potential and thus it was considered necessary to determine the change accurately. In addition, by plotting the changes of resting potential in different concentrations of potassium, some value for the actual potassium concentration around the muscle fibres in potassium free solution can be obtained. This concentration will also be the effective potassium concentration around the nerve terminals which will govern their behaviour in potassium free solution.

A range of potassium concentrations between 0mM and 10mM was used. Determinations were made in three separate diaphragms and measurements were taken from approximately fifty fibres in each solution after allowing 5 min for equilibration. The procedure took about 15 min for each solution and about 4 hr overall. Determinations in potassium free solution were made, both at the start and at the end of the range of concentrations. Thus, any loss of internal potassium throughout the period could be determined. The length of time taken for the procedure, although relatively long for this sort of determination if changes in internal concentrations are to be avoided, was similar to that taken for the mechanical response experiments. Thus, any slowly occurring changes in these experiments would also have been occurring in the resting potential determinations and would possibly show in variation in resting potentials obtained at

the start and finish of the investigation. There was however no significant difference between the hyperpolarisations obtained once the preparation had equilibrated with the solution. This suggests that after an initial loss of internal potassium, after dissection and placing in the oxygenated Krebs solution, no further change occurred.

A steady increase in resting potential between 10 mM external potassium and 0 mM was obtained as expected. In potassium free solution some particularly high (120-130 mV) values were observed and also some very low (30-50 mV) resting potentials, indicating a wide effect of the potassium free solution on the permeability characteristics of the muscle fibres. The majority of the fibres in the sample hyperpolarised to between 90-100 mV within 3-4 min of solution change, thereafter remaining at a constant potential.

The observations were pooled and the resting potential plotted against the logarithm of the external potassium concentration producing the graph in Fig. 8.1.

The graph shows the change in resting potential observed between 10 mM and 0 mM external potassium concentration in the bathing solution. The solid line is a theoretical change of resting potential assuming 2.2% sodium permeability and 149 mM internal potassium concentration. These values were arrived at by fitting the series of points to the observed resting potentials. Creese (1954) gives values of 158mM for in vivo internal potassium and 149mM for in vitro potassium, in preparations of the rat diaphragm. The in vitro concentration was shown to be dependent on oxygenation, quite small changes in oxygenation tension having a marked effect on potassium loss. An internal concentration of 50 mM for intracellular sodium was used, also given by Creese (1954).

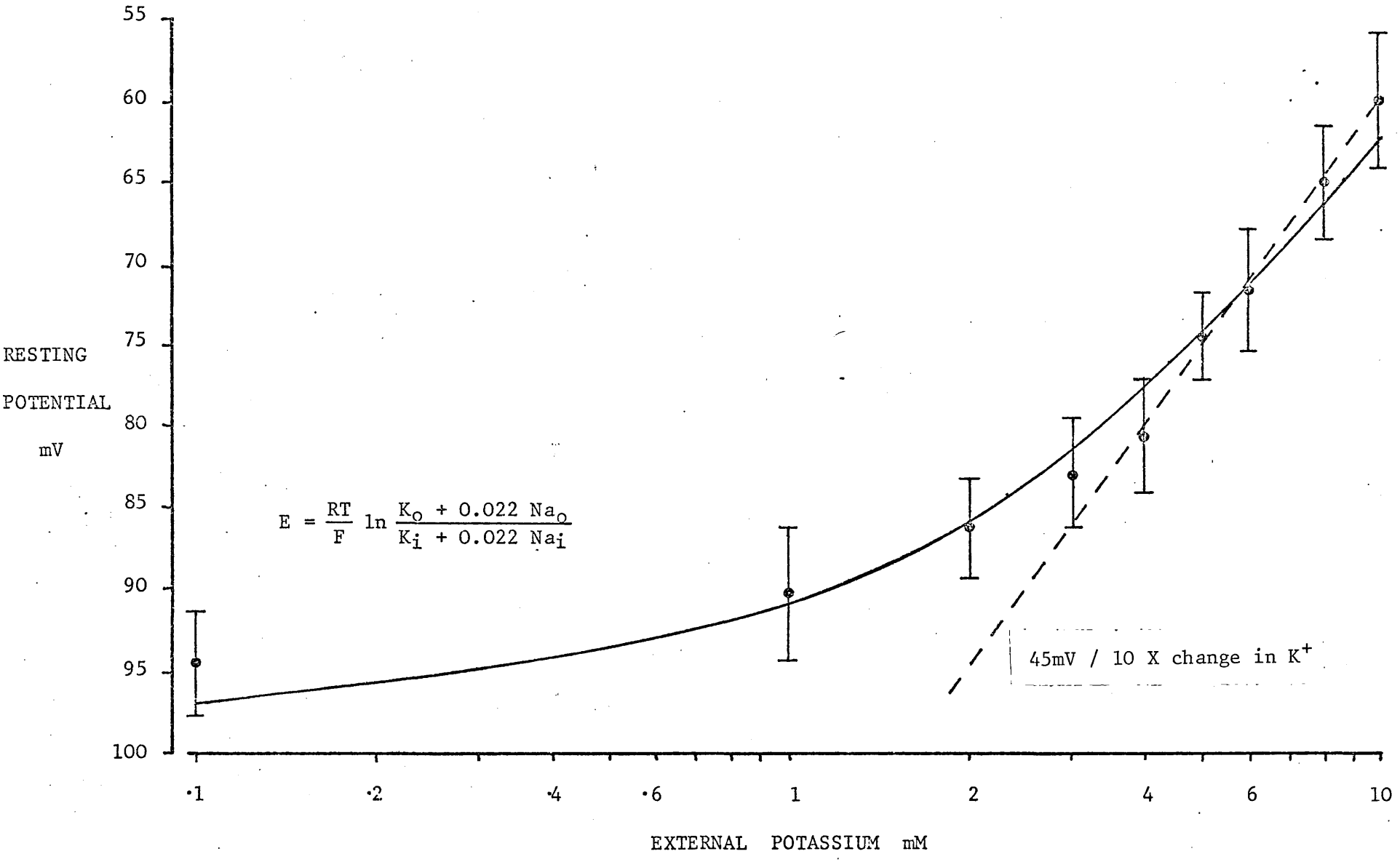

55
60
65
70
75
80
85
90
95
100
RESTING
POTENTIAL
mV
$E = \frac{RT}{F} \ln \frac{K_o + 0.022\ Na_o}{K_i + 0.022\ Na_i}$
45mV / 10 X change in K^+
·1
·2
·4
·6
1
2
4
6
10
EXTERNAL POTASSIUM mM

Fig. 8.1. Resting potential of diaphragm muscle fibres in Krebs solutions of potassium concentration ranging from no added potassium up to 10 mM. Points plotted are mean $\pm$ S.D. Data from 160 fibres in 3 muscles. Broken lines fitted by least squares to points between 4 mM and 10 mM. Solid line calculated from equation shown, (Hodgkin & Horowicz 1959). Ordinate: resting potential of fibre in mV. Abscissa: logarithmic scale potassium concentration.

Using these values, a resting potential in normal solution of 74.5mV is predicted, which agrees with the observed value if 2.2% of the potassium permeability for sodium is accepted. This value is higher than the data for frog muscle would suggest, but in the absence of figures for the diaphragm muscle, the fit to the observed values must be considered a good approximation. Using this theoretical resting potential curve it would appear that the effective potassium concentration of the fluid surrounding the nerve terminal in potassium free solution is approximately 0.5mM. It is certainly no greater than this. It may be less if the low external potassium concentration reduces the potassium permeability, thus making the sodium permeability relatively larger and more effective in modifying the resting potential. The addition of Ouabain 1.5×10^{-5} g/ml or Digoxin 1.5×10^{-5} g/ml had no effect on the resting potential increase. Because these drugs block the sodium pumping mechanism in the membrane it is unlikely that the sodium transport mechanism is involved in producing the hyperpolarisation observed.

Between 10 mM and 4 mM the resting potential is linearly related to the logarithm of the external potassium concentration, as shown by the broken line in Fig. 8.1. At these potassium concentrations the potential is determined by the extracellular potassium and changes by 45mV for 10 fold change in K^+ concentration. Below 4 mM the potential deviates from a direct dependence on potassium, and the effect of the sodium permeability becomes significant, causing the slope of the dependence on potassium to reduce and eventually tend to level off below 1 mM.

In order to ascertain whether any changes occurred after prolonged exposure to potassium free solution, a second series of measurements were obtained. The resting potential was determined in

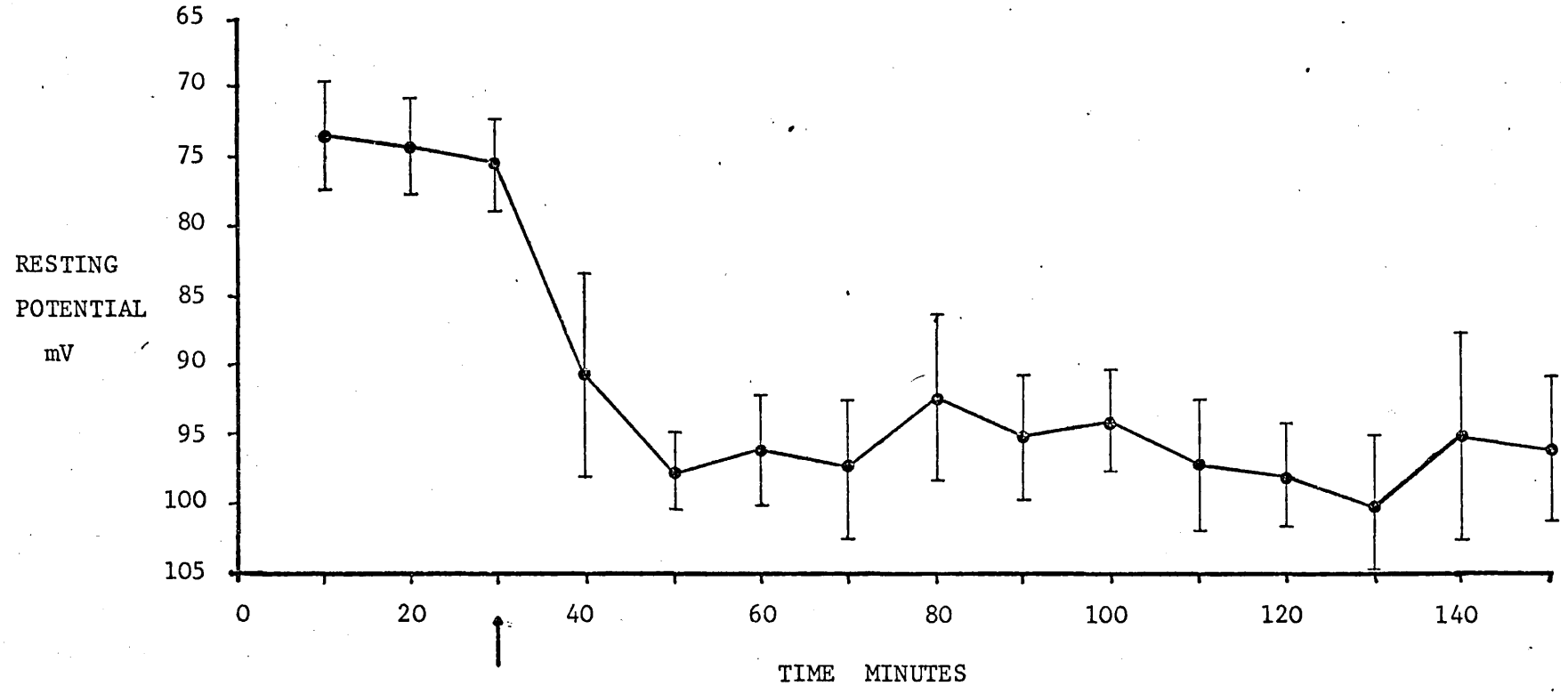


Fig. 8.2. Resting potential of muscle fibres in potassium free solution plotted against time of exposure. Solution was changed at time indicated by arrow. Points are mean ± S.D. Values calculated from a minimum of 100 fibre measurements per point, data from 4 muscle preparations.

normal solution. The solution was then changed to potassium free and measurements were made for a period of 2 hrs. This period is longer than the time for which the preparation was exposed in any other procedure to potassium free solution with no change. Measurements were made in four muscles. Recordings were made continuously and the measurements then grouped into 10 min intervals. About 30 separate fibres could be measured accurately in this interval. The results from the different muscles were then pooled and the resting potential plotted against time. From Fig. 8.2. it can be seen that after equilibration with the solution, there is little change in the resting potential attained. It is evident therefore that no rapid loss of internal potassium occurs resulting from removal of the external potassium. This observation makes unlikely the possibility that there is an increase in the potassium permeability of the fibres, and possibly a decrease may occur.

The change in resting potential described will have a marked effect on the e.p.p. amplitude produced by the action of the transmitter on the postsynaptic membrane. Fatt and Katz (1951) have shown a linear relationship between e.p.p. amplitude and resting potential. By electrically polarising the muscle fibre membrane they obtained increases in effective resting potential and showed that a parallel rise occurred in the e.p.p. amplitude. This relationship was shown to hold over a wide range of resting and e.p.p.s. By knowing the e.p.p. and the resting potential in the normal state, predictions may be made of the changes expected at different membrane potentials.

At normal resting potential levels the maximum attainable e.p.p. E_e is the transmitter equilibrium potential. From considerations of the relevant ion equilibrium potentials, a value for the potential

attained, even though the membrane is completely porous to all cation species (during the effect of the transmitter), may be derived. (Nastuk & Hodgkin 1950). A value of about 15mV is usually accepted for normal solution concentrations. The driving potential V_d, effective during the action of the transmitter is therefore the difference between the resting potential and the transmitter equilibrium potential.

$$V_d = (E_r - E_e)$$

Thus, the driving potential will determine the amplitude of the e.p.p. and a larger driving potential will lead to an increase in e.p.p. produced.

One of the reasons given by Krnjevic and Miledi (1958) for transmission failure during prolonged stimulation was an increase of muscle end-plate threshold. The increase in resting potential produced by the potassium free solution could be overcoming this rise in threshold. Thus, with a larger driving potential the increase in e.p.p. will be such that during a tetanic train of impulses, the reduction in size caused by potassium build up will be counteracted and more e.p.p.s will be above threshold and therefore able to excite the muscle fibre. Even in the case of lower transmitter output which is probably the cause of the decline in tension during the period of the tetanus, the e.p.p.s will be maintained at a larger than normal amplitude by the hyperpolarisation of the postsynaptic membrane. The proportion of e.p.p. above threshold then will always be greater in the potassium free solution.

The characteristics of the m.e.p.p. and the e.p.p. were therefore investigated under both the normal and potassium free conditions.

Miniature end-plate potential measurements

A change in muscle end-plate sensitivity in potassium free solution at rest should be revealed by the recording of larger than normal m.e.p.p.s. The basic quantal unit of the e.p.p. has been shown to remain at a relatively constant amplitude under a variety of different conditions and no authors have yet shown an actual increase in quantal size due to polarisation or lower potassium concentration. Katz and Thesleff (1957) demonstrated that the amplitude of the intracellularly recorded m.e.p.p. was not constant when the results from different junctions were compared. It was also shown however, that this variation in amplitude at different junctions was caused by a parallel variation in muscle fibre size, and therefore passive cable properties. Thus, the amplitude of the recorded m.e.p.p. will depend on the diameter of the fibre innervated. At any one end-plate however, the size of the quantal unit should remain constant throughout, assuming no change in the muscle fibre cable properties during the period of the recording. Assuming this constancy of quantal size, the m.e.p.p. should be an ideal natural index of e.p.p. sensitivity without the interference of any agents such as blocking drugs.

The m.e.p.p.s were recorded on moving film by photographing the oscilloscope screen. The frequency was also monitored independently by counting occurrences during a set length of time. The period varied with discharge frequency but it was endeavoured to always count at least one hundred events for frequency determination.

The m.e.p.p. amplitudes were plotted against the number in each class and distribution plots obtained as shown in Fig. 8.3.

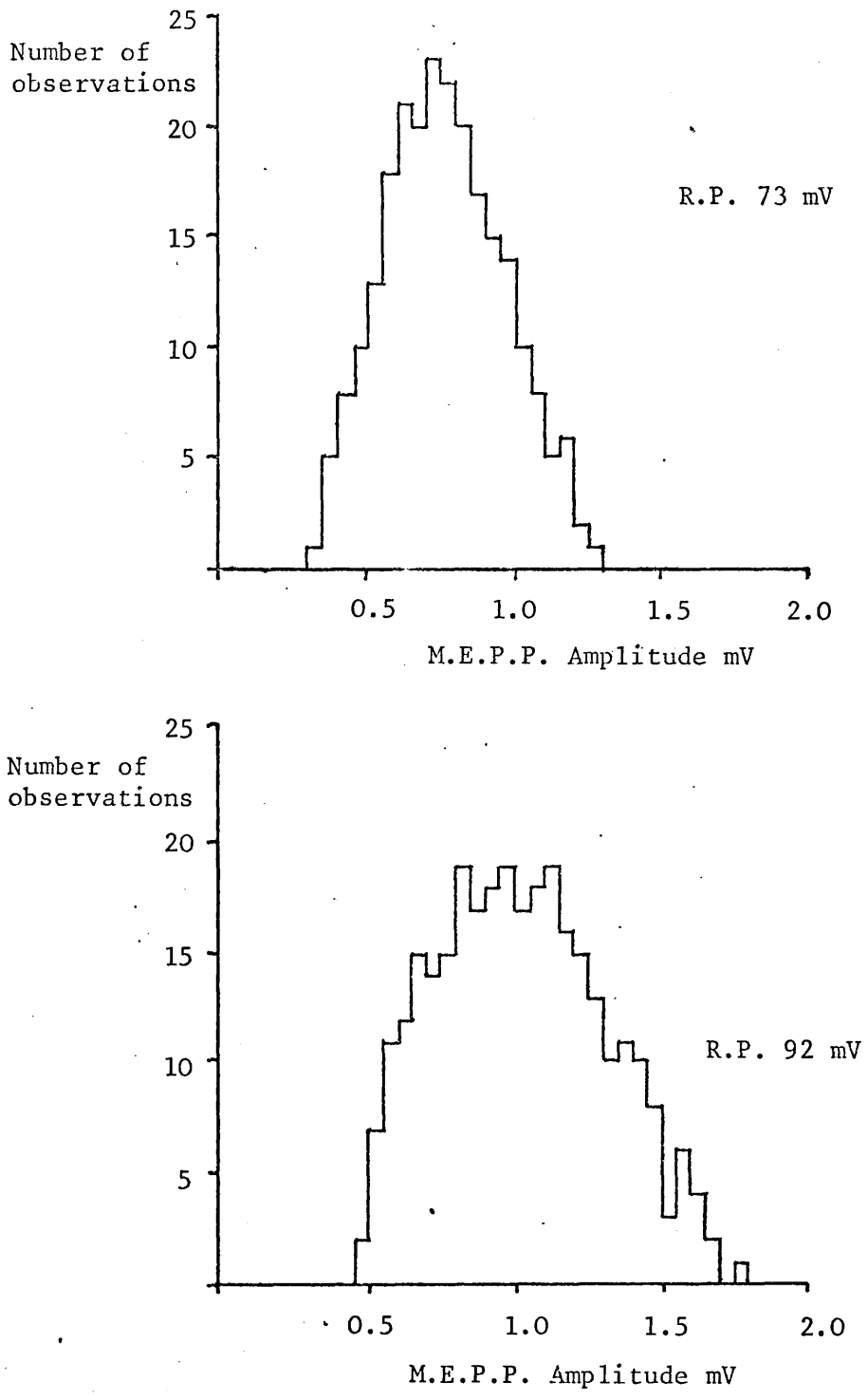


Fig. 8.3. Histogram plot of m.e.p.p. distribution. Upper figure is amplitude in mV plotted against number of m.e.p.p.s of that amplitude in normal solution. Lower figure is distribution plotted in same manner from same end-plate in potassium free solution.

The method used for recording m.e.p.p.s from junctions in the two solutions was to impale the muscle fibre at regions thought to be nerve muscle junctions, searching for normal shape and size m.e.p.p.s. When such a region was found, where m.e.p.p.s could be recorded with amplitudes of approximately .5mV or greater, recordings were made of about 100 events and a careful note was made of the position. The procedure was repeated until ten or twelve junctions were located and a map was obtained of the muscle surface. The electrode was kept positioned at the last located junction and the solution changed. The junctions were then re-located and further recordings made in the second solution. Thus, during each solution change, one electrode position had been left undisturbed. When the results were analysed, large variations in mean amplitude were obtained and no consistent trend in frequency changes could be seen. If, only those junctions where the electrode had been left undisturbed were used for analysis, trends in both amplitude and frequency changes were detectable. Further experiments were therefore carried out, each time only using one junction and leaving the electrode undisturbed. Small variations in electrode tip position were thought to be the cause of the variability in the first experiments of this type, even though no obvious change was seen on returning to the junctional site. Results obtained from junctions in which the electrode was left undisturbed between solution changes are given in Table 8.1.

The mean rise in resting potential obtained in the potassium free solution was 20mV. This increase in resting potential should cause a rise in the mean m.e.p.p. amplitude of 150 uV, if the relation between rise in resting potential and rise in e.p.p. holds. (Fatt & Katz 1954). From Table 8.1. it can be seen that a rise of m.e.p.p. has occurred at a number of junctions. The variation in m.e.p.p. amplitude appeared to be larger when the muscle was immersed in potassium free

Miniature End-plate Potentials in Normal & Potassium Free Solutions

End-Plate	Normal Krebs Solution			Potassium free solution		
	RP mV	Amp. mV	Frequency Events/sec	RP mV	Amp. mV	Frequency Events/sec
A	75	0.74	0.93	93	1.0	0.72
B	74	0.56	0.63	99	0.78	0.58
C	75	0.63	0.86	95	0.77	0.83
D	76	0.80	1.8	98	1.0	0.5
E	70	0.59	0.73	89	0.61	0.28
F	73	0.71	0.4	96	0.73	0.3
G	74	0.68	1.5	92	0.87	0.4
H	69	0.55	2.9	89	1.0	2.0
I	71	0.66	1.2	94	0.87	1.0
J	73	0.51	0.97	97	0.75	0.94
K	75	0.84	1.3	98	1.2	0.8

Table 8.1. Comparison of amplitude and frequency of occurrance of m.e.p.p.s in normal and potassium free bathing solution. All results were obtained from single end-plates with no disturbance to the recording electrode during solution change. Determinations of mean amplitude and frequency of occurrence were made from not less than 100 successive events.

solution. This increase in variation may have obscured such a small rise of 150 uV at some end-plates.

In no case was there a very large increase in m.e.p.p. amplitude, all of the increases being within the range predicted from the increase in the resting potential. The results suggest that potassium free solution does not cause an increase in muscle end-plate sensitivity to acetylcholine (under resting conditions) and so lead to larger e.p.p.s by an enhancement of the transmitter action on the postsynaptic membrane.

The frequency of m.ep.p. occurrence is also set out in Table 1 and it is evident that there is a reduction in potassium free solution. This effect is similar to that obtained by Lilley (1956), Takeuchi & Takeuchi (1961) and Hubbard & Willis (1962) under conditions in which both lowered potassium and hyperpolarisation of the nerve end was used.

A relationship between m.e.p.p. frequency and the logarithm of the external potassium concentration was demonstrated. It is well known that an increase in the external potassium concentration, leading to depolarisation, increases the m.e.p.p. frequency. Decreases in frequency under conditions of hyperpolarisation above the normal resting potential have not been substantiated (Landau 1969). When the junction is hyperpolarised from an initially depolarised state, a reduction of frequency is observed, this reduction then reaches a limiting value. It was suggested by Landau that a decrease in frequency caused by hyperpolarisation would only occur if the nerve terminal was initially marginally depolarised, even if in normal Krebs solution. As it is now considered that spontaneous generation of m.e.p.p.s is under the control of intracellular free calcium ions (Baker 1974), reduction in frequency has to be explained by some mechanism involving this ion.

The nerve terminals in the present experiments may have been initially depolarised, although in normal bathing solution, enabling the hyperpolarising effect of the potassium free solution to return the resting potential to normal. The result obtained suggests that the resting potential in the nerve terminal does rise when exposed to potassium free solutions, though whether or not it is initially depolarised is difficult to decide.

Measurement of end-plate potentials

Following the investigations into the effects of potassium free solutions on the amplitude and frequency of m.e.p.p.s, the amplitude of evoked e.p.p.s was determined in normal and potassium free solutions. d-Tubocurarine was used to block meuromuscular transmission and a level of between 1.8 and 2.0ug/ml was usually used. The blocking agent was added to the Krebs solution and care was taken to keep the level in both solutions constant throughout an experiment.

Measurements were made, at a stimulation frequency of 1 Hz, at a number of junctions in the preparation, after the muscle had ceased to twitch. The solution was then changed to one containing no potassium and measurements were made at the same junctions. The statistical fluctuations due to difference in quantal content are small in the curarised preparation, however, an average of at least 10 e.p.p.s was used to determine the amplitude at each junction.

The e.p.p.s in the potassium free solutions were found to be consistently larger than those obtained at the same junctions in the normal Krebs solution. Fig. 8.4. In some cases the enhancement of the e.p.p. was such that the threshold for the muscle fibre was

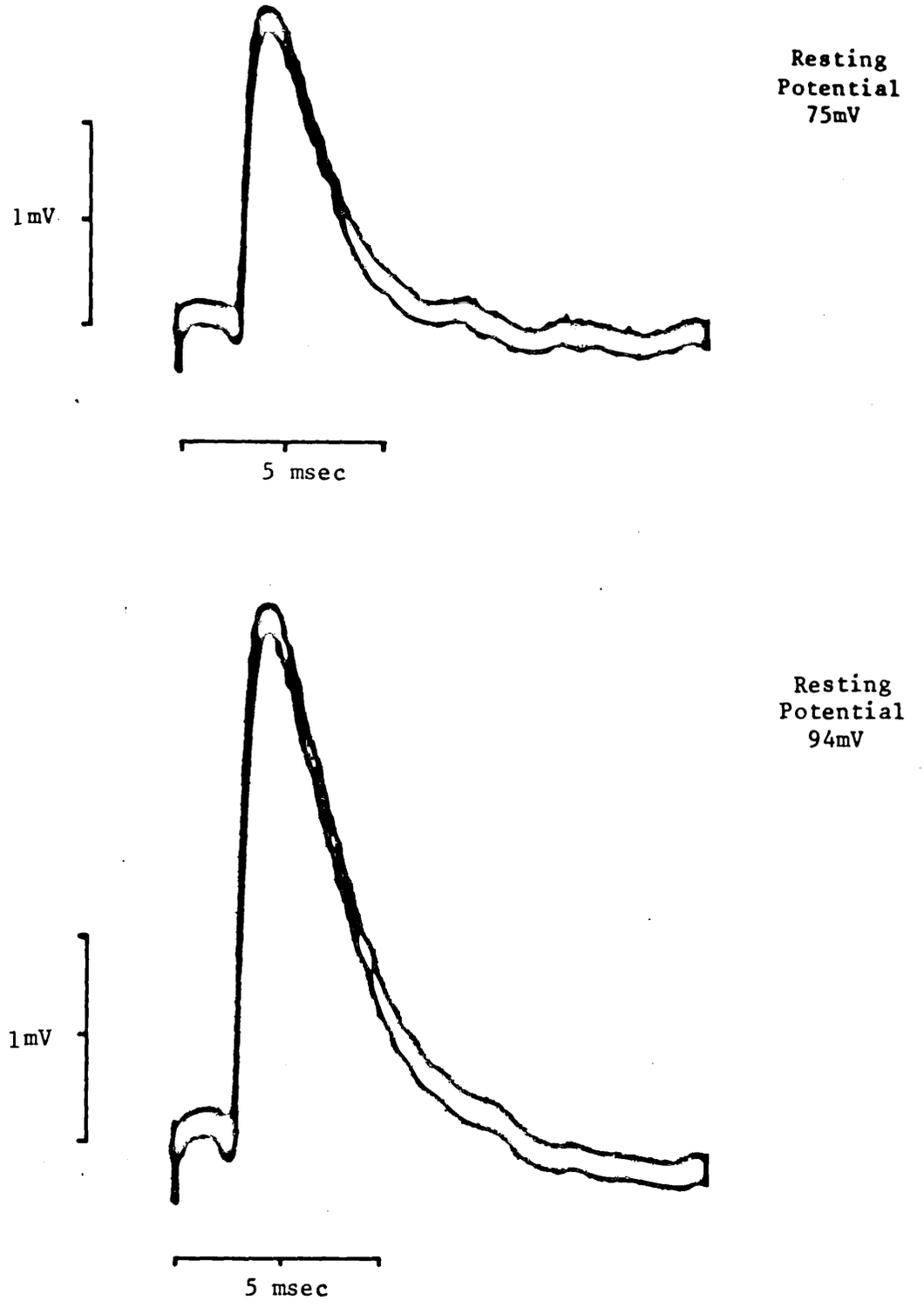


Fig. 8.4. End-plate potentials recorded from the same end-plate in normal and potassium free solution. Traces were photographed from the oscilloscope screen at the same scale. Upper trace recorded with resting potential 75mV in normal solution. Lower trace recorded with resting potential 94mV in potassium free solution. Increase in resting potential 25%. Increase in end-plate amplitude 64%. d-Tubocurarine 1.0×10^{-6} g/ml.

exceeded and a conducted action potential occurred. The resulting contraction often broke the recording electrode, releasing potassium locally and affecting the conditions of the experiment. To overcome this problem, a small increase in curare concentration was made and the preparation allowed to re-equilibrate. This necessitated starting with a smaller e.p.p. in normal solution.

If a large number of e.p.p.s recorded from a single junction are compared with a similar number recorded from the same junction in the potassium free solution, the rise is very clear-cut with little deviation from the average size. Fig. 8.5. is a result from one such junction. Fig. 8.6. is the pooled data from nineteen junctions in one preparation.

The e.p.p. amplitude increases linearly with resting potential. Thus, if the resting potential increases from 74 mV to 94 mV, an increase of 27%, the e.p.p. amplitude would also be expected to increase by the same amount. The end-plate in Fig. 8.4. shows an increase of 67%. The increases in amplitude caused by the potassium free solution at different end-plates varied between 45% and 80%.

The effect was found to be essentially reversible after return to normal solution, although amplitudes in excess of control amplitudes often persisted for some minutes after the resting potential had returned to normal.

In Table 8.2. are listed the results obtained from end-plates in six separate diaphragm preparations. It can be clearly seen that in all cases, the amplitude increase is considerably in excess of that predicted by the resting potential hyperpolarisation. The data in this Table was obtained from junctions where the electrode was undisturbed

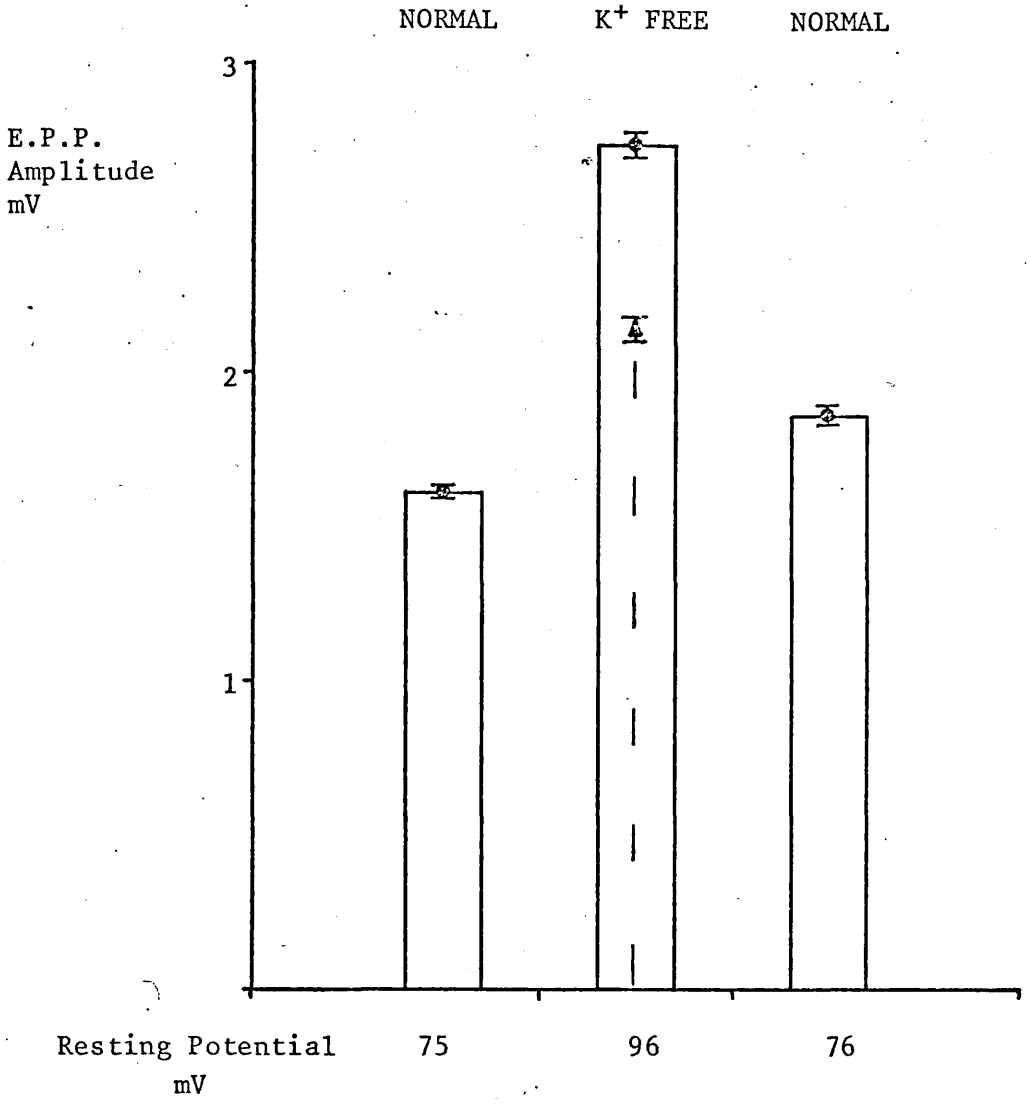


Fig. 8.5. Changes in e.p.p. amplitude occurring at one end-plate with no electrode disturbance after change of bathing solution potassium concentration. ● mean changes occurring in mV ± 2S.E. ▲ corrected mean increase in amplitude in potassium free solution. All changes significant with p less than 0.001 (Student's t test).

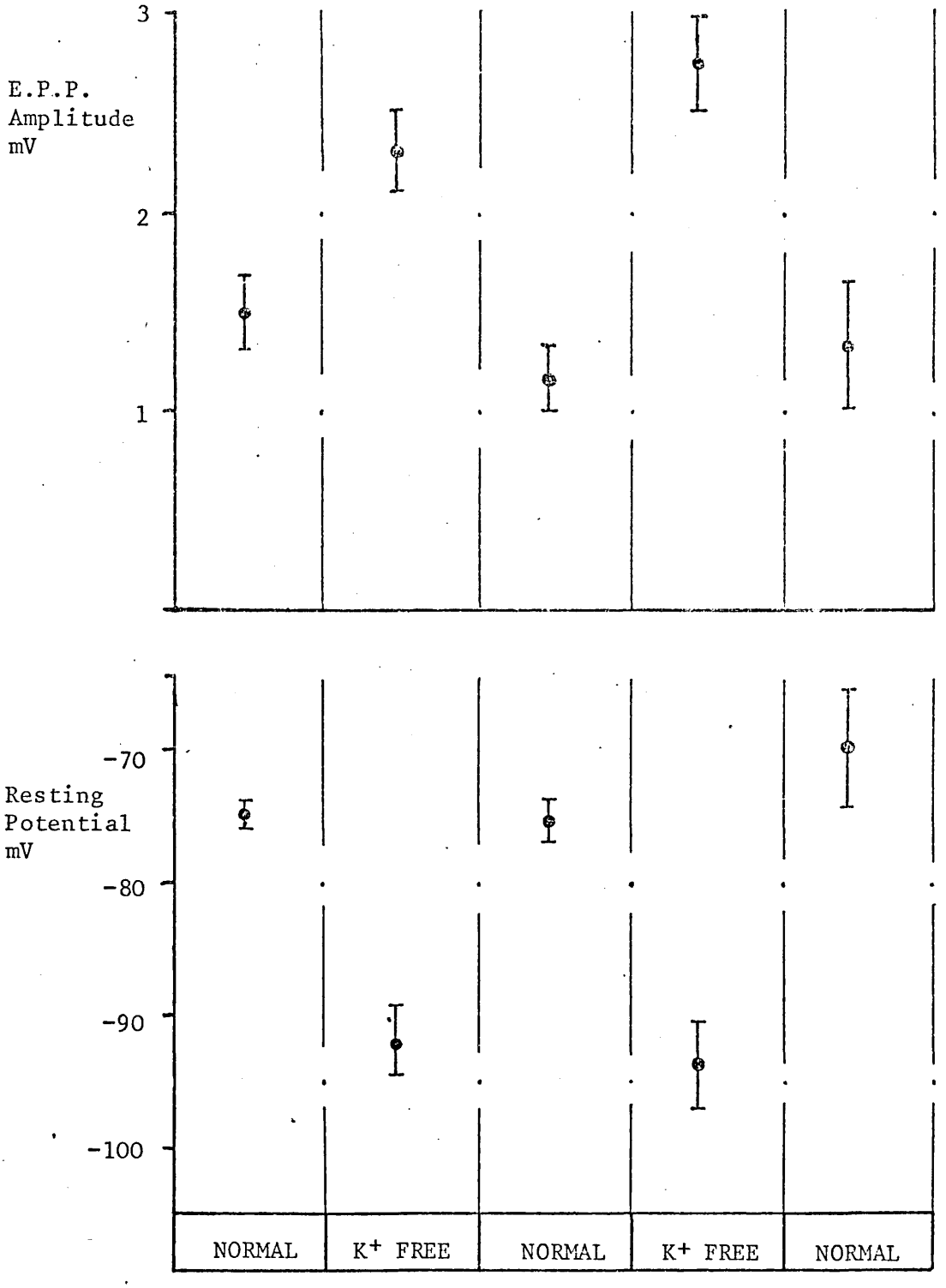


Fig. 8.6. Changes in e.p.p. amplitude and resting potential in normal and potassium free solutions. Data pooled from measurements made at 19 junctions in each of the solutions. 10 min was allowed for equilibration after solution change each time. Points are mean ± 2S.E. All changes are significant with p better than 0.001. (Student's t test).

End-plate Potentials in Normal and Potassium Free Solutions

Diaphragm	End-Plate	Normal Krebs solution		Potassium free solution			
		R.P. mV	Mean E.P.P. Amp. mV	R.P. mV	Mean E.P.P. Amp.	R.P. Inc.%	E.P.P. Inc.%
1	A	75	3.27	94	5.46	25	67
	B	72	2.0	91	3.3	26	65
2	C	75	1.6	96	2.75	28	72
	D	69	1.48	87	2.21	26	49
	E	76	2.1	95	3.34	25	59
3	F	74	1.6	98	2.5	32	56
	G	78	2.3	97	3.98	24	73
4	H	74	3.25	93	4.75	26	46
5	I	75	1.9	101	3.4	35	79
6	J	73	1.7	96	3.0	32	76

Table 8.2. Comparison of mean e.p.p. amplitudes recorded in normal and potassium free Krebs solutions. Data were obtained from experiments where the electrode was undisturbed during recording from end-plate. Results are from 6 diaphragms. 30 min was allowed for equilibration in normal solution after exposure to potassium free solution. Means were obtained from a minimum of 10 e.p.p. amplitude measurements. Curare concentration varied between diaphragms, but was in the range 0.7 to 1.4×10^{-6} g/ml.

during the solution change and in all of these results there is no possibility of an error caused by change of recording position.

A comparison of the change in e.p.p. amplitude with that of resting potential is made in Fig. 8.7. This summarises all the results obtained from junctions in the two solutions. The data for both resting potential and e.p.p. amplitude has been pooled and a clear increase in mean e.p.p. amplitude, greater than the increase in mean resting potential would predict, can be seen.

The changes occurring due to removal of potassium after preliminary investigation may therefore be summarised as: (i) increase in resting potential, (ii) a small predictable increase in m.e.p.p. amplitude, (iii) decrease in m.e.p.p. frequency and (iv) increase in e.p.p. amplitude, larger than that predicted by the increases in resting potential.

A number of hypotheses may be advanced to account for the increase in e.p.p. amplitude. There is the possibility that an increase in the postsynaptic receptor sensitivity could account for the increase. This sensitivity increase should affect all transmitter coming from the nerve terminal equally. Therefore it should have been demonstrable by an increase in m.e.p.p. amplitude larger than that predicted by the increased resting potential. However, no such increase was found in any of the junctions investigated. The increase in resting potential should cause a proportional increase of e.p.p. The average resting potential increase is from 74mV to 94mV, an increase of 27%. Thus, a similar increase in e.p.p. would be expected. The average e.p.p. increase in Fig. 8.5. is 73%. This is more than twice that predicted from the linear relationship between resting potential and e.p.p.

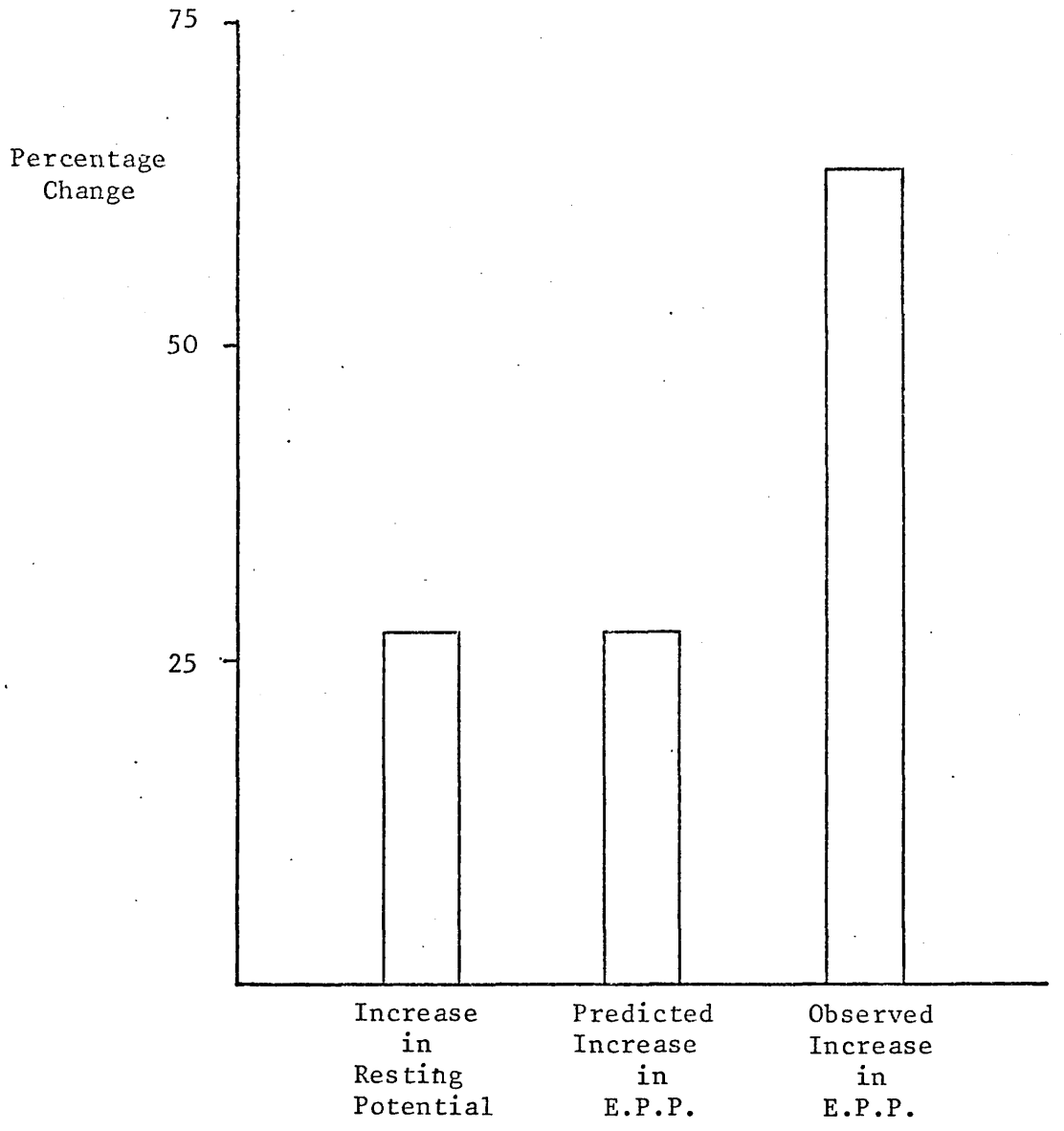


Fig. 8.7. Increase in amplitude of e.p.p. in potassium free solution compared with increase predicted from the change in resting potential. Mean values were obtained from measurements made at a total of 200 end-plates in 18 diaphragms. Actual values, for increase in resting potential 27.5%, increase in e.p.p. 64.2%.

Two presynaptic factors could affect the amplitude of the e.p.p.; an increase in the number of quanta released (quantal content) or an increase in quantal size.

An increase in quantal size should be obvious as a change in m.e.p.p. amplitude. This was not seen to change significantly after correction was made for the change in resting potential. A change in some property of the nerve terminal leading to an increase in quantal content could also be expected to show as an increase in the frequency of m.e.p.p. at rest. This was not the case however. It must therefore be concluded that any presynaptic effect on the nerve terminal leading to a greater output of transmitter must only occur during evoked release and not be manifest at the resting junction. The effect is therefore possibly on the depolarisation-releasing mechanism rather than simply a non-specific action on the nerve ending.

Chapter IX

Further End-Plate Potential Investigations

Use of paired stimuli

Experiments were devised in an attempt to investigate which of the parameters determining the size of the e.p.p. were responsible for the enhancement observed.

As set out in the section of the introduction dealing with acetylcholine storage and release, it is considered that the acetylcholine is released from a small 'immediately available' store. The number of quanta contained in this store may be defined as N. This then is the total releasable acetylcholine at any one time. From this store, a fraction, p, may be defined as released by a nerve impulse, depending on the probability of release of each of the quanta. The number of quanta released therefore is Np. This is also the quantal content m.

$$m = pN$$

A change in p will therefore result in a corresponding change in m, and therefore the amplitude of the e.p.p. If N is a finite size compared to p, i.e. if a depletion, m, causes a significant change in N, then change in N will also be reflected in subsequent quantal contents. In the rat diaphragm the immediately available acetylcholine is only about 1/500 of the total releasable acetylcholine contained in the nerve terminal. (Krnjevic & Mitchell 1961). A mobilisation of the stores into this compartment is required to keep N at a constant level. An increase in the mobilisation therefore could be expected to produce an enhancement of e.p.p. amplitude, via a change in N.

Birks & MacIntosh (1961) suggest that one quarter of the acetylcholine in sympathetic ganglion terminals is available for release at any one time and only 1/1000 of this is released per impulse at this synapse and a lack of depression, such as occurs at the neuromuscular junction, at higher rates of stimulation, appears consistent with the larger store of releasable acetylcholine in this terminal.

The changes in N and p will cause changes in e.p.p. amplitude at a constant quantal size, q. However, if the size of the quantum changes, this will also result in a change of the amplitude of the e.p.p. Few agents have been shown to have an effect on this parameter. HC3 and prolonged stimulation can be shown to reduce the quantal size at the point of exhaustion of transmitter, (Birks & MacIntosh 1961, Elmqvist & Quastel 1965), but there is little evidence to show that the quantal size can ever be increased. Changes in this factor however must not be overlooked.

One method of investigating variations in N and p is to use pairs of pulses as a means of stimulation of the motor nerve.

When two e.p.p.s are elicited from the neuromuscular junction at intervals greater than 5msec, the second e.p.p. is invariably smaller than the first. This depression of the second e.p.p. is considered to be the result of a reduction of transmitter output. (Lilley & North 1953, Lundberg & Quilish 1953, Thies 1965). Changes in the postsynaptic sensitivity may be discounted as it has been shown (Otsuka, Endo & Nonomura 1962) that following stimulation of the nerve, the e.p.p. amplitudes are depressed, but sensitivity to iontophoretically applied acetylcholine remains the same. Most

of the evidence favours a reduction in the available acetylcholine; the release fraction staying constant. (Thies 1965).

Postsynaptic effects should thus be evident as similar changes in the amplitude of both e.p.p.s resulting from twin pulses, and have an equal effect on both.

To determine if a change in N or p was occurring, the twin pulse technique was used in this investigation. Experiments were carried out with pulses spaced 600 msec apart. It was found by trial and error that this value gave maximum depression of the second pulse in normal solution. The pairs were repeated at 15 sec intervals. This repetition frequency causing no observable fatigue of the neuromuscular junction during the period of the investigation.

A comparison of the results obtained in normal and potassium free Krebs solution at a single junction shown in Fig. 9.1.

The quantal content fluctuations in the curare depressed preparation were very small and the conditioning e.p.p. amplitude remained constant. However, a number of pairs were allowed to build up a trace on the screen of a storage oscilloscope; an average of about 8-10 pairs were obtainable before the line became unduly thick.

The amplitude of the second e.p.p. was expressed as a fraction of the first e.p.p. amplitude. Thus, if the first e.p.p. control amplitude is arbitrarily made 100 the size of the second e.p.p. is obtained as a percentage. A test e.p.p. of the same size as the conditioning e.p.p. will be 100%, and increases in test e.p.p. amplitude are shown as increases in percentage values.

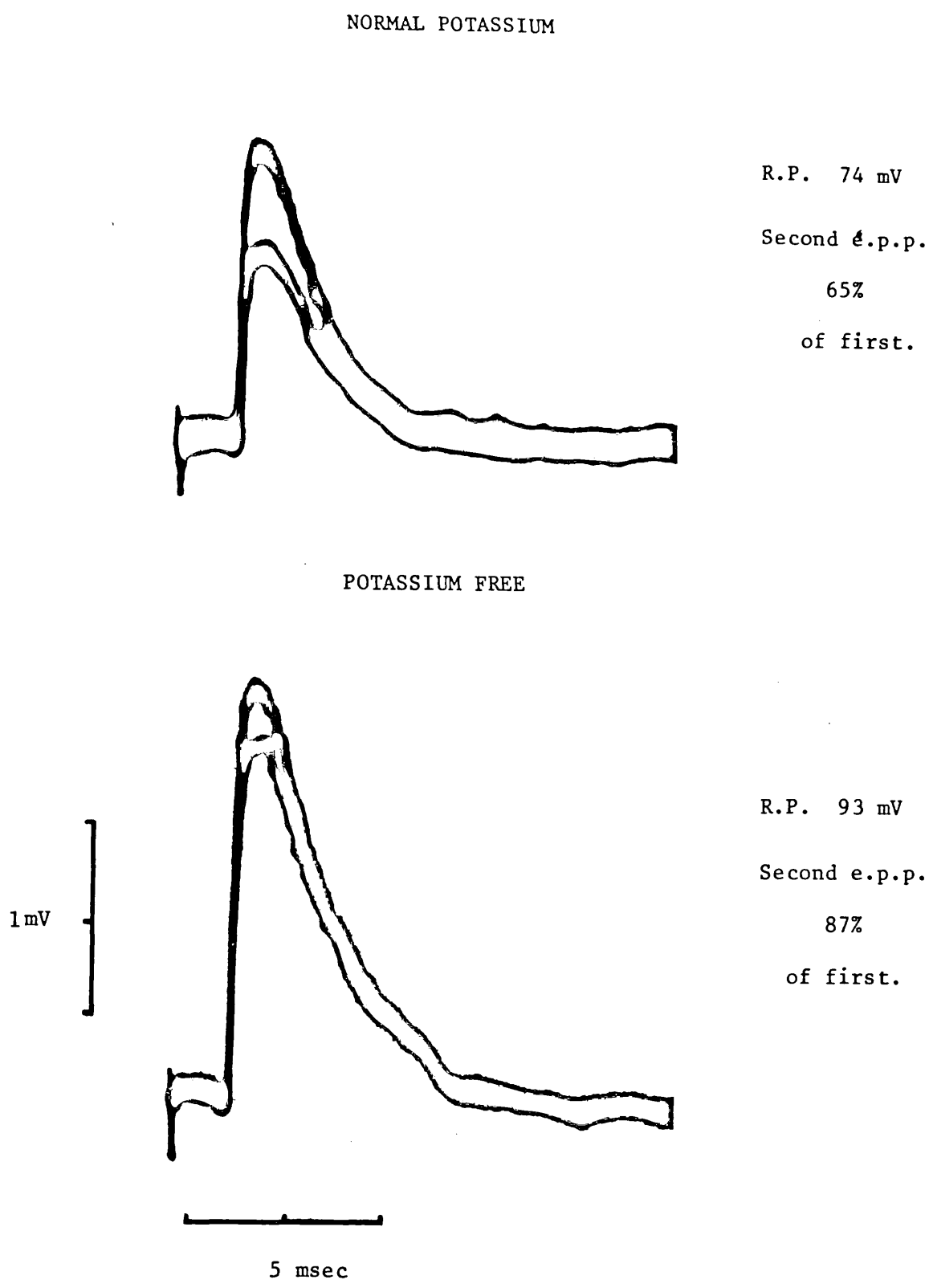


Fig. 9.1. Superimposed pairs of e.p.p.s, recorded in normal and potassium free Krebs solution, at one end-plate, with 600 ms interval between pulses. Both traces photographed from oscilloscope screen at same scale.

In normal solution, the second e.p.p. is depressed to approximately 70% of the control amplitude. Measurements were made at many junctions in normal solution to ascertain the maximum range that could be expected. The greatest depression obtained was 52% and the least depression to 86% of the control e.p.p. amplitudes. The mean amplitude of the second e.p.p. at the 600 msec interval was found to be 74% ± 6.4% of the control e.p.p. amplitude. This value was calculated from measurements made at ninety two junctions in normal solution.

When the solution was changed from normal to potassium free, the amplitude of the conditioning e.p.p. increased in a similar manner to that of the single e.p.p. The second e.p.p. of the pair however, was found to have been depressed to a lesser degree than it had been in normal solution and the amplitude was closer to that of the conditioning e.p.p.

A simple increase of both e.p.p.s in potassium free solution could be explained as an increase in postsynaptic sensitivity caused by a direct effect on the receptors for acetylcholine, or by a prolongation of acetylcholine action. The differential effect on the second pulse is strongly suggestive of a presynaptic mechanism enhancing transmitter output.

If the enhancement were due to an exclusive change in p, this would account for the increase in conditioning e.p.p. amplitude. However, the reduction of N due to this, occurring without any increase in mobilisation, would lead to a smaller second e.p.p. of the pair. It would appear therefore that what is occurring is an increase in the value of N for the second pulse of the pair.

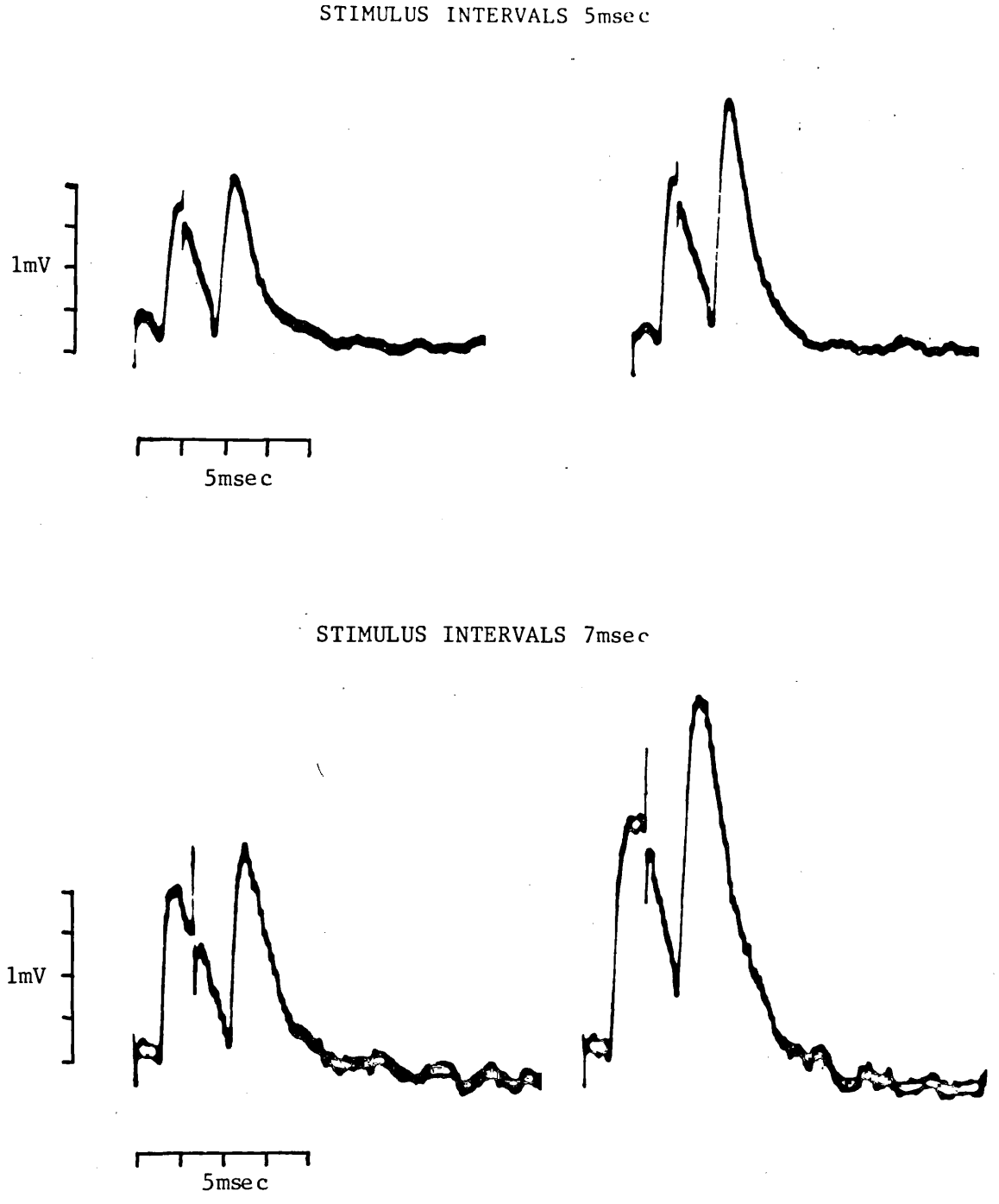


Fig. 9.2. Pairs of e.p.p.s recorded in normal and potassium free solution. Upper pairs recorded from same end-plate at 5ms stimulus interval. Lower pairs are from different end-plate at 7ms interval. d-Tubocurarine 1.0×10^{-6} g/ml.

At a stimulus interval of only 2 or 3 msec, the second e.p.p. of a pair is often potentiated under normal conditions. (Hubbard 1963). At greater intervals the potentiation reduces and changes to depression. As the interval further increases, the depression of the second e.p.p. becomes larger until it is maximum at the interval of about 600-700 msec used previously. The experiment was repeated to determine the effect of potassium free solution at shorter intervals between stimuli. Greater potentiation of the second e.p.p. amplitude compared with the first was found at all intervals tested. An example of a pair of responses obtained at 7 msec spacing is shown in Fig. 9.2. Under normal conditions the two e.p.p.s were approximately the same amplitude. After the change to potassium free solution the second e.p.p. can be seen to be potentiated more than that of the first.

Data was obtained from pairs spaced at various intervals between 5 msec and 600 msec apart. The depression of the second pulse was calculated and the mean values plotted against the time interval. Two sets of points showing the depression at each interval in normal and potassium free solutions are shown in Fig. 9.3. The result for the normal solution is similar to that obtained by Hubbard (1963) and is typical for the rat diaphragm. It is evident that at all intervals, in potassium free solution the amplitude of the second e.p.p. of the pair has been enhanced compared to that amplitude in normal solution.

The decrease in depression must mean that more transmitter is released by the second pulse in potassium free solution than in normal solution. The agent known to have a marked effect on the probability of release is calcium. Increase in the extracellular concentration causes an enhancement of transmitter release by acting

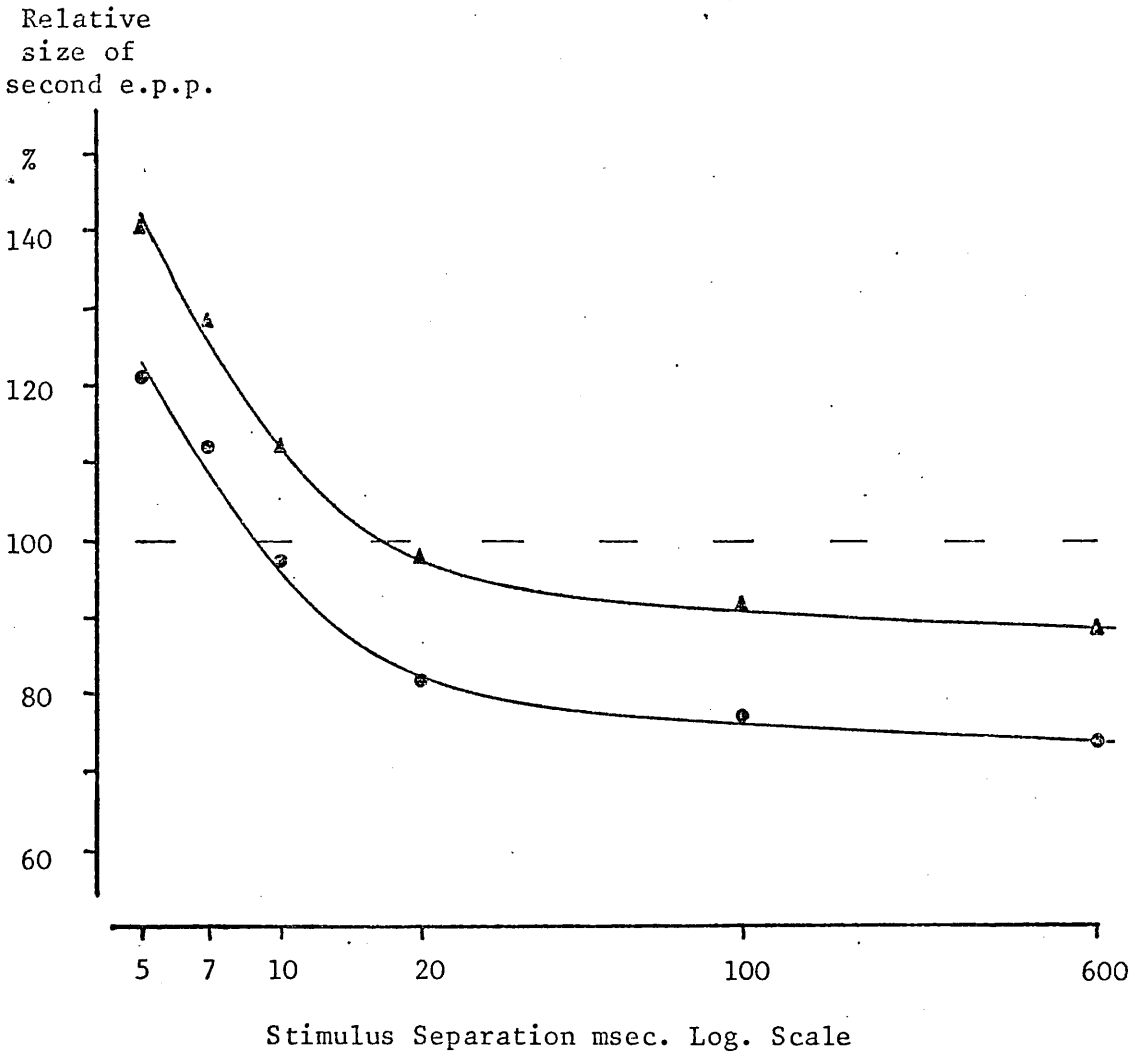


Fig. 9.3. Amplitude of the second e.p.p. of a pair as a percentage of the preceding e.p.p. amplitude plotted against stimulus interval. ● mean depression or potentiation in normal solution. ▲ mean depression or potentiation in potassium free solution. Stimulus interval plotted on logarithmic scale. All points are means obtained from at least 20 end-plates in each solution up to that interval.

on the nerve terminal and increasing the probability of release of the quanta. Thus, an increase in the amount of calcium in the bathing solution will have the predictable effect of increasing p. (Cooke, Okamoto & Quastel 1973, Baker 1974, Dodge & Rahamimoff 1967). Increase in calcium ion concentration was therefore used to compare the enhancement obtained in potassium free solutions and the effect of a known increase in p.

Depression at the normal calcium concentration of 2mM was compared with that obtained at 4mM and with that obtained with 4mM calcium and no potassium. If the effect obtained with potassium removal was acting through an increase in p, calcium should potentiate this effect. The results obtained with calcium are shown in Fig. 9.4. The effect obtained with an increase of calcium from 2mM to 4mM is similar to that obtained by other workers. (Lundberg & Quilish 1953, Thies 1965). The first pulse is potentiated but the second is greatly depressed. The effect of calcium is to facilitate release by increasing the probability of release (p) of any of the quanta. Thus, the first impulse has a larger quantal content than usual due to increased likelihood of release of all the quanta. However, the second impulse, although the release probability may remain high, has to effect release from a smaller presynaptic pool (N), i.e. from one which has been reduced by the first larger than normal release, and as yet not replenished. Thus, with a higher than normal probability of release and even a normal replenishment rate, the junction will exhaust very quickly. The effect of the potassium free solution however was to return the depression of the second pulse towards a more normal amplitude. If potassium free solutions caused an increase in release of transmitter the effect in addition to that of added calcium would have been an even greater depression of the second pulse. Fig. 9.4b.

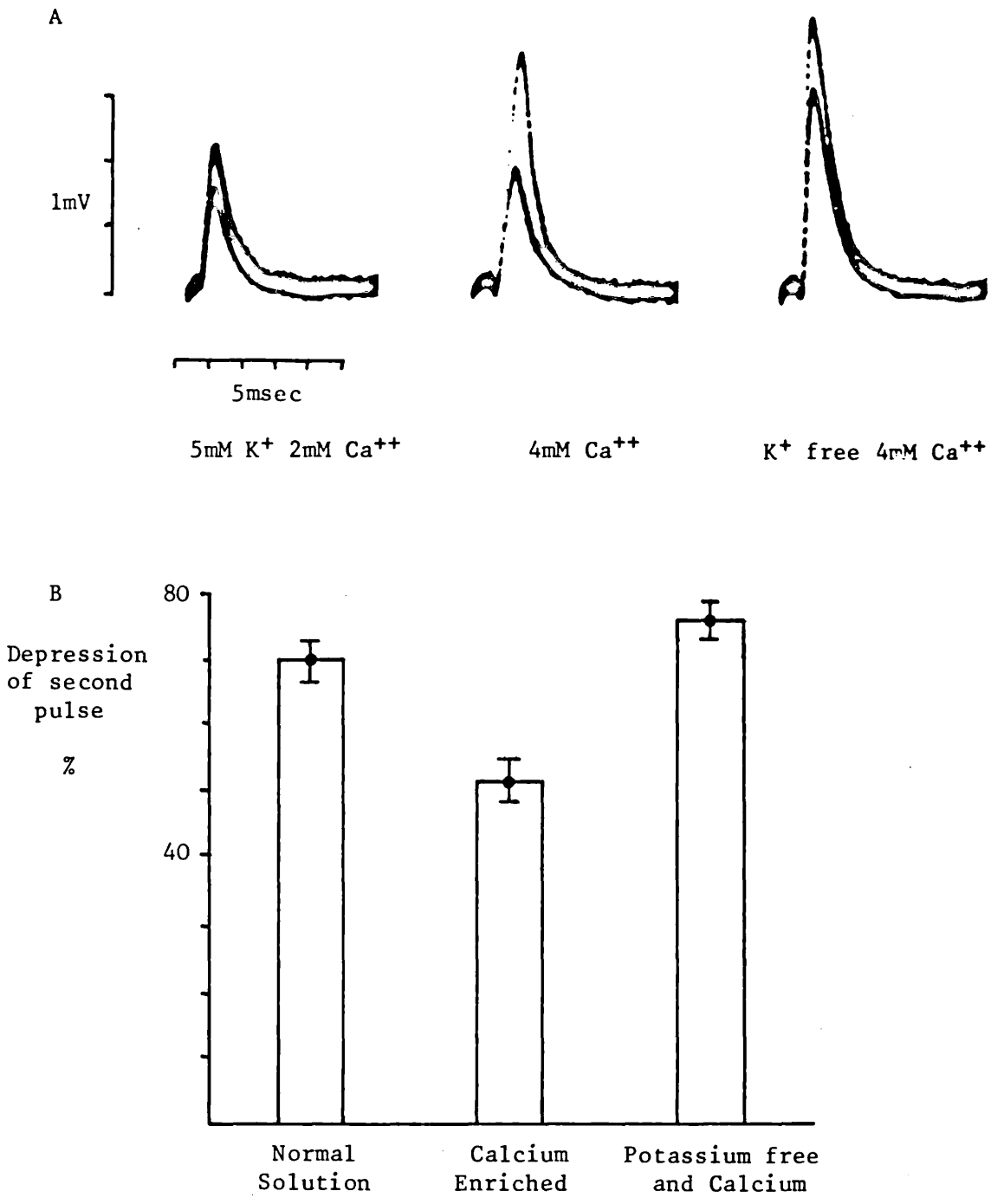


Fig. 9.4. A. Pairs of e.p.p.s recorded in normal solution, calcium enriched solution and potassium free calcium enriched solution. Stimuli spaced 600 msec apart and e.p.p.s superimposed. B. Depression of the second e.p.p. of the pair expressed as a percentage of the first in each solution. Values are means of response obtained from five diaphragms. ± 2S.E. Changes are significant at p = 0·001 (t test).

illustrates the results obtained from experiments of this type.

It can be seen from Fig. 9.4. that the effect of potassium lack is to reverse the effect of increased calcium. If potassium free conditions caused only a reduction in the probability of release there would then be less difference between the two amplitudes. However, the first pulse would now be smaller compared with the amplitude under conditions of increased release probability. This is not in agreement with the result obtained with low potassium alone, where the first e.p.p. is larger than normal. If a simple increase in presynaptic store was to occur, this would increase the size of both potentials but the probability of release would remain constant and therefore the relative sizes would not change. The explanation which best fits the observed results is that an increase occurs in the store population from which the second e.p.p. is released. One mechanism whereby this could occur would be through an increase of mobilisation of transmitter immediately following the first impulse, leading to a larger releasable pool. Mobilisation is thought not to occur for about 100 ms as can be seen from experiments using trains of stimuli. The other alternative is that an increase occurs in the synthesis of releasable transmitter, caused by the reduction in potassium concentration. The fact that the potassium free enhancement reverses the depletion caused by increased calcium suggests that there is an improvement in terminal transmitter stores. The output is increased compared with normal conditions, but also maintained at this level with no depletion. This therefore must be accompanied by an increase in transmitter synthesis. This would maintain the size of the store and prevent the increased release caused by calcium from depleting it. With no extra calcium and potassium free solution this would also lead to the observed increase in the second e.p.p. of a pair.

From these results it must be concluded that a change in some parameter other than p has occurred during the exposure to potassium free solution. As the output from the available store is better maintained, a possible hypothesis is that the size or replenishment rate of this store has been increased. Thus, the fractional release per impulse has less effect on the store size. An increase in N would therefore explain the observed effect with twin pulses better than an increase in p.

Use of Trains of End-Plate Potentials

The original observation on muscle contraction, using mechanical recording, was one of enhancement under conditions of tetanic stimulation of the motor nerve. A change in the parameters of release would be expected to have most effect under the conditions of rapid stimulation when transmitter turnover is high. An increase in the value of p, thus leading to an increased fractional release, would rapidly lead to a depletion of available transmitter within the nerve terminal, a decline of e.p.p. and eventual neuromuscular block. An increase of release need not be excluded from a hypothesis if the increase in available store size is large enough to overcome any reduction caused by this increase. It must be concluded however, from the above reasoning, that if the effect of potassium removal is mediated through a change in quantal output, then there must be an appreciable increase in the amount of available transmitter. With the hypothesis that the effect of potassium free solution was to alter the magnitude of N, changes in the size of the available store were further investigated.

Wedensky demonstrated in 1903, inhibition of tetanic stimulation with high frequency activation of the muscle by the nerve.

Both he and Hofmann (1903) demonstrated that the neuromuscular transmission was more sensitive to this than was the muscle or the motor nerve. Since this time, inhibition produced by high frequency stimulation has been referred to as Wedensky inhibition. After the advent of intracellular recording, which led to a better understanding of the e.p.p., it was shown that the e.p.p.s produced by a train of stimuli, at rates greater than 0.5 Hz, decline in amplitude until a plateau is reached. The rate of fall and final amplitude attained are dependent on the stimulation rate. The effect being increasingly rapid with higher rates of motor nerve stimulation. (Lilley & North 1953, Elmqvist & Quastel 1965, Capek, Esplin & Salehmoghaddam 1971). It has also been reported (Hubbard, Jones & Landau 1971) that between the frequencies of 25 Hz and 200 Hz there is no change in the estimate of store size with frequency of stimulation. The determination of the store size also depends on the rate of eliciting tetanic trains of impulses and this frequency is not always made clear.

The use of trains of impulses is simply an extension of the twin pulse technique, which just uses the first two pulses of such a train, but therefore has also less effect on the store. By investigating the rate of decline of the e.p.p. amplitude from its initial value to its final plateau value, much information may be obtained about the storage and release of transmitter in the nerve terminal.

If the transmitter were released from a small store with no replenishment into it, the amount released by each impulse would get progressively less as each impulse reduced the total store size. This would continue, less and less being released each time up to the point of effective exhaustion. The total amount released in this case would be the total available store or N. Its size could be

calculated by summing all the preceding quanta released from the store. Under normal conditions, this state is complicated by replenishment of the available store from a reserve store, by the process of mobilisation or synthesis, or both. With rates of stimulation above about 25 Hz, the replenishment process can be seen to be delayed for the first three or four e.p.p.s. No significant effect of the replenishment process on release appears to occur for about 100 msec. At 50 Hz, the amplitude of the e.p.p.s often drops below the eventually maintained plateau level before the replenishment process is able to keep up with the now reduced rate of release. Thus, the initial release from the readily available store may be studied at this frequency with little complication from the processes contributing to its maintenance. The fall in amplitude of the first 4-5 e.p.p. in a train will be exponential as the store is decreased with each impulse. The sum of all the e.p.p.s released, assuming no replenishment, will be equal to the original presynaptic store. This can thus be calculated by plotting the amplitude of the e.p.p.s against the sum of all previous e.p.p.s in the train. The slope of the line drawn through the points is the release fraction and the intercept of the extrapolated line is equal to the original store size. In practise, the e.p.p. amplitudes reach a plateau when the replenishment and release are in equilibrium. The amplitude of this plateau is representative of the mobilisation ability of the terminal.

The technique of using short trains of e.p.p. has been used for determining the various parameters of storage, release and turnover of transmitter at the neuromuscular junction. (Capek et al 1971, Elmqvist & Quastel 1965). The initial decline rate appears to be greater, and the plateau level smaller with increasing curare concentration.

(Lilleheil & Naess 1961, Galindo 1971, Hubbard & Wilson 1973). No definite explanation is available for this at present although it has been suggested that the changes may be due to a presynaptic action of curare on the nerve terminal.

The same method was employed in the present investigation to investigate changes in the immediately available store caused by potassium free conditions. The motor nerve was stimulated by short trains of stimuli (50 pulses) at a frequency of 50 Hz at intervals of 20 sec. The muscle contraction was prevented as before by producing a partial neuromuscular block with curare at a concentration of 1·6-2·0 x 10^{-6} g/ml. At this level of curare block, large enough to prevent the first e.p.p. from exceeding threshold, the amplitude of the plateau part of the e.p.p. train became very small and difficult to measure satisfactorily. Estimates of quantal content by the method of e.p.p. variance were found to be too unreliable to be useful. Had this not been the case, the quantal content of the store could have been determined directly.

Under conditions of potassium lack, the amplitude of the e.p.p.s were larger, as expected. The rate of decline also appeared to be often less than that occurring in normal solution. In addition, the final plateau amplitude also appeared to be larger. Examples of the trains of e.p.p.s obtained at 50 Hz in normal and potassium free solution, are shown in Fig. 9.5. In practise it was found difficult to prevent muscle movement after change to potassium free solution unless a high concentration of curare was used. This, as noted previously, caused the plateau e.p.p. in normal solution to be small. It was also found difficult to follow the change from normal to potassium free solution in one junction, and obtain the enhancement

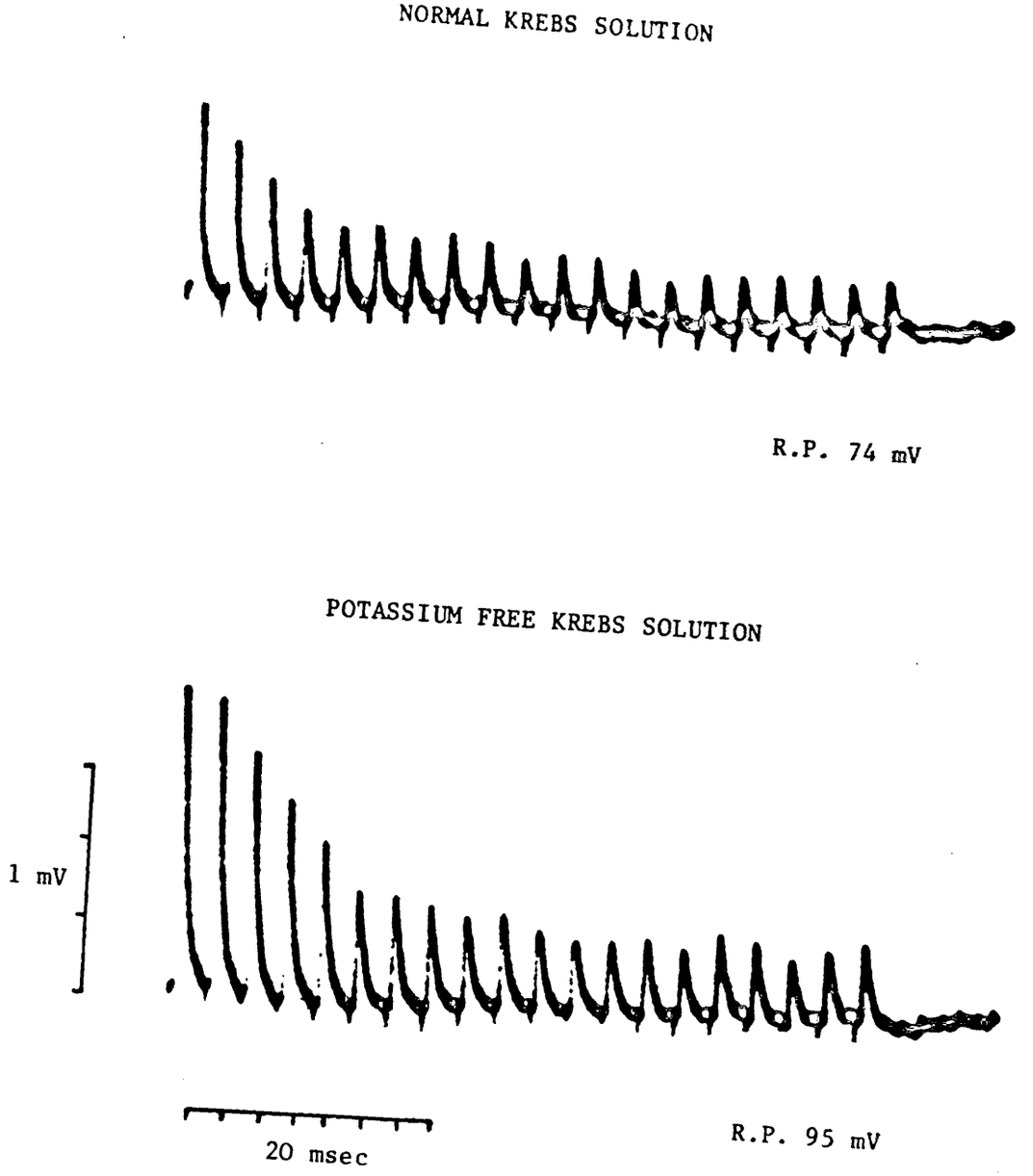


Fig. 9.5. Trains of e.p.p.s recorded at the same junctional site in normal and in potassium free, Krebs solutions. Stimulation frequency 50 Hz. d-Tubocurarine 1.2×10^{-6} g/ml.

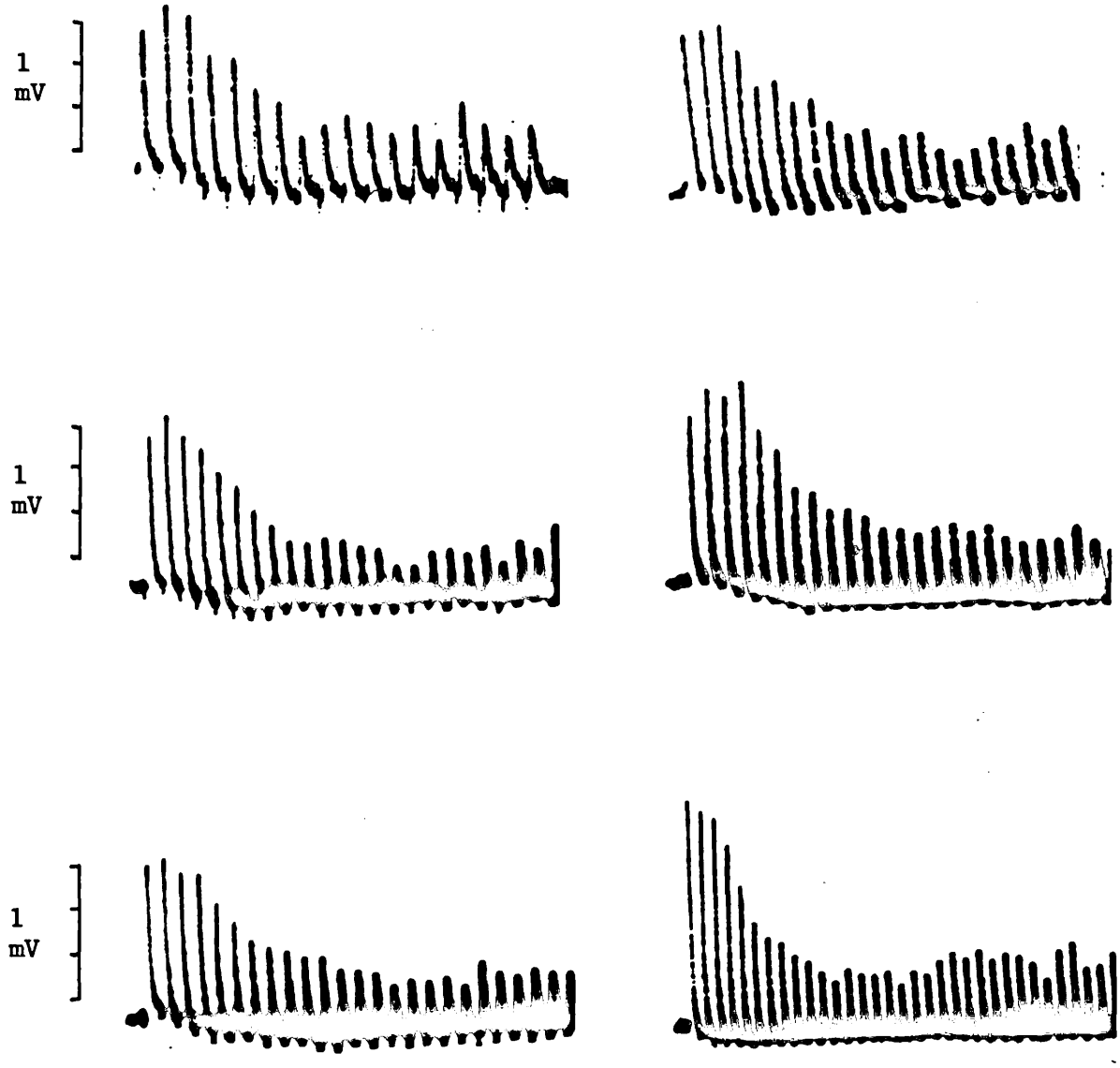


Fig. 9.6. Trains of e.p.p.s recorded from junctions in potassium free bathing solution. Stimulation frequency 50 Hz. Traces have different time scales but similar voltage scales. d-Tubocurarine concentration approximately 1.0×10^{-6} M in all cases. These trains should be compared with the result for normal solution shown in Fig. 9.5.

shown. However, after removal of the electrode and impalement at another junctional site, it was found that a change had occurred in the decline rate compared to that usually expected. Examples of such trains of e.p.p.s are shown in Fig. 9.6. Calculation of the store size under such conditions showed a marked increase over the usual result obtained in normal solution at most junctions. In addition, the change in decline rate suggests either a reduction in release probability and a much larger store, or an increase in replenishment rate, thus keeping the store closer to its initial value and the e.p.p. amplitudes larger. The effect on the e.p.p. trains of removing potassium was more variable from junction to junction, than it had been in the paired stimulation experiments. In many cases however it was possible to follow the change from normal to an enhanced state in potassium free solution without electrode disturbance.

The amplitudes of the e.p.p.s were measured and corrected for resting potential changes to a standard resting potential of 75 mV. Amplitudes over 3 mV were corrected for non-linearity (Martin 1955). This enabled some comparison to be made between store sizes obtained at different end-plates and under different conditions. The e.p.p.s were then plotted against the sum of the e.p.p.s preceding. A regression line was then fitted to the first 5 points, or points 2·5 if the first two e.p.p.s were similar in size as often occurred in potassium free solution. This line when extrapolated to the axis gives an approximate value for the size of the immediately available store, assuming no replenishment to have taken place. (Elmqvist & Quastel 1965). This method is illustrated in Fig. 9.7. Fig. 9.7b. summarises the change in presynaptic store size obtained at 15 junctions where the electrode was left undisturbed between normal

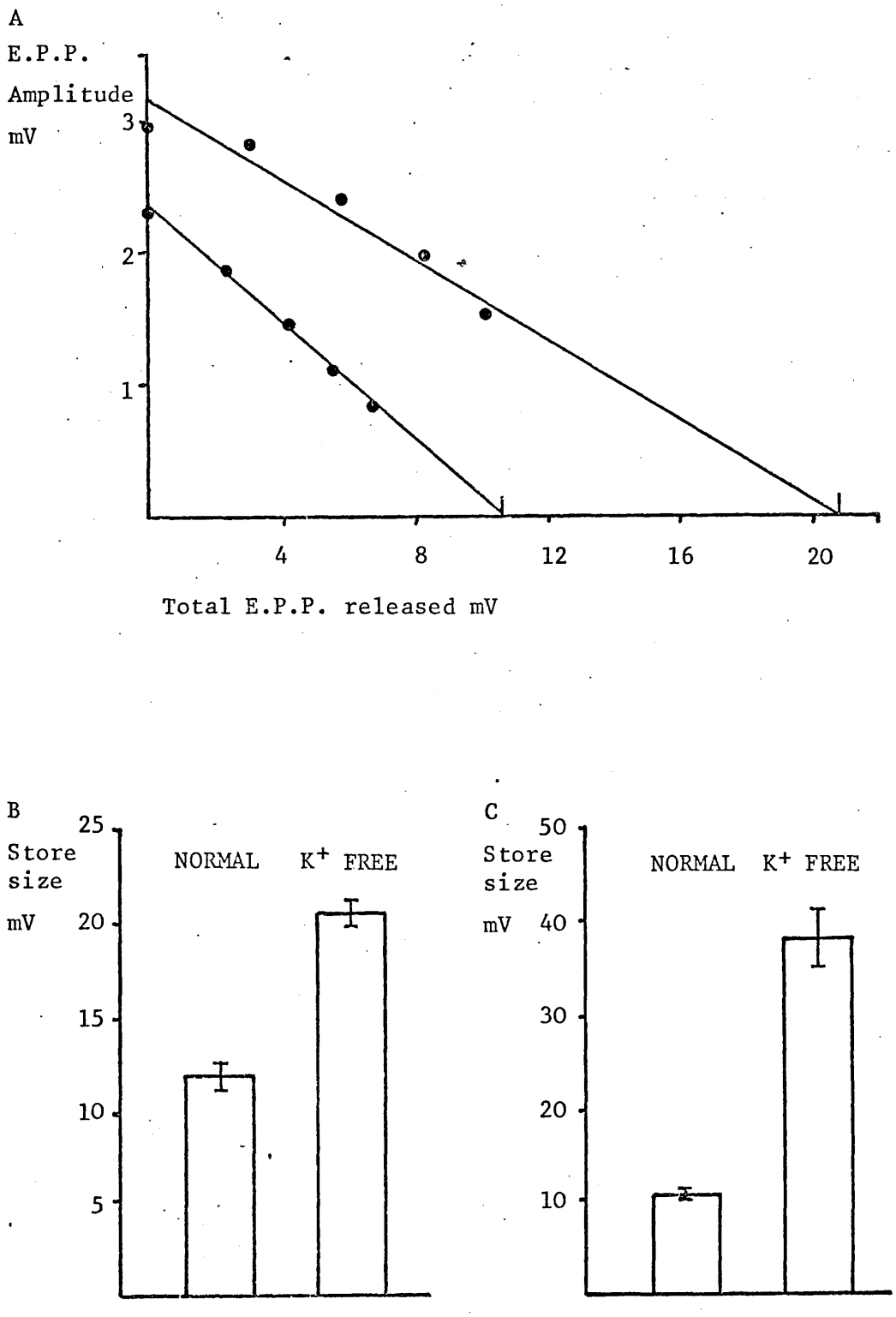


Fig. 9.7. A. Estimate of initial store size. E.p.p. amplitude corrected to 75 mV resting potential plotted against total released transmitter in mV. Upper line for end-plate in potassium free solution, lower line in normal solution. B. Comparison of values obtained at 15 end-plates in the two solutions. C. Store size at junctions such as those in Fig. 9.6. compared with mean store size obtained from pooling all estimates in normal solution. Points are mean $\pm$ SE.

and potassium free recordings. Fig. 9.7c. shows the mean store size obtained at junctions under potassium free conditions for which no control is available recorded at the same junction under normal potassium conditions. This is compared with the store size obtained from determinations at 52 junctions in 18 diaphragms under normal conditions. The estimates of store size obtained were from junctions where the reduction of decline in amplitude was particularly noticeable, producing results such as those illustrated in Fig. 9.6. These results are therefore the maximum which might be expected under these conditions. At these junctions, there was presumably no leakage of potassium caused by damage due to impalement for the time required to record in normal solution, possibly allowing a greater effect to be observed. End-plate potential trains such as these were never observed in normal solution under normal potassium conditions.

The values for initial store size obtained under these conditions were 40-50 mV. It should be noted that the initial e.p.p. amplitude of the trains is not five times larger which could be predicted from the relationship between N, p and release. The subsequent amplitudes are usually better maintained suggesting a maximum limit for the amount of transmitter released. Alternatively the store may be replenished more rapidly during release thus maintaining output. This would cause later e.p.p.s in the train to be larger compared with the initial e.p.p. amplitude but have less effect on the first e.p.p.

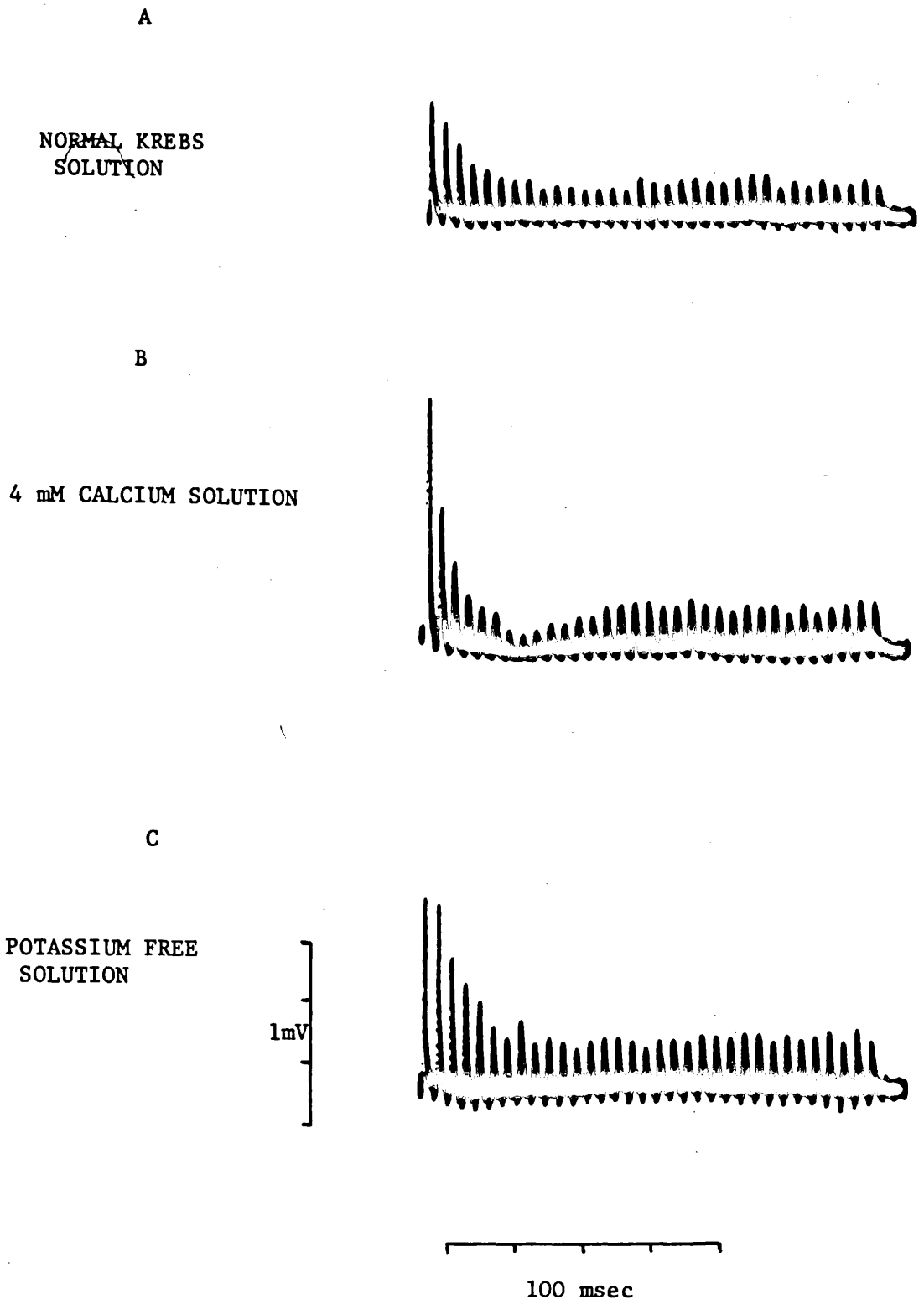


Fig. 9.8. Three e.p.p. trains recorded from the same junctional site. A under normal conditions, B with 4 mM calcium in the bathing solution and C with no potassium in the bathing solution. d-Tubocurarine 1 x 10^{-6} g/ml. Stimulation frequency 50 Hz.

Calcium and release

Trains of pulses were also used to investigate the effect of an increased calcium ion concentration in a study similar to the experiments with twin pulses. The calcium concentration used was again 4 mM. The effect of increased calcium was similar to its effect on the pairs of pulses. A typical e.p.p. train in calcium enriched solution is shown compared with trains in normal solution and potassium free solution at the same end-plate in Fig. 9.8.

The release is facilitated to the extent of causing the depletion of the immediately available store more rapidly than normal. Thus, although the initial e.p.p. amplitude is larger, the depletion of the store by an increase in fractional release, causes the subsequent pulses to be considerably smaller. Calcium then, may be considered in this case to be acting at the level of release of transmitter, but not on the replenishment of, or the mobilisation into, the immediately available store.

A comparison of the effect of calcium was also made using the mechanical response of the muscle and the cyclic stimulation procedure. The type of response pattern obtained is shown in Fig. 9.9. The main effect is seen to be to increase the decline in tension during the period of tetanic stimulation. It is suggested that the effect on the e.p.p. and the observed effect on the tetanic sustaining power are connected. Calcium is thus facilitating transmitter release to the point, where under conditions of rapid stimulation, the replenishment is unable to keep up with output and the transmission begins to fail. It should also be noted that the effect of calcium increase on the tetanic tension maintenance is opposite to that of potassium free solution, as it is on the e.p.p. train amplitudes.

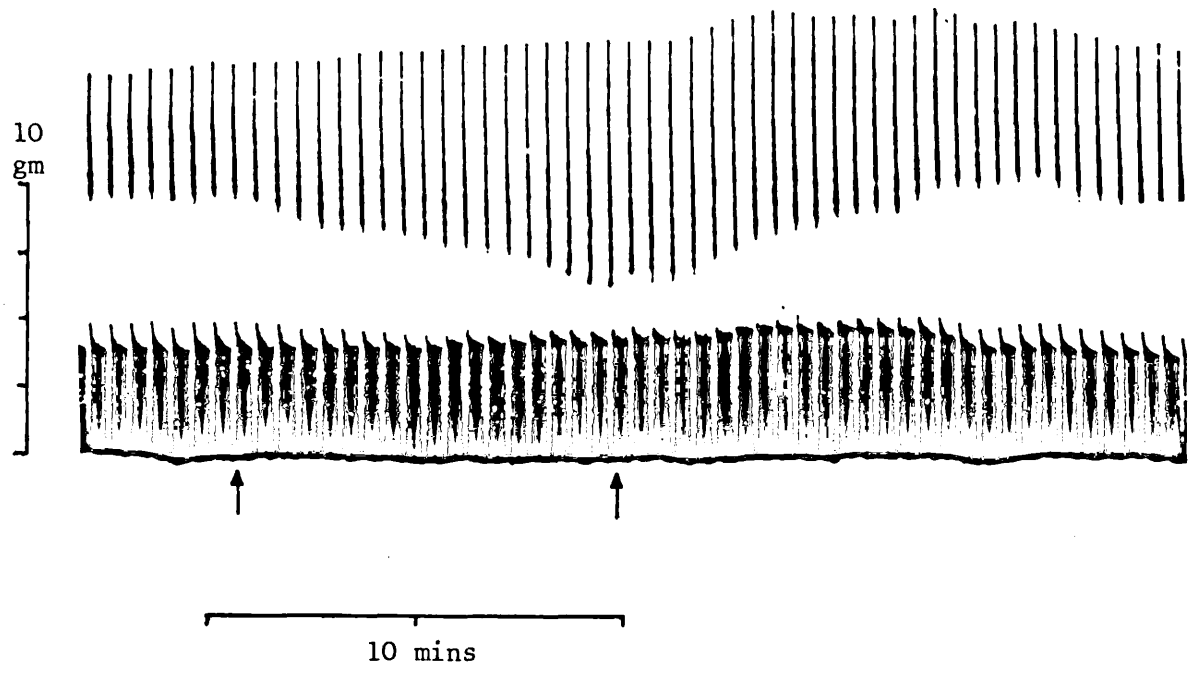


Fig. 9.9. Change in mechanical response after addition of calcium to bring the bathing solution concentration to 8 mM. Calcium was added at the first arrow and the solution washed out at the second arrow. Stimulation was 1 Hz for 55 seconds and 50 Hz for 5 seconds.

Block by Magnesium Ions

The recognised method for investigation into changes of quantal output of transmitter is to use a preparation blocked by an excess of magnesium ions with or without a decrease of calcium ions in the bathing solution. An increase of magnesium in the bathing solution from the normal concentration of 1 mM to 10-15 mM reduces the transmitter output to a few quanta per impulse and allows the investigation to proceed free of postsynaptic complications. At the same time it is possible to measure individual quantal amplitude both as m.e.p.p.s and as components of the residual e.p.p. which fluctuates in a quantal manner. Using this method, a number of experiments were conducted to investigate the effect of potassium free solutions on quantal output. It was hoped that by using the method, investigations of quantal variations would show in what manner the increase of transmitter output was taking place.

The results were not completely conclusive. It was found to be more difficult to obtain consistent effects at the junctions studied. The increases in e.p.p. were not as clear as those in the preparations blocked by curare. Stimulation frequency was 1 Hz, the same as used in the curare blocked investigation. Magnesium was added to solution until e.p.p.s with a mean quantal content of about 3 were obtained. Recordings of both m.e.p.p.s and e.p.p.s in normal and potassium free solution were made with no disturbance of the electrode. The results from fifteen end-plates are listed in Table 9.1. The effects on resting potential were as before and indicate the expected change. Quantal content was estimated by using both the mean m.e.p.p. and e.p.p., and also by the method of failures. These procedures were explained in Chapter II.

Recordings in high magnesium concentrations

NORMAL				POTASSIUM FREE			
Mean M.E.P.P. Amp. mV	Mean E.P.P. Amp.mV	Mean M	R.P. mV	Change in R.P. %	Change in M.E.P.P. %	Change in E.P.P. %	Change in M %
.44	0.99	2.58	74	28	77	49	-17
.51	1.22	2.6	75	24	61	48	-12
.48	1.25	2.75	74	27	65	34	-24
.45	0.99	2.15	74	30	69	38	-11
.49	1.23	2.65	76	30	71	63	- 6
.54	1.67	3.15	75	25	31	23	- 3
.58	1.33	2.25	76	33	38	50	16
.62	1.74	3.0	73	27	42	1	-28
.75	1.8	2.3	74	26	32	-1	-17
.46	1.01	2.45	71	34	46	40	-16
.64	1.79	3.0	73	26	30	25	-17
.66	1.72	2.75	72	31	32	42	9
.45	1.31	3.05	71	35	36	-7	-28
.59	1.59	2.8	74	28	51	64	14
.70	2.24	3.05	72	28	43	56	33

Table 9.1. M.e.p.p.s and E.p.p.s recorded under conditions of high magnesium block. Values under 'normal' were obtained from junctions bathed in normal Krebs solution. Mean M (quantal content) is mean of E.P.P./M.E.P.P. and M derived from failures. Under potassium free are listed the changes which occurred after solution change at the same end-plate. - sign indicates decrease. Data from 15 end-plates in 12 diaphragms.

At certain end-plates, clear increases in the mean m.e.p.p. amplitude were obtained. At these junctions, the increase in e.p.p. amplitude may be accounted for by this increase. The increase in m.e.p.p. amplitude is in contrast to the result obtained at resting junctions, where there was no effect on quantal size, beyond that produced by the change in resting potential. Increase in quantal content occurred at some end-plates, but the value calculated from the e.p.p. amplitudes is not always borne out by the value calculated from the failures. The pattern of the results in Table 9.1. suggests that some factor in the experimental procedure was not held constant and variations in this unknown factor were responsible for the anomalous changes occurring at certain end-plates.

If the increases in m.e.p.p. amplitude are not an artefact, then the potassium free solution is having a direct effect on quantal size. When the m.e.p.p. amplitudes obtained at resting junctions in normal and potassium solution were corrected for resting potential changes no change in quantal size was obtained. A possible explanation is that the effect on quantal size is related to transmitter turnover. Thus, no effect would be seen at the low release rates occurring with spontaneous release at the resting junction. Under conditions of more normal release, (as in the curare blocked preparation), the effect appears to be greater. Two things occur under these conditions. Firstly, the immediately available store is depleted. If the stimulus for mobilisation is the depletion itself, potassium lack could act on this and increase the size of the store. Secondly, with more transmitter released, more choline will be available for reabsorbtion. The effect of low external potassium or the resulting increased resting potential may act on this part of the mechanism and cause an enhancement through increased reabsorbtion of choline, or increase of synthesis of acetylcholine.

If the effect of potassium free solution is indeed related to release of transmitter, the effect would be reduced in a magnesium blocked preparation where the presynaptic reduction of transmitter stores is low.

Chapter X

Experiments on the Involvement of Choline and Choline Re-uptake

End-plate potential investigations

The hypothesis, that low potassium solutions result in a maintained increase in transmitter output, requires that at some stage an increase in synthesis must occur. The effect may be mediated through an increase in mobilisation into the immediately available store from other compartments of pre-synthesised acetylcholine; in which case attempts at experimental changes in synthesis will have little obvious immediate effect. If this is not so, then the system should be affected by an increase in the availability of substrate, i.e. choline, assuming the supply of acetyl-CoA is not a limiting factor. If low potassium solutions exert their effect by promoting re-uptake of choline, then addition of choline should potentiate the action and block of choline uptake should prevent it from occurring.

In preliminary experiments using trains of stimuli, only small and inconsistent changes in the e.p.p. amplitudes and decline rate were found after changing from normal to potassium free conditions. At the time, the working hypothesis was that potassium free solutions were facilitating release, and choline had been added to the bathing solution to help maintain the transmitter stores to ensure sufficient transmitter availability for enhancement of release to be evident, if present. In fact, what probably occurred was that the increased choline in the bathing medium was already saturating the uptake capability of the terminal. Reduction of potassium concentration therefore would have caused less increase under these conditions if it was acting by facilitating choline uptake. The train

experiments for the purpose of investigating the action of potassium free solution were therefore repeated with no choline in the bathing solution.

The effect of choline on the preparation was investigated directly and comparisons were attempted between trains of e.p.p.s in normal solution, in potassium free solution and in potassium free solution with added choline (2×10^{-5} M). In only two diaphragms in five attempts was it possible to follow the changes in store size at one end-plate. In these experiments, definite increases in presynaptic store were found and the effect of choline and potassium free solution appeared to be additive. In the two successful experiments, reversal to normal store size was achieved after returning the preparation to normal bathing solution. The result obtained at one end-plate is shown in Fig. 10.1. The store increased by 76% when the solution was changed to potassium free, and by a further 30% in potassium free and choline solution. At other end-plates under conditions of no potassium and added choline, further potentiation was found as in the earlier low potassium experiments. Examples of these e.p.p. trains are shown in Fig. 10.2. Often trains of e.p.p.s recorded at end-plates after incubation in solutions containing no potassium but with added choline showed very little decline in amplitude during the first pulses compared with that found at all end-plates in normal solution. The improvement in maintenance of transmitter output to such an extent suggests an increase of the available store during the release process. It is difficult to differentiate between increase in synthesis of immediately released transmitter and an increase in mobilisation of preformed transmitter. However, the effect of choline on maintenance suggests an increase in synthesis. This hypothesis however does require that the junction is able to preferentially release new

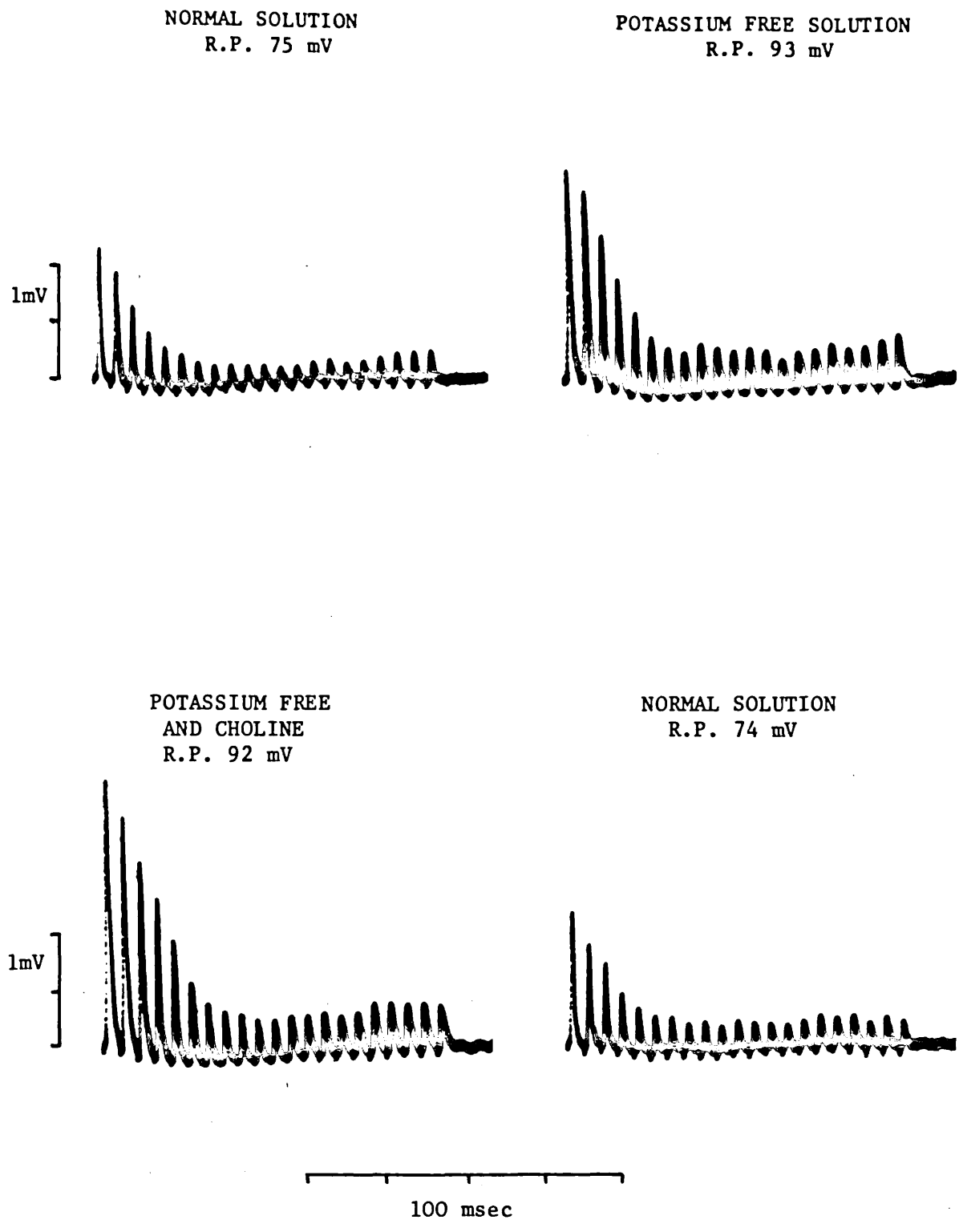


Fig. 10.1. Trains of e.p.p.s demonstrating the increase in Presynaptic store size caused by potassium removal and addition of choline chloride in the bathing solution. d-Tubocurarine concentration 1.2×10^{-6} g/ml. Final train in normal solution was recorded 10 minutes after return to potassium containing solution.

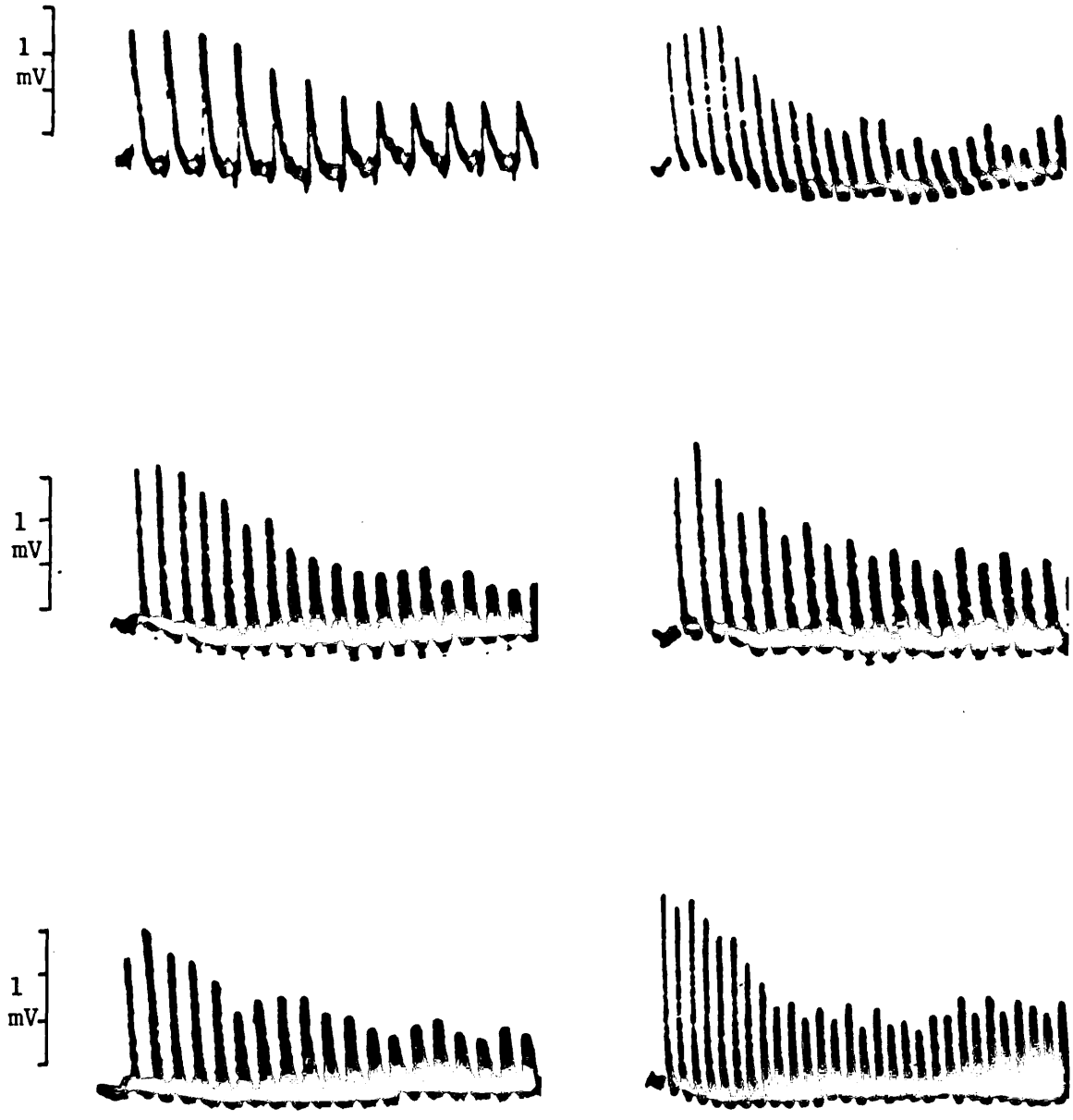


Fig. 10.2. Examples of trains of e.p.p.s, recorded in choline containing potassium free bathing solution, and showing exceptional maintenance of transmitter output. Stimulation frequency 50 Hz. Traces have different time scales but vertical amplitudes are the same in all cases.

synthesised transmitter. The hypothesis formed from the above observations therefore, is that reduction of potassium, with, or without the concurrent increase in resting potential enables the junction to reabsorb choline more readily from the synaptic space. Thus, its effect on transmitter release could be expected to be reduced in cases of lower transmitter turnover, which does appear to be the case.

If re-uptake of choline is involved in the effects being investigated, the action of blocking agents known to interfere with choline uptake in the nerve terminal, should antagonise the effect of potassium free solution. The principle choline uptake antagonist known is hemicholinium-3. (Schueler 1955, Birks & MacIntosh 1961). The effects of this compound were described in the relevent section of the introduction to this work.

The experimental procedure was to record from junctions under conditions of curare block and ascertain the size of the releasable store by analysis of the e.p.p. trains as in the previous experiments. The bathing solution was then changed to a potassium free medium with HC3 added. The store size was again determined for comparison with the normal effect of potassium free solution. The concentration of HC3 was 10^{-6} M, this concentration having been shown to produce block of choline uptake with little postsynaptic effect. At higher doses, the effect becomes predominantly postsynaptic, similar to that of curare. Under these conditions there were no increases of e.p.p. amplitude after change to potassium free conditions. However, it was also found that the combination of curare and HC3 depressed the e.p.p. amplitudes in normal solution. This depression was of such an extent that it was found impossible to record satisfactorily from the junctions after addition of HC3 under either normal or low potassium conditions.

This problem could have been overcome by reduction of the HC3 concentration. However it was then not certain whether or not the uptake was being blocked completely. The result of these experiments was therefore rather inconclusive.

From the results obtained with the two drugs, it appeared that both curare and HC3 had similar actions and that their combined effect was similar to a larger concentration of either, alone. A further investigation was made to compare the effects of curare and HC3 using the mechanical response of the non-blocked preparation. The object was to ascertain any similarities of response by examining the pattern obtained when using the cyclic stimulation procedure. For details of the method refer to Methods II.

Investigation using cyclic stimulation procedure

The action of HC3 is known and at low doses considered to be a presynaptic block of choline uptake. By investigating the response obtained with HC3 and comparing the response obtained with curare, it was hoped that some insight into the mode of action of curare could be obtained.

The effect of curare at a concentration of 1×10^{-6} g/ml can be seen in Fig. 10.3. This concentration is approximately that used to block the transmission during the recording of e.p.p.s. A rapid effect on maximum tetanic amplitude, coupled with a slower effect on the single twitch response is seen. These effects develop with increasing time. An additional obvious feature of the record is that the sustaining power of the tetanic contraction is reduced. In the portion of the record shown with an expanded time scale, it is clear that the tetanus only maintains its tension for the period of about one twitch response. Following the tetanic train, the twitch response is depressed and slowly recovers. This level of curare

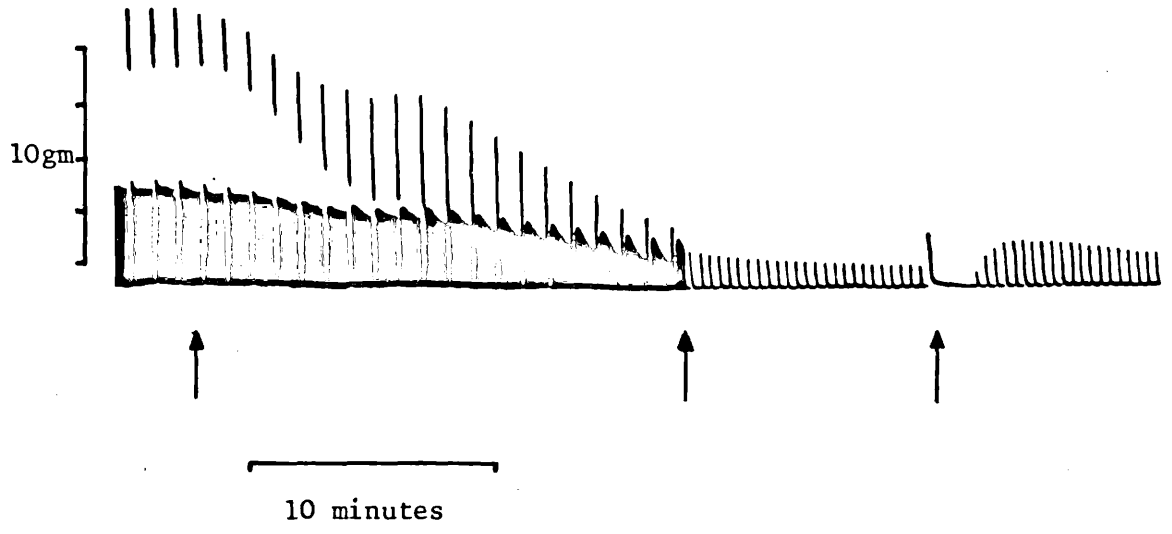


Fig. 10.3. The effect of d-Tubocurarine at a concentration of 1×10^{-6} gm/ml on the mechanical response of the indirectly stimulated diaphragm. Curare was added at the first point indicated. As the block progresses the tail of the tetanic response on the record runs into the twitch stimuli. At the position shown by the second arrow the chart speed was increased. The tetanic contraction, third arrow, can now be seen to be maintained only for a short period following which there is depression of the twitch response. Stimulation throughout was 1 Hz for 55 seconds and 50 Hz for 5 seconds repeated.

block is usually only just sufficient to completely block the mechanical responses at a frequency of stimulation at 1 Hz. From this result it is evident that the curare has a greater effect at higher rates of transmitter turnover, as occur during tetanic stimulation.

The effect of calcium on the response at the preparation under these same conditions of stimulation has already been described. (Chapter IX. Fig. 9.8.). Calcium, it is well known, facilitates transmitter release. When replenishment of stores can keep pace with release this leads to larger e.p.p.s. Under conditions of rapid stimulation, the store is depleted and thus smaller e.p.p.s are produced. When the calcium concentration was increased above normal, under the cyclic stimulation conditions, little effect was seen on the twitch amplitude or maximum tetanic amplitude, the predominant effect was on the sustaining power of the tetanic contraction. This leads to the suggestion that the effects on the sustaining power of the tetanus are an indication of presynaptic action, whereas changes in twitch and tetanus amplitude are related to postsynaptic events, when occurring at low drug concentrations and with short time courses.

To further test this hypothesis the effect of nicotine was investigated. This should have a different action to that of curare and also be postsynaptic in its mode of blocking. The effect of three different concentrations are shown in Fig. 10.4. The main effect can be seen to be on the amplitude of the maximum tetanic response. With the longest concentration (1×10^{-5} g/ml), an effect on twitch amplitude is also seen. However, at this concentration of drug, little effect in the sustaining power is evident compared with the depression of twitch amplitude. When the concentration is reduced below that required to affect the twitch and tetanic amplitude there is also little

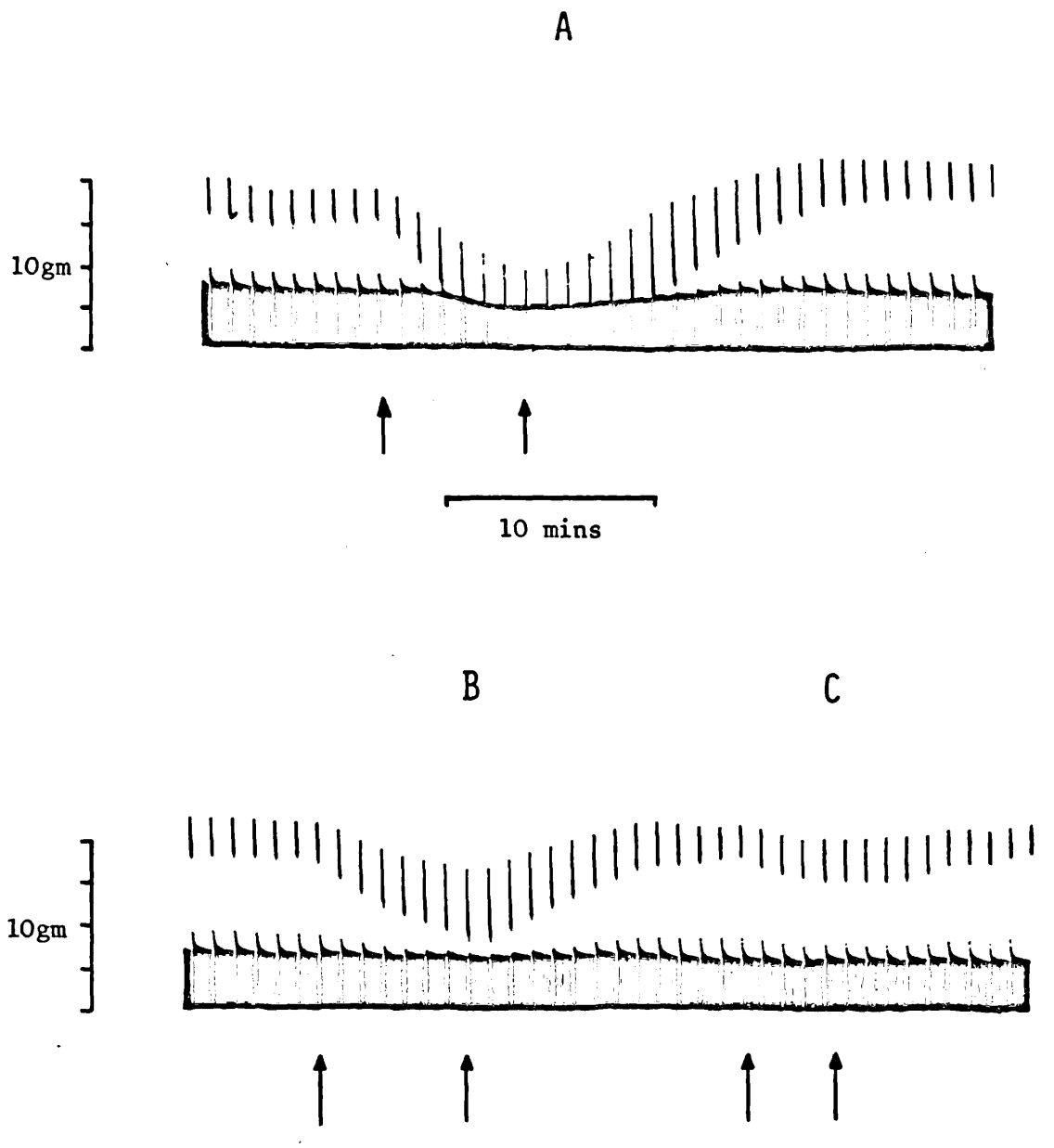


Fig. 10.4. Changes in isometric tension produced by three concentrations of nicotine. A. Nicotine was added at the first arrow to produce a bathing concentration of 1 x 10^{-5} gm/ml. The bathing solution was changed for fresh Krebs solution at the second arrow. B. A concentration of 6 x 10^{-6} gm/ml was produced. C. A concentration of 3 x 10^{-6} gm/ml was produced. Stimulation was uninterrupted during solution changes and was 1 Hz for 55 seconds and 50 Hz for 5 seconds.

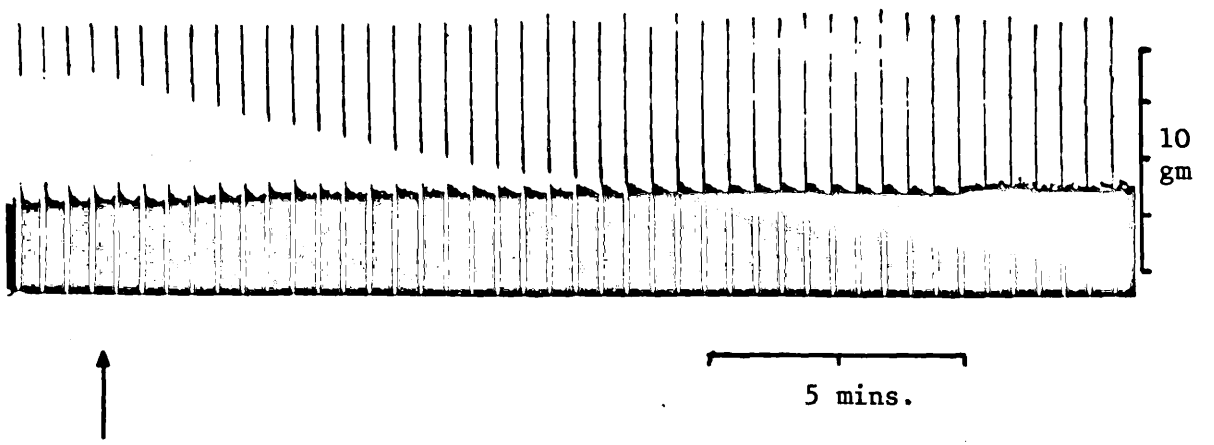


Fig. 10.5. Mechanical response change which occurs after the addition of d-Tubocurarine to the preparation when stimulated via the phrenic nerve at 1 Hz and 50 Hz alternately. d-Tubocurarine was added to the bathing fluid to produce a concentration of 10^{-7} g/ml at the point indicated at the arrow. The line produced by the tetanic response becomes longer as the tension is less well maintained throughout the period of tetanic stimulation until it merges with the twitch response as in the later part of the record.

or no effect on the sustaining power. From these results it is considered that the actions of curare and nicotine are fundamentally different at low concentrations.

The minimum concentration of curare found to have any effect when using the technique of cyclic stimulation was 5×10^{-8} g/ml. It was found however that the preparation had to be left for some time before this effect was seen. It was found that below 10^{-7} g/ml there was little change in either peak tetanic tension or twitch amplitude. The main effect was seen to be on sustaining power and post-tetanic potentiation. The effect of curare at 10^{-7} g/ml is shown in Fig. 10.5.

The changes in both sustaining power and post-tetanic potentiation suggest an effect on transmitter release and thus an action of curare which is not purely postsynaptic at these low concentrations. The experiments were repeated using HC3. Very similar results were obtained at comparable concentrations.

At concentrations of 10^{-5} M and above, HC3 is considered to have a predominantly postsynaptic effect. (Martin & Orkand 1961). Below 10^{-5} M, the action is thought to be mainly presynaptic, (Birks & MacIntosh 1961, Potter 1970), and below about 5×10^{-6} M there is little or no postsynaptic effect. The same workers have suggested that the action is mainly on the choline re-uptake mechanism in the nerve terminal. The effect of curare on the preparation at a concentration of 5×10^{-7} M is shown in Fig.10.6. The similarity of response when compared with that of curare suggests that curare may be acting through the same mechanisms as HC3 at low concentrations.

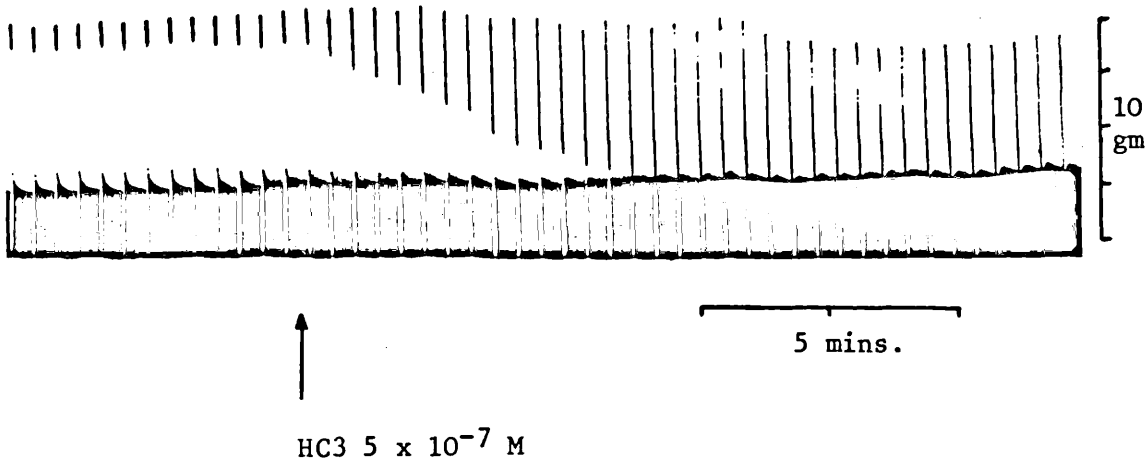


Fig. 10.6. Change in tension produced by the addition of 5 x 10^{-7} M HC3 to the bathing solution of the rat diaphragm when stimulated via the phrenic nerve at 1 Hz and 50 Hz. HC3 was added at the time indicated by the arrow. The tetanic tension (upper trace) declines during the period of 5 second 50 Hz stimulation progressively during the action of the drug. Associated with this is a change from post-tetanic potentiation of the twitch response to post-tetanic depression. This figure should be compared with Fig. 10.5. (the effect of d-Tubocurarine).

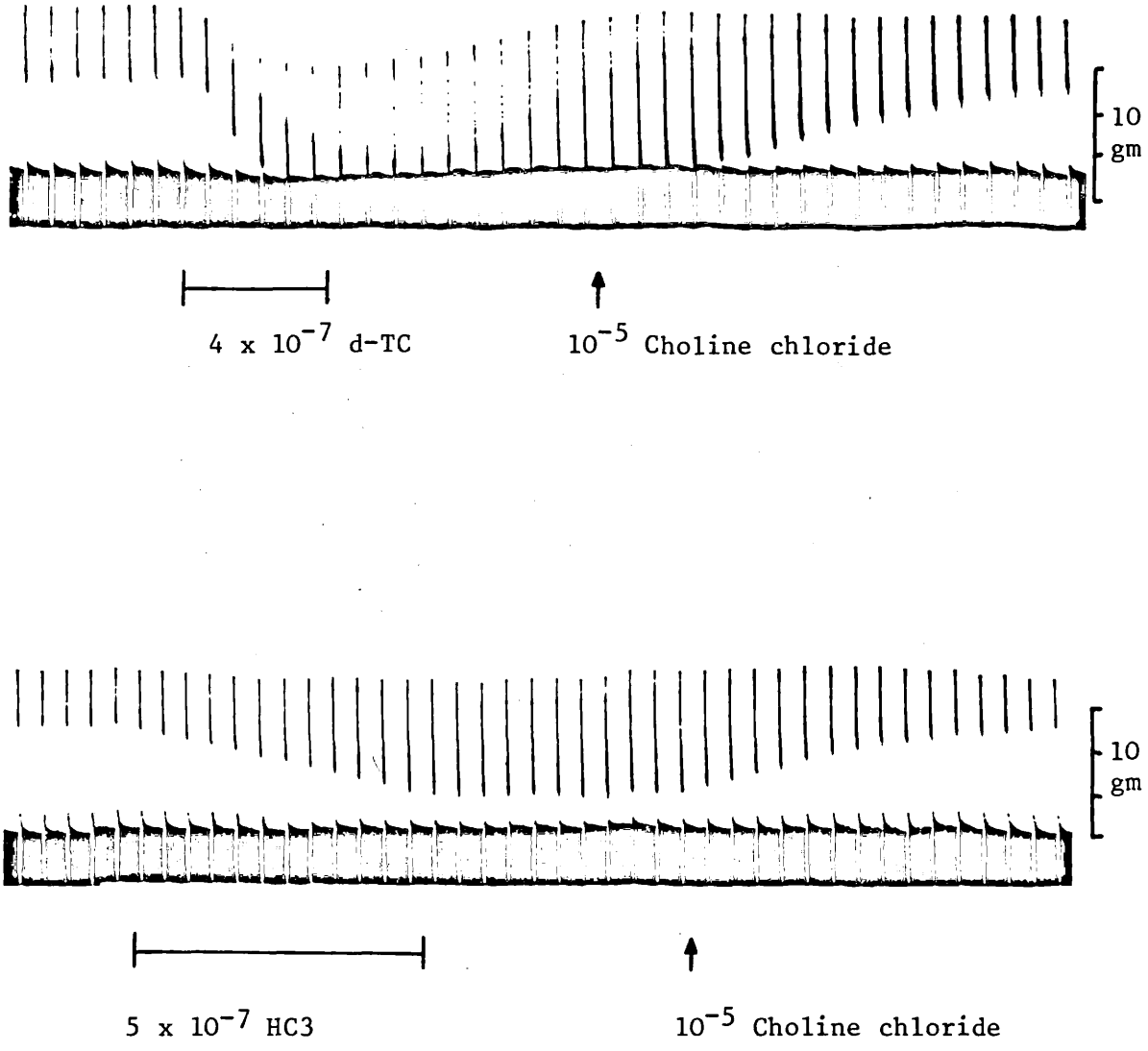


Fig. 10.7. Recovery of mechanical response after block by d-Tubocurarine (upper record) and HC3 (lower record). Drug, as indicated, was added to produce the required bathing concentration for the periods indicated by the bars. Choline chloride 10^{-5} M was added at the point indicated to reverse the block in each case. Stimulation as in Fig. 10.5. and Fig. 10.6.

When the preparation was washed with normal Krebs solution, the decline due to the blocking agent was halted. The response remained constant with little recovery. Addition of choline chloride to the bathing solution eventually produced complete recovery. Levels of choline chloride greater than about 5×10^{-8} M caused block of transmission and the amount given had to be kept below this concentration. Recovery to normal amplitude could only be achieved with the addition of choline chloride after block by curare or HC3, suggesting that a depletion of transmitter store had taken place. Recovery from both curare and HC3 block is shown in Fig. 10.7.

The effects of both curare and HC3 at low concentrations appeared to be opposite to that of the potassium free solution when the responses to the tetanic stimulation were compared. This, coupled with the fact that choline addition facilitated recovery from such block and also appeared to potentiate the effect of potassium free solution on the e.p.p., suggests that choline addition itself may cause an augmentation of the response. The effect of choline addition to the bathing fluid was investigated on preparations which had been previously exhausted. The type of exhaustion was found to be unimportant. A response was obtained which showed poor maintenance of tension during tetanic stimulation. The preparation was then washed with normal solution and left to stabilise for at least 30 min. Thus, a preparation was obtained with poor maintenance of tetanus but with no trace of blocking agents or suggestion of normal recovery. Choline chloride was then added to the bathing solution. An example is shown in Fig. 10.8. It was found that the choline had to be washed out of the preparation before the full effect was seen, and also that this procedure had to be repeated to produce full recovery. Often it was found that the

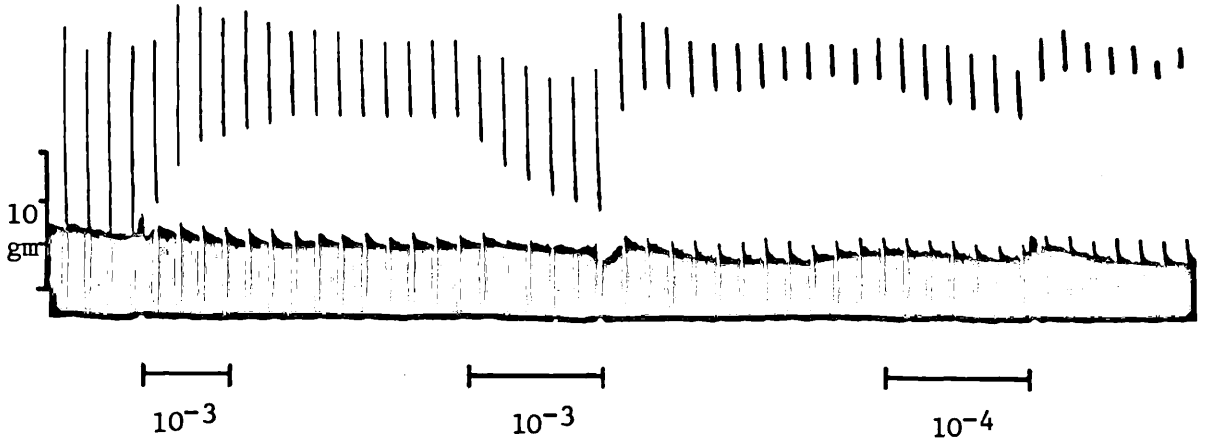


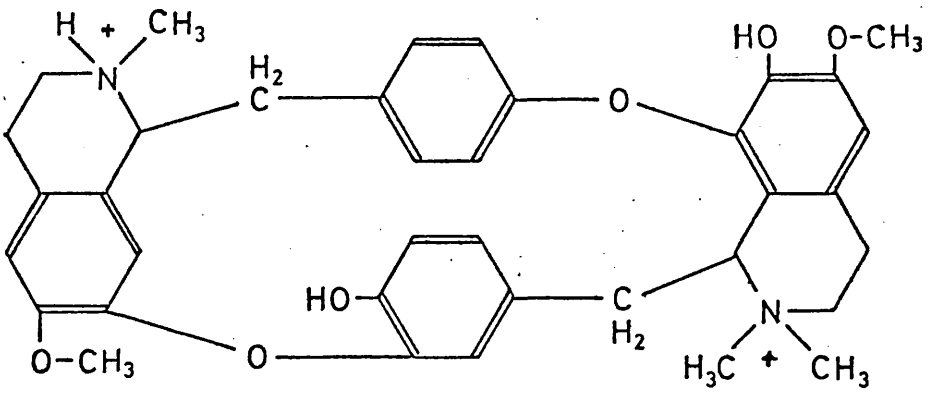
Fig. 10.8. Demonstration of the action of choline in promoting recovery from poor tetanic tension maintenance. Choline chloride was added to the bathing solution to produce the concentrations stated for the periods indicated by the bars. At other times the fluid was normal Krebs solution. In addition to aiding recovery it can also be seen that choline may produce block during the same period, as in the second application of 10^{-3}. Stimulation 1 Hz and 50 Hz at 1 minute cycle.

preparation response could be enhanced above that obtained at the beginning of the experiments.

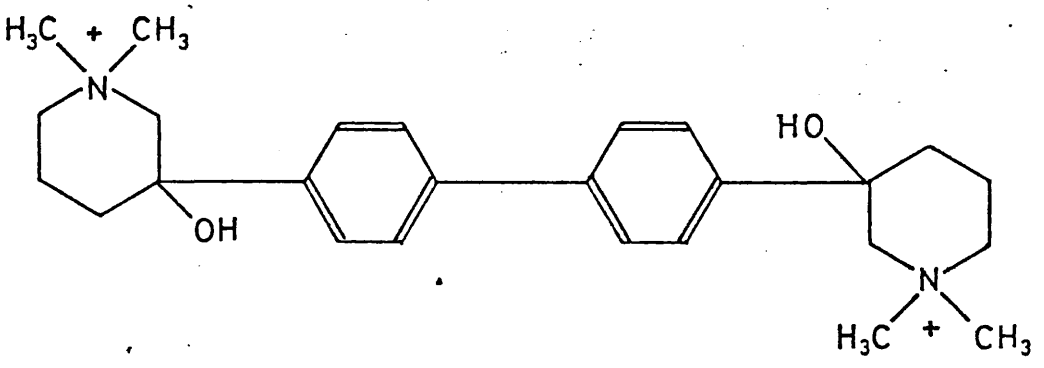
It was often found that at the end of an experiment when the preparation was in a generally poor state, that by changing the bathing fluid between potassium free and normal and adding small amounts of choline chloride, the response could be returned to normal and maintained for some time. It was concluded from observations such as these that the main cause of the decline in tetanic tension was exhaustion of transmitter, and that the nerve ending was able to resynthesise this, given favourable conditions.

The necessity for choline replacement after block by both HC3 and curare, emphasises the similarity of action of the two drugs. HC3 is known to block choline uptake and the similarity suggests that curare may do also. At the levels of curare block used in the experiments on the e.p.p., at least ten times greater, it must be concluded that a considerable reduction in transmitter output was taking place, especially during the experiments using trains of stimuli where a high turn over of transmitter would be expected.

HC3 is known to reduce the conversion of choline into acetylcholine at some point in the chain of events following transmitter breakdown. This point is probably the reabsorbtion of choline back into the nerve terminal. If the structure of both HC3 and curare are compared, (shown in Fig. 10.9.), a certain similarity can be seen. The quaternary ammonium groups bind to the choline reception sites on the postsynaptic membrane, when at concentrations great enough to produce postsynaptic block. If these



d-Tubocurarine



Hemicholinium-3

Fig. 10.9. Chemical structure of d-Tubocurarine and Hemicholinium-3.

same groups bind to choline uptake systems, then it could be expected that curare would bind similarly to HC3. Under most conditions of curare block this factor may not be of importance, however, under the present conditions of use, when the possibility of choline uptake is involved, it is of extreme importance and could have been modifying the experimental results.

Summary of chapter X

1. Investigations using trains of e.p.p.s as an index of transmitter output suggest that uptake of choline may be relevant to the present investigation and that the effect of potassium free solution may be linked with choline transport mechanisms. Electrophysiological experiments designed to directly investigate this were found to be particularly difficult, it appeared from the experiments that d-Tubocurarine and Hemicholinium were having similar effects at the end-plate, at the concentration used.

2. The actions of Curare and HC3 were compared by recording the mechanical response of the diaphragm muscle whilst using the cyclic stimulation procedure for activation. The response pattern obtained at low doses of each of these drugs was remarkably similar.

3. The response pattern obtained was also compared with the response in increased calcium concentration - known to facilitate release, and with that produced by nicotine - known to be a postsynaptic blocking agent. From comparison with these and HC3 the results obtained when using curare suggest a possible presynaptic role for this drug similar to that of HC3.

4. Choline can be shown to antagonise the effects of the block by both curare and HC3. In addition the response pattern of the preparation when in a poor state, after the addition of choline, is very similar to the response produced in potassium free solution.

SECTION IV
DISCUSSION

Chapter XI

The action of Potassium Free solution

The initial hypothesis at the beginning of this investigation was that reduction of the potassium concentration in the extracellular bathing solution surrounding the rat diaphragm phrenic nerve preparation, should enhance its response to indirect tetanic stimulation. This was found to be the case. The problem investigated therefore, was what changes occur, to cause such an improvement?

The potassium ion is so central to the activity of excitable tissues that changes in distribution, which are bound to result from an artificial lowering of the extracellular concentration, would be expected to have widespread effects on their functioning. This investigation has shown that a number of the properties of neuromuscular transmission do indeed change, when subjected to this alteration in ionic environment, in a way which could possibly improve the overall response of the preparation. Some, or all, of these changes may be directly relevant to the enhancement observed, others may be secondary effects occurring as an indirect result of action on some other part of the system

The synaptic cleft forms a natural dividing line both structurally and functionally between the nerve and the muscle. The presence of this division suggests the initial question of whether the effect is presynaptic, postsynaptic, or possibly both in origin.

All the investigations reported have necessarily involved the postsynaptic membrane and structures functionally related to it. Physical limitations prevent access to the presynaptic structures. In all experiments therefore, measurements made with the intention of investigating presynaptic action are measurements of postsynaptic responses or responses which have involved the postsynaptic membrane. There may or may not be a true reflection of presynaptic changes when, and if, any occur. Because of the overriding effect of changes in postsynaptic properties, the effects of reduction of the potassium in the bathing solution on these structures and their characteristics will therefore be considered first.

Effects of Potassium free solution on the muscle side of the synaptic cleft

A major factor to be considered under this heading is the direct effect of the low extracellular potassium on the contractile properties of the muscle itself. An enhancement of muscular contraction would be a simple explanation for the phenomena observed, from a mechanical point of view. An initial practical investigation of this using curare blocked neuromuscular junctions and direct stimulation of the muscle, revealed that there was little difference between the observed effects in either normal or potassium free solutions.

Furthermore, from a theoretical standpoint, a reduction in tetanic amplitude would be expected. Goffart & Ritchie (1952) have shown the active state of the muscle to be affected by extracellular potassium levels. A change in the active state would affect tetanic tension more than twitch tension, as the active state of the muscle is virtually over before an isometric twitch is recorded. During a

rapid tetanic stimulation therefore, a change in the active state ought to be seen as a change in tension. Increased potassium has been shown to increase the active state; removal then would be expected to decrease it. The records demonstrate an increase of tetanic tension with low extracellular potassium, and so a direct effect on the active state may be excluded.

As the electrical polarisation or resting potential of the nerve and muscle cells is determined by the distribution of potassium between the inside and outside of the cell, a primary effect of lowered extracellular potassium will be a change in this polarisation. A reduction of the extracellular concentration will result in a larger gradient between the inside and outside and thus lead to hyperpolarisation.

The relationship between the potassium distribution and the polarisation has been extensively studied in various tissues (e.g. Cowan 1934, Adrian 1956, 1960), by altering the external potassium and also in squid giant axon, where the internal fluid is accessible, by an alteration in the internal potassium. (Baker, Hodgkin & Shaw 1962). The results of these investigations have shown that in a general way the resting potential is related to the potassium equilibrium potential and may be predicted by the Nernst equation.

At values of external potassium below 10mM, the Nernst relationship ceases to be a reliable indicator of the actual resting potential. The membranes studied show that the effects of other ions, such as sodium and chloride, need to be taken into consideration. It has been shown that a more accurate prediction of

the resting potential may be made from the Goldman-Hodgkin-Katz equation derived from membrane constant field theory. This takes into account the permeabilities of the other ions and so their effects on the resting potential. At low levels of extracellular potassium and thus increased resting potentials, the shunting effect of other ions such as sodium, is particularly important.

In the present experiments, the measurements made of muscle fibre resting potentials in potassium free solutions demonstrated a hyperpolarisation to about 95mV from 74mV. It was also found that the Nernst type of relationship held reasonably well from 10mM potassium to 4mM potassium. From 4mM the linearity ceased and eventually the cell hyperpolarised no further. Hodgkin and Horowicz (1959) suggested that the anomalies of resting potential hyperpolarisation in frog muscle occurring at low external potassium could be explained by a resting permeability to sodium. 1% that of potassium. It would also appear that potassium permeability is reduced under conditions of hyperpolarisation. (Stampfli 1959, Baker, Hodgkin & Shaw 1962). Both of these factors would tend to lower the maximum hyperpolarisation achieved by a lowering of the extracellular potassium.

The values of the resting potential in various concentrations of potassium in the present experiments could be predicted by assuming a permeability to sodium, about 2% that of potassium. This is larger than that suggested for frog muscle fibres and may suggest a difference between the two species. Similarly, comparisons with other tissues of the effects of low potassium are pointless as there appears to be little similarity between species or tissues in their behaviour when deprived of external potassium. Akiyama and Grundfest (1971) reported little effect of low potassium on the resting potential of single semitendinosus

muscle fibres. They obtained hyperpolarisations in one third of their fibres but attributed this to an unbalanced pumped efflux of sodium. Mammalian Purkinje fibres are known to depolarise when exposed to a potassium free medium but not when sodium is also absent. (Carmeliet 1961). This contrasts with the effect in lobster muscle fibres, where the hyperpolarisation remains even after twenty four hours exposure to potassium free solution. (Gainer, Reuben & Grundfest 1967).

The results obtained with ouabain tend to suggest that the increase in resting potential obtained in the present work is a purely passive effect. Thus, the changes observed are entirely dependent on the potassium distribution and do not result from an acceleration of the sodium pumping mechanism.

Because anomalous changes occur in tissues exposed to low potassium it is difficult to predict an accurate value for the external potassium, in the vicinity of the cell membrane, from the value of the resting potential. This knowledge is important if it is required to calculate the value of the potassium equilibrium potential. It is well known that an increase in resting potential leads to a corresponding increase in the amplitude of e.p.p.s or m.e.p.p.s. (Fatt & Katz 1951, Hagiwara & Tasaki 1958). As the increase in permeability produced by the transmitter action of the neuromuscular junction is a permeability increase for cations, the depolarisation so produced will depend on the driving force behind each ion. Thus, the equilibrium potential for the transmitter will be changed by changes in the equilibrium potentials of the ions free to move. For the neuromuscular junction, Fatt and Katz (1951) have shown that the increase in e.p.p. is linear with

respect to increases in resting potential, and thus, the change in e.p.p. can be predicted when the resting potential is known. For muscle fibres therefore, the resting potential increase is known and the equilibrium potentials do not need to be calculated; this method being the most reliable for predicting increases in e.p.p.

The amplitudes of e.p.p.s recorded in the present investigation under potassium free conditions were found to have increased more than twice that which would be predicted on the basis of the increase in resting potential for the same fibres. It may be concluded therefore that although the resting potential increase could account for a percentage of the augmentation of the e.p.p.s, some other factor must also be involved.

To obtain a more accurate estimate of the increase in e.p.p. which should occur, the transmitter equilibrium potential must be known. This is the residual potential when the membrane is completely shunted by the effect of the transmitter. (Nastuk & Hodgkin 1950). For normal ionic concentrations a value of -15 mV was proposed. This has since been verified by other workers (Takeuchi 1963) by extrapolation from end-plate currents plotted from a curare blocked preparation. The change in potassium distribution will produce a change in its equilibrium potential and thus a corresponding shift in the transmitter equilibrium potential. For accurate prediction of the increases in e.p.p. in potassium free solution, the transmitter equilibrium potential would have to be derived from the use of end-plate currents in a similar manner.

The increases in m.e.p.p. amplitude were found to be of

the order predicted from the increases in resting potential. As the increases in e.p.p. were larger than the increases in m.e.p.p., it is unlikely that the increase in e.p.p. was caused by a single rise in equilibrium potentials. However, difficulty was experienced in measuring the small amplitude changes of the m.e.p.p.s accurately and this may have led to an anomalous result.

Muscle receptor sensitivity

Changes in end-plate sensitivity would be expected to affect all transmitter release equally. It was found that e.p.p.s were increased in amplitude while m.e.p.p.s were not. Furthermore, it was found that with pairs of pulses there were different effects on the two e.p.p.s. It has been shown for the neuromuscular junction that depression of a second e.p.p. of a pair is not due to receptor desensitising but to a reduction of quantal content of output. (Castillo & Katz 1954, Thies 1965, Elmqvist & Quastell 1965). It has also been demonstrated by Otsuka, Endo & Nonomura (1962) that at frog end-plates, the size of an iontophoretically applied pulse of acetylcholine following a depressed train of e.p.p.s is not itself depressed, showing that receptor sensitivity is not altered. This is assumed to hold for rat also.

The experiments reported here involving the use of twin pulses show that although both e.p.p.s were potentiated, it was not to the same degree. The second of the pair was facilitated more than the first when the bathing solution was changed to that containing no potassium. This result alone removes the possibility that the potentiation is due to an increase of receptor sensitivity in the potassium free solution. It would be very difficult to put

forward an hypothesis based on receptor changes which would take this observation into account.

In view of the above, it is considered that the increase in e.p.p. amplitude cannot be mediated by either an increase of receptor sensitivity or similarly an antagonism of a desensitising action of the transmitter on the postsynaptic membrane. This concludes the discussion of possible postsynaptic changes which could cause the augmentation of response. A further explanation was sought in a presynaptic mode of action. Two main lines of argument were followed: one based on the change in potassium ion distribution or the resulting electrical effects affecting the amount of previously stored transmitter released, the other based on a change in the amount of acetylcholine synthesised and thus resulting in a larger amount released.

Factors relating to the effects of potassium free solutions acting on the nerve terminal and modifying release of transmitter

The exact state of the presynaptic terminal under conditions of low potassium in surrounding fluid cannot be known. Unfortunately the nerve terminals are too small to impale with micro-electrodes and no direct measurement of the resting potential is possible. It is not known if the nerve membrane behaves in similar manner to that of the muscle under these conditions. If the permeability of the nerve terminal does not change, the potassium equilibrium potential must be increased, and therefore the resting potential, although the extent of this increase is unknown.

Evidence does exist to support the theory that hyperpolarisation takes place, as a result of low extracellular potassium concentration,

in the presynaptic terminals of the phrenic nerve. (Grundfest & Gasser 1938). In their experiments, assessment of excitability of nerve terminals was used to obtain an indication of changes in the polarisation of the terminal membrane.

It is well known that following a single spike potential, the nerve fibre may be hyperpolarised for a variable time. This effect summates, and its magnitude can be shown to be proportional to the number and the interval of the stimuli. (Ritchie & Straub 1956, Connelly 1959, Meves 1961 and Hubbard & Schmidt 1963). Two hypotheses have been advanced as an explanation for the increase in resting potential. Firstly the effect may be electrogenic in origin due to either pumping of sodium only, (Connelly 1959, Straub 1961, Holmes 1962) or to a depletion of potassium in extracellular space due to increased exchange pumping. (Ritchie & Straub 1956). Alternatively, the potassium permeability, which increases during the spike, does not return to normal immediately, leading to an increase of potassium equilibrium potential and thereby increasing the resting potential. (Hodgkin & Huxley 1952). This theory has been extended to cover the after hyperpolarisation, following a train of action potentials. (Meves 1961, Ito & Oshima 1962).

The hyperpolarisation has been investigated by Gage & Hubbard (1966a,b) in the rat diaphragm preparation and shown to be linked to changes in the potassium permeability. These workers demonstrated, by using the excitability of the nerve terminals as an index of hyperpolarisation, that potassium free solution at first increased, and then decreased their level of polarisation, as judged from their responsiveness to activation. The decrease,

which was not apparent for thirty minutes, they suggested was due to a slow decrease in intracellular potassium, caused by an increase in potassium permeability, probably coupled with a reduction in active ion transport, and the increase in potassium gradient out of the cell. These other workers have demonstrated that under normal conditions, the mammalian nerve terminal does hyperpolarise when exposed to potassium free solutions. Furthermore, the hyperpolarisation so produced does not affect the potassium permeability dramatically, at least not in the short term. If this was not the case, then the post-tetanic hyperpolarisation so investigated would not have been potentiated on exposure to potassium free solution.

It may be reasonably assumed therefore that in the present experiments, the nerve terminals are hyperpolarised to some degree. As described previously, following a single spike the potassium permeability will be increased. During a tetanic train of impulses this increase will be larger and more sustained. Thus, more potassium per impulse will be lost during a train than during a single activation. Low extracellular concentrations of potassium would therefore initially decrease the effect of accumulation of potassium around the nerve terminal caused by a train of impulses. Against this must be set the effect of an increased potassium gradient out from the nerve, due to the potassium free solution, coupled with the permeability increase, causing loss of potassium from the nerve fibre, and build up in the extracellular space. Post-tetanic depolarisation noted by Gage and Hubbard (1966a) did not occur for about thirty minutes after the start of the testing. This result suggesting a long time course for critical loss of potassium from the nerve, build up in the surrounding fluid, or possibly a marked increase in permeability. This time interval is far in excess

of the time during which potassium free effects were studied in the present work and tends to suggest that this factor is not complicating the experiments under consideration to any great extent.

There is some evidence, obtained from frog neuromuscular junctions, that inhibition of the sodium pump by ouabain or ouabain and potassium free ringer can result in facilitation of transmitter release. (Elmqvist & Feldman 1965, Birks & Cohen 1968, Baker & Crawford 1975). The time course appears to be similar to that for the change from a hyperpolarised state, to that of depolarisation, in the mammalian nerve terminals described above; about thirty minutes being required for increases in m.e.p.p. frequency to be seen. It would appear that this facilitation of release is mediated through changes in quantal content of output, which slowly increases during exposure until the terminals become totally inexcitable after a further thirty minutes.

Facilitation of transmitter release by an increase of potassium was also noted by Gage and Quastel (1965) in the rat. A dual action of potassium was suggested, to explain the rapid facilitation of m.e.p.p. release by potassium, (Lilley 1956) and the effect described above which has a much slower time course.

An hypothesis could be formulated suggesting that due to an increased potassium gradient across the cell caused by low extracellular potassium, the cell would lose potassium by leakage. This state would be aggravated by little exchange pumping and thus a facilitation of output could occur, probably caused by depolarisation of the terminal.

This hypothesis, as a possible explanation for the present results, can be rejected in view of the time course of the events.

Enhancement in all properties recorded were obtained immediately following solution changes. In addition, the effect reported in these experiments is also seen to remain constant over periods of 20 minutes as evidenced by the cyclic stimulation experiments. Lastly, the phenomena were all rapidly reversible on return of the bathing solution to normal potassium concentration. If a slow depolarisation of the nerve terminal was responsible then the effects would be delayed in onset. The changes observed would tend to be more variable due to the different leakage properties of the junctions studied. The nerve terminal would probably become inexcitable and this was never seen. Finally, the change back to a control solution containing more potassium would be expected to aggravate the depolarisation and therefore cause a rapid enhancement of the effect before a return to normal could be expected.

The alternative hypothesis is that in which the nerve terminal is considered to be hyperpolarised. There is a considerable body of evidence, dating back to the early intracellular work on neuromuscular transmission, linking release of transmitter and the degree of presynaptic depolarisation. Castillo & Katz (1954b) have shown that applied presynaptic depolarisation can augment transmitter release, as evidenced by an increase in the m.e.p.p. discharge frequency. Liley (1956a) investigated the same phenomena at the mammalian neuromuscular junction in the rat diaphragm. A logarithmic dependence of discharge frequency on extracellular potassium concentration was demonstrated. This was shown to be valid only for concentrations of potassium which were normal or greater than the standard Krebs solution values. Thus, the relationship holds where the membrane potential is governed by the potassium distribution and not at the lower levels of potassium where there is a large difference

between the potassium equilibrium potential and the resting membrane potential. These experiments led to a simple model involving the amplitude and time course of the nerve action potential which in some way regulated the output of transmitter; this being similar to a sudden increase in resting discharge. Thus, hyperpolarisation of the presynaptic nerve ending could be expected to modify the presynaptic action potential, and so the amount of transmitter released.

The hypothesis is supported by data from workers relating the amount of transmitter released to the measured presynaptic action potential at the squid giant synapse. (Hagiwara & Tasaki 1958, Takeuchi & Takeuchi 1962, Bloedel et al 1966, Katz & Miledi 1967a,b,c). In this preparation, which appears to have many properties similar to the vertebrate neuromuscular junction, electrodes may be inserted to record the presynaptic potential. Hyperpolarisation caused an increase in the presynaptic action potential which resulted in a greater post-synaptic potential by facilitation of transmitter release.

Doubt has been expressed about the validity of this hypothesis by workers obtaining results suggesting that the amplitude of the presynaptic action potential does not regulate the amount of transmitter released. (Katz & Miledi 1965, Braun & Schmidt 1966). These results were determined from frog neuromuscular junctions which are known to behave differently from those of other species under conditions of current application. In particular, sudden increases in the frequency of m.e.p.p.s occur under conditions of strong hyperpolarising current. This may underline a difference between rat and frog, in that the neuromuscular junction of the frog

may have around it a greater barrier to diffusion than does the rat. However, this phenomena of 'anodic breakdown' has been reported to occur under certain conditions in the rat. (Landau 1969). In the experiments of Braun and Schmidt (1966), repetitive stimulation was shown to reduce the size of the presynaptic action potential, the reduction being due to an increase in potassium permeability. No relationship was found however between the amplitude reduction and release. Braun and Schmidt suggested that the amplitude of the presynaptic action potential did not play a significant part in regulating release in the frog as it appears to do in the rat.

There is also other evidence suggesting that all the transmitter released may not be entirely due to the depolarisation of the presynaptic terminal. (Liley & North 1953, Takeuchi & Takeuchi 1961, Gage & Quastell 1965, Cooke & Quastell 1973). The relation between depolarisation and release may be different at different levels of depolarisation. A variable contribution to output may be made by resting release. This spontaneous release slowly increases when the terminal becomes depolarised and can make interpretations of results difficult. Thus, it is not valid to correlate quanta released with active depolarisation and also with resting potential derived from the external potassium concentration. The slow time course of changes in resting release making impossible an accurate estimate.

Further supporting evidence for depolarisation regulated release comes from work on squid giant synapses. Here, experiments have demonstrated that neither tetrodotoxin (TTX), which is known to block sodium conduction changes, nor tetraethylammonium ions

(TEA), which affects potassium channels, interferes with the depolarisation release response. (Katz & Miledi 1967a,b,c,d, 1969a,b, Kusana, Livengood & Werman 1967). Furthermore, TTX does not alter the effect of presynaptic polarisation on release of m.e.p.p.s. (Landau 1969). Katz and Miledi have made extensive use of terminal polarisation in studies on release of transmitter in TTX blocked preparations and so in the absence of nerve impulses. These experiments have led to a theory that the depolarisation governs the calcium movement across the membrane and it is this which determines the amount of transmitter released. The possibility of calcium being involved in the present work has been considered and will be discussed later.

Presynaptic hyperpolarisation therefore, when it occurs, most probably ultimately affects the calcium concentration within the nerve terminal membrane so affecting the release probability of the individual quanta. Whatever the underlying explanation, hyperpolarisation, when resulting from electrical application, has been shown to increase transmitter output in a number of instances: At the frog neuromuscular junction (Castillo & Katz 1954), cat motor neurone synapses (Eccles, Kostyuk & Schmidt 1962), squid giant synapse (Hagiwara & Tasaki 1958, Takeuchi & Takeuchi 1962), and rat diaphragm (Lilley 1956, Hubbard and Willis 1962).

Takeuchi & Takeuchi (1961) in a study of presynaptic polarisation of nerve ends at the frog neuromuscular junction, have suggested that the facilitating effects of the polarisation may be due to induced changes of extracellular potassium concentration in the close vicinity of the nerve terminals. As m.e.p.p. frequency was used as an index of potassium concentration, the results are open to the criticism that due to the slow effect of potassium on

the membrane, the m.e.p.p. frequency does not truly reflect the potassium concentration. (Gage & Quastell 1965). This does not affect their main finding however that effects of current could be explained by changes in potassium concentration. In their preparations they also obtained no reduction of m.e.p.p. frequency when the potassium concentration was lower than normal. Again, this may be a basic species difference between frog and rat.

The phenomenon of facilitation by hyperpolarisation was investigated using the rat diaphragm preparation by Hubbard & Willis (1962). An increase in the amplitude of the e.p.p. was demonstrated but no change in amplitude of the m.e.p.p. Because the m.e.p.p. amplitude stayed constant, the facilitation of the e.p.p. was considered to be due to an increase of quantal output. This could either be from a larger amount of transmitter available or due to an increase in releasing ability of the nerve action potential. The most likely possibility was considered to be an increase in the mobilisation of the transmitter in the terminal, brought about by the hyperpolarisation and possibly the resulting potassium distribution changes.

The work was extended by Hubbard and Schmidt (1963) who also used the rat diaphragm preparation. The presynaptic nerve action potential was recorded and increases in the amplitude of this were shown to occur together with e.p.p. amplitude potentiation as a result of hyperpolarising current flow. The experiments were carried out to investigate the effect of repetitive stimulation on the nerve action potential. It was found that under these conditions there was a sustained increase. It has been shown (Rahaminoff 1968, Landau 1969, Katz & Miledi 1970, Weinreich 1971, Cooke et al 1973), that the facilitation due to repetitive stimulation is probably linked to calcium movement through the presynaptic membrane

and formation and breakdown of calcium complexes. The experiments of Hubbard and Willis (1962) and Hubbard and Schmidt (1963) however, underline the link between nerve terminal hyperpolarisation and increase of transmitter release. The mechanism for the facilitation may be either an increased mobilisation of actual transmitter as suggested by Hubbard and Willis (1962) or an increase in mobilisation of calcium which must be considered in view of the knowledge now available about calcium and release. The two possibilities would however have entirely different characteristics when investigated and this will be discussed later.

In all these investigations the workers noted a decrease of m.e.p.p. frequency when using hyperpolarising current, as did Lilley (1956). This has since not been confirmed (Landau 1969). In the experimental work reported here, a variable effect of potassium free solution was obtained on the m.e.p.p. frequency. The suggestion was made by Landau (1969), that to account for the previously reported decreases in m.e.p.p. frequency by hyperpolarisation, the nerve terminals may have been in a state of partial depolarisation before the current application. A plateau effect would be expected on this basis and no further reduction in frequency occurring below a certain potassium concentration producing a defined membrane potential, 'anode breakdown' occurring at potentials more negative than this. (Castillo & Katz 1954b, Katz & Miledi 1965, Landau 1969). No breakdown effects were observed with potassium free solutions in the present work. At some junctions, a depression of frequency was observed and at others some facilitation, indicating that the effects are not predictable. Changes in the permeability to both potassium and calcium due to potential changes may have been produced, so

explaining the variable results.

Reduction of potassium permeability may possibly occur at low levels of extracellular potassium. As E_k increases, so the membrane conductance decreases in some cases, (Carmeliet 1961), at low levels the sodium - potassium exchange will also reduce, allowing the internal sodium to increase. Thus, the activity of the postulated sodium - calcium pump, (Baker 1972), might be expected to increase, leading to an increased calcium entry. If this effect only occurred at specific points within the preparation where the structure of the terminal made the system more sensitive to potassium, then the variable effects could be explained. However, in many cases a reduction in m.e.p.p. frequency was obtained and thus hyperpolarisation must be assumed at those endings, and either local damage or permeability changes at the others.

Although the effects on e.p.p.s described by other workers using hyperpolarisation were obtained by passing current, there are certain similarities with the present work in which any hyperpolarisations achieved resulted from changes in extracellular potassium. The main effect, that of increase in e.p.p. amplitude, was obtained by a change in potassium distribution, directly in the present experiments, and indirectly as a result of current flow in those of the other workers. There is therefore, strong support for an hypothesis which would require a common mechanism for the results described in this work and those of workers using hyperpolarising currents. Possibilities are: A change in the presynaptic spike amplitude, mobilisation of calcium, mobilisation of transmitter. Any of these three may be linked. Conceivable mechanisms of action for presynaptic facilitation are discussed in the following chapter.

There is one further factor which although presynaptic would point to the results being a non-specific effect of potassium concentration rather than a specific effect on transmitter release. It is known from studies using electron microscopy, (Birks 1962), that after inhibition of the sodium pump in sympathetic ganglia, and in nerve terminals in frog skeletal muscle, the fibres are seen to swell. If it were assumed that potassium free solutions could inhibit the exchange pump for sodium and potassium, then this swelling could explain some of the effects. It is known that stretching of the presynaptic nerve membrane renders the terminal leaky and m.e.p.p. frequency increases. (Fatt & Katz 1952, Hutter & Trautwein 1956). These experiments also demonstrated that stretch of the muscle fibre, reducing the diameter, also gives rise to larger m.e.p.p.s due to changes of membrane constants. There was no change of fibre diameter in the present experiments, but the membrane could be rendered more leaky by stretch. Many of the results would be difficult to explain on such a mechanism. A simple non-specific increase in transmitter output would have been expected. This did not occur. The effect on e.p.p.s reported may be modified by choline or calcium. In addition, a differential effect on pairs of e.p.p. was seen. The second e.p.p. of a pair would not be facilitated in the potassium free solution, to a greater extent than the first, if the effect was so non-specific. It is also unlikely that the sodium - potassium exchange is completely blocked as it would be when using drugs to achieve this state. The fact that in some junctions a reduction of m.e.p.p. frequency was noted is, in itself, an indication that the effect is not of any great significance under the experimental conditions used. The possibility of terminal swelling contributing to the facilitation observed has therefore not been further considered.

Consideration of the points in this chapter leads to the conclusion that a small potentiation of amplitude of e.p.p. will occur postsynaptically due to changes in resting potential. This will affect all responses equally. This is not in accord with some results presented, especially those responses obtained using twin stimuli, trains of stimuli, calcium ions and choline. These effects can be better explained by a mechanism involving a change in presynaptic release of transmitter. The possibility exists that facilitations demonstrated here may be similar to that seen in the electrical hyperpolarisation experiments of Hubbard and Willis, and of other workers, and that both involve presynaptic facilitation of transmitter release. It is now necessary to establish a possible mechanism for the potentiation of the transmitter which is in accord with the observed results.

Chapter XII

Discussion of Mechanisms leading to Augmentation of transmitter output

Increase in synthesis

To postulate how a modification of acetylcholine synthesis could occur, and thus increase the store of transmitter available for release, some of the junctional physiology needs to be reconsidered.

The transmitter, once its effect in depolarising the muscle end-plate receptors has been achieved, is broken down by the enzyme acetylcholinesterase. This occurs in the synaptic cleft and results in the production of free choline and acetate. The free acetate is of no account in the present consideration and it will be assumed that it is removed from the cleft by the process of simple diffusion. The choline must be considered to be under the influence of both concentration gradients, and, because of the presence of the charged quaternary group, under the influence of electrical gradients.

There is a considerable body of evidence to support the fact that the choline is reabsorbed by a specialised uptake mechanism into the nerve terminal for resynthesis into usable transmitter. Many examples of choline uptake systems are now known to exist in a variety of tissues. Human red blood cells (Martin 1967, 1968), loligo giant axons (Hodgkin & Martin 1967), rat diaphragm (Chang & Lee 1970), and mouse brain slices (Bhatnager & MacIntosh 1965). Potter (1968) has described uptake of choline into rat cerebral cortex. Birks and MacIntosh have extensively demonstrated uptake of choline in superior cervical ganglion cells and have described many of the limiting factors. (Birks & MacIntosh 1961, MacIntosh 1963). In all these systems, transport of choline appears to be active against a concentration gradient for

choline. It has also been shown to be inhibited by hemicholinium in all these cases. An interesting further fact to emerge is that in most instances dependence of uptake on the extracellular sodium concentration had also been demonstrated.

With this evidence available in support of choline uptake systems it would be reasonable to assume a similar system for the nerve terminals under investigation in the present work, although as yet this has not been conclusively proven. Chang and Lee (1970) have demonstrated an active uptake system in the diaphragm but not specifically in the nerve terminals. Potter (1970) has presented evidence which indirectly suggests an active uptake system. By investigating turnover of acetylcholine in the nerve terminals of the rat diaphragm it was shown that a large percentage of the choline used was recycled, and newly synthesised transmitter was released immediately. The recycling may be inhibited by hemicholinium. In addition an efficient mechanism for choline capture has been demonstrated by the procedure of constantly stimulating the rat diaphragm muscle via the phrenic nerve for three days, superfused with nothing other than Krebs solution containing the normal amount of glucose. (Present work unreported). It was found that the preparation was affected more by bacterial infection than it was by transmitter lack! However, if the preparation was deprived of glucose the transmission failed in about 4-5 hours. The output of acetylcholine from preganglionic terminals was studied by Bennett and McLachlan (1972a,b). They suggested that the main substrate for acetylcholine synthesis is recaptured choline from hydrolysed transmitter. From this they went on to suggest that the synthesis is thus controlled by the release rate.

Involvement of other ions with a carrier system for choline or dependence on electrical gradients could both be affected by a change in potassium distribution or the effects resulting from it.

Hyperpolarisation could be expected to act directly on transfer of choline across the membrane even if it were only diffusing down a concentration gradient. Choline contains a positively charged group and if at times the membrane became specifically more permeable to choline, an increased negative charge within the nerve terminal would facilitate entry. If the membrane permeability to sodium was low and to choline and potassium equal, then by removing potassium, choline entry would be improved both by removal of competing potassium and by the increase of resting potential.

Entry of choline is not the only factor to be considered; the synthesis of acetylcholine may also be affected by changes in the ionic environment. Birks (1963) has presented data to support a theory that the acetylcholine synthesis in sympathetic ganglia and in frog myoneural junctions is a function of sodium concentration within the nerve terminal. A mechanism such as this would have obvious practical advantages during prolonged activity when the intracellular sodium will inevitably rise. According to this theory, synthesis would be stimulated to keep pace with release, which is maintained by the rise in intraterminal sodium concentration. Birks found that removal of extracellular sodium inhibited the synthesis. By using procedures for blocking sodium pumping such as cardiac glycosides with or without potassium free solutions, it was shown that as the intraterminal sodium rises, the effect of each nerve impulse is to release a larger amount of acetylcholine.

Reduction of external potassium could indirectly produce a rise in the intraterminal sodium concentration. Reduction of exchange pumping, and an increased electrical gradient for sodium into the cell will eventually cause the intracellular sodium concentration to increase. The stimulation of acetylcholine synthesis by this rise

in intracellular sodium could therefore explain the increase in presynaptic store observed in the present work.

The question of whether the nerve terminals contain sodium pumping aparatus is not resolved. However it has been demonstrated by electronmicroscopy (Birks 1962) that after exposure to digoxin, intended to block pumping, cholinergic nerve endings in sympathetic ganglia and frog skeletal muscle are seen to swell. This swelling may be prevented by replacing chloride with a larger non-penetrating anion such as sulphate. This is precisely that which would be expected if the nerve ending posessed sodium pumping properties. Because the volume of the terminals is small, a small exchange of ions will result in large concentration changes within them. Thus, during activity, the loss of potassium and gain of sodium which occurs would rapidly lead to depolarisation and block of function. This is not seen. The terminal must therefore have some method of removal of sodium ions and this is probably an exchange pump.

Any increase in acetylcholine production will have a rapid direct effect on the total number of quanta available for release. There will also be an indirect effect on choline transport. Stimulation of synthesis will inevitably reduce the intraterminal choline concentration thus increasing the gradient for choline across the membrane. This would then lead to an increase in uptake from the synaptic cleft.

Rise in intraterminal sodium concentration could be a possible way of regulating the acetylcholine synthesis normally, to enable the store replenishment to keep pace with transmitter release during activity. If this was the case then the system could be expected to respond to small changes in sodium concentration as would occur in the conditions under discussion.

At no time during the experimental procedures was there any block of transmission from the nerve to the muscle. Even during the longest exposures to potassium free solutions, no end-plates investigated demonstrated a failure to conduct action potentials to the terminal, and to produce e.p.p.s in response to stimulation. This fact may be cited as evidence for their being no complete block of the sodium pumping activity. If the small fibre size and pump inhibition produced a rapid change in intracellular concentration, it would be expected that with complete inhibition and constant stimulation that some fibres would become too depolarised to conduct. As this was never seen it may be assumed that the effect of potassium free solution was not eventual depolarisation because of pump inhibition. The system remains physiologically active over long periods of exposure and the enhancement remains stable, the process whereby this is achieved must therefore not be detrimental to normal mechanisms active within the terminal. A complete block of pumping would eventually have such an effect.

One other possibility which cannot be ignored is that the sodium continues to be ejected in potassium free conditions but not in exchange for potassium. The other cation in high concentration during periods of activity is choline. If the pump could exchange sodium and choline then the intracellular sodium would be kept low and choline transported across the membrane. There would be obvious advantages to the nerve terminal with such a system during normal activity. If a pump existed whereby potassium and choline could be exchanged for sodium, a perfect mechanism would exist for accumulating choline from the synaptic space during activity. In the absence of any further data on sodium pumping, no precise hypothesis is possible but the possibility does exist.

Martin (1972) has shown that the choline carrier mechanism in human red cells can react with other monovalent cations and that potassium has a higher afinity as a competitor than sodium. Reduction of the potassium concentration would therefore favour choline uptake if a similar mechanism existed in nerve endings. Little is yet known of choline carrier systems in excitable tissues, but one great difference from a system such as the red cell contains is that the choline is transported with an electrical gradient as well as against a concentration gradient. Removal of potassium may well be removing a competitive ion but will also facilitate transport by hyperpolarisation of the cells involved.

Further evidence that the choline re-uptake mechanism is involved may be drawn from the experiments using cyclic stimulation on the mechanical response of the preparation. The effect of potassium free solution under these conditions was to increase the 'holding power' of the tetanus. When the choline uptake blocking drug HC3 was used, the effect on the preparation was exactly the opposite to that of potassium free Krebs solution. The ability of the preparation to sustain the tetanic contraction becomes consistently reduced, the effect becoming progressively greater during the period of exposure. The application of choline chloride to the preparation can reverse this effect. The actions of potassium free solution and that of choline on this preparation also appear to be very similar if the conditions are correct for the choline to exert its effect. One observation for which an explanation could not be found was that to demonstrate the effect of choline it had to be applied to the preparation for several minutes and then washed out. When the preparation was returned to normal Krebs solution after washing, an enhancement was observed very similar to that obtained when using potassium free Krebs solution.

The available evidence therefore points to an enhancement produced by potassium free solution acting through an increase of acetylcholine synthesis produced by direct ionic stimulation, increased uptake of choline or both. This would lead to an increase in terminal transmitter stores and thus an increase in output by increasing the number of quanta available for release.

Possible mechanisms for enhancement of transmitter release under the effect of potassium free solution

Two main factors control the release of acetylcholine from the presynaptic terminal. Firstly, there is the fraction of transmitter, (of the total stored), which is available for release at any defined time and secondly, the probability that any of it may be released by a single activation. Thus the same effect will be generated by a large supply and small probability or by small supply and a large probability. The long term effects on the nerve terminal will however be very different. A large probability of release will lead to a depletion of the available store and thus a drop in the actual amount released after the passage of only a small number of impulses. Thus, to keep pace with release, synthesis would have to be increased considerably if serious depletion were not to occur. In the opposite situation where a large store is postulated, release could continue for some time without serious depletion of transmitter and subsequent drop in output. It is however known that the pool of transmitter from where the quanta are released is small, about 300-1000 quantal units. (Birks & MacIntosh 1961, Elmqvist & Quastel 1965). No assumption may be made that release has little effect on the store size, a fraction of between one third and one tenth being released for each impulse. Some mechanism of replenishment must exist to replace this lost transmitter. This must be either synthesis of new transmitter for

release or mobilisation of previously synthesised transmitter from a reserve store into the immediately available store.

There is evidence to suggest that newly synthesised transmitter is released preferentially. (Birks & MacIntosh 1961, Potter 1970, Bennett & McLachlan 1972b). By radioactive labelling it was shown that there was a rapid turnover of the transmitter during activity. In addition, it was shown that HC3 could block the turnover of the stores by preventing re-uptake. In view of these results an action on synthesis would have an immediate effect. A change of mobilisation could possibly be expected to have less effect.

The mechanism of release is governed by calcium ion concentration changes. The low potassium solution could possibly change the calcium movement into the cell during activity and so increase the probability of release. A facilitated or prolonged entry of calcium may occur, or a change in the level of intracellular bound calcium might be produced as a result of the potassium distribution, or polarisation changes. An increase in release probability will not lead to consistently larger e.p.p.s. The first e.p.p. of a train is facilitated, this causes a reduction in store size which results in subsequent e.p.p.s released with rapidly diminishing quantal content. This was demonstrated in the experiments on pairs of e.p.p.s and trains of e.p.p.s in calcium enriched solutions.

The results lead to the conclusion that there is an increase in the size of the presynaptic, immediately available store. Only this, can produce the continued enhancement demonstrated. There may also be a coupled increase in probability but it would always be an effect secondary to increase in available transmitter. If the increase in store size results from a larger number of quanta, it can be considered that this state could be achieved in two ways: (a) increase of mobilisation

from the larger, long term store of acetylcholine, already produced within the terminal, (b) increase in storage of newly synthesised transmitter, by a mechanism involving stimulation of synthesis by potassium free solutions.

It is difficult to postulate a mechanism for the first possibility as the morphological correlate of the immediately available store is not known. If it be assumed that the layer of synaptic vesicles close to the membrane is indeed this, as suggested by Hubbard and Kwanbunbumpen (1968) then an attraction of vesicles toward the membrane under the influence of an electric field is a possibility. A similar mechanism was suggested by Hubbard and Willis (1962) to explain the results obtained with electrical hyperpolarisation of the nerve membrane.

As the morphological basis for the transmitter stores is tenuous, further speculation about movement of vesicles is pointless. It is difficult to imagine bulk movement of vesicles in the number required in such very short times. However, if a purely field type of effect is occurring in actively moving acetylcholine in one form or other closer to the membrane then this would be a possible explanation for the effect of potassium free solution. It would also explain the similarity between these results and those obtained with electrical hyperpolarisation. (Hubbard & Willis 1962).

In view of the time course of the events and also experimental results suggesting preferential release of newly synthesised transmitter, (Birks & MacIntosh 1961, Potter 1970), the second of the two alternatives seems more likely. This postulates an increased synthesis of transmitter. In the present experiments this may result from a change in concentration in potassium ions on the outside of the membrane or from a change in

electrical field across the membrane based on this ion. These changes will either lead to a direct stimulation of synthesis, or an increase in the availability of choline for resynthesis into active transmitter.

Chapter XIII

Discussion relating to the use of curare as a postsynaptic blocking agent in these experiments

During the investigations extensive use has been made of the drug d-tubocurarine as a postsynaptic blocking agent. Since the work of Dale, Feldberg & Vogt (1936) the effect of this drug has been considered to be due to a competition between acetylcholine and curare for the postsynaptic receptor sites. These authors studied the amount of acetylcholine released at low rates of stimulation and compared that released in paralysed and non-paralysed neuromuscular junctions. They found no difference and thus under their experimental conditions the action of curare was considered to be on the postsynaptic membrane. These conditions were low frequency stimulation (about 5 Hz) and a short experimental procedure. Under other conditions of investigation curare may not be having a single postsynaptic effect. There is evidence that at greater stimulation rates or over a longer time course, curare may have a presynaptic effect at low concentrations.

Wedensky demonstrated in 1903, inhibition of transmission by repetitive stimulation at high frequencies. He also showed that the neuromuscular junction was more sensitive to the effect than was the nerve or the muscle. This inhibition he found to be increased by curare. Since the advent of e.p.p. recording, a similar inhibitory effect has been demonstrated, which has also come to be termed Wedensky inhibition. This is the decrease in e.p.p. amplitudes under conditions of tetanic stimulation described by a number of authors. (Eccles, Katz & Kuffler 1941, Liley & North 1953, Lundberg & Quilish 1953a, Elmqvist & Quastel 1965b). It is now generally agreed that this decline in amplitude is due to a reduction of acetylcholine stored in the

nerve terminal. This conclusion involves the assumption that the curare used to block the transmission in all of these experiments was having a postsynaptic effect.

In experiments where there was no use of blocking drugs it has been shown that there is very little Wedensky inhibition of e.p.p. amplitudes, but that it is possible to produce this decline in amplitude by addition of small concentrations of curare to the preparation. (Lilleheil & Naess 1961, Hubbard, Wilson & Miyamoto 1969, Galindo 1971, Hubbard & Wilson 1973). The concentration of curare used was of the order of 10^{-8} - 10^{-7} g/ml in some cases. These authors thus suggest that curare can exert a presynaptic effect at very low doses, and also presumably at higher ones.

It is of note however that rapid decline of e.p.p. amplitudes under tetanic conditions is dependent on the frequency, of stimulation, or the interval between pulses, in addition to the action of curare. (Thies 1965, Hubbard, Llinas & Quastel 1969, Capek, Esplin & Salehmoghaddam 1971). It can also be shown that the depression of the second e.p.p. can be correlated with the height of preceding e.p.p., suggesting that depletion of quanta in a store can produce variations in the quantal content of subsequent e.p.p.s. (Elmqvist & Quastel 1965b). From these observations, the conclusion must follow that if curare is having a presynaptic action, it is only adding to an already existing mechanism in the presynaptic terminal which can cause depression of acetylcholine output.

It has been suggested (Thesleff 1959) that the decline in e.p.p. amplitude during rapid stimulation may be caused by a change in the postsynaptic sensitivity of the end-plate. Krnjevic & Miledi (1958) demonstrated a change in sensitivity after a long period of

stimulation by acetylcholine. This explanation for the short term decline is not supported however by the results of Hutter (1952), who demonstrated no change in sensitivity following tetanic stimulation. Otsuka & Endo (1960) applied acetylcholine iontophoretically on the motor end-plate following rapid stimulation and also demonstrated no reduction in sensitivity.

In contrast to this decline in e.p.p. amplitudes at the neuromuscular junction, it is known that in sympathetic ganglia (Eccles 1955) and motor neurones (Curtis & Eccles 1960), stable synaptic potentials may be recorded at relatively high frequencys/ transmission (40-50 sec) with no significant decline in amplitude. Curtis & Eccles suggest a mechanism whereby each impulse causes a depletion of transmitter and at the same time a mobilisation of new transmitter. In this way high frequency transmission may be maintained at rates of up to 250 per sec. At times increases may be seen due to over compensation of the mobilisation process at lower rates. The increases in e.p.p. at the beginning of a tetanus in frog during curarisation has been described by Eccles, Katz & Kuffler (1941). There is probably an increase in release of acetylcholine per impulse in this preparation and no rapid depletion as assumed for the mammalian preparation. It would appear that the mammalian neuromuscular junction is an exception in that the e.p.p. amplitudes decline with rapid stimulation.

The theory that curare acts on the presynaptic terminals has been criticised by a number of authors. Auerback & Betz (1971) have suggested that the results of Hubbard, Wilson & Miyamoto (1969) could be explained by a change of space constant in the muscle fibres. Some studies on acetylcholine release by nerve stimulation have shown that d-tubocurarine has no effect on output. (Chang, Cheng & Chen 1967,

Fletcher & Forrester 1975). The work of Chang et al did not confirm that of Beani, Bianchi & Ledda (1964) who demonstrated a reduced output with curare when rapid stimulation was used. The work of Fletcher & Forrester was with low frequency stimulation. There have also been reports from workers, investigating e.p.p.s, that curare has no presynaptic action. (Beranek & Vyskocil 1967, Bowen & Merry 1969, Bauer 1971). These all suggest that the action of curare on the e.p.p. amplitudes is by postsynaptic block. Against this may be quoted the work of Blaber (1970) who demonstrated an effect on the quantal content of the e.p.p. with the use of curare.

The evidence at present would appear to favour some type of presynaptic action at the mammalian neuromuscular junction in addition to the well known postsynaptic effect. This may be in addition to an inbuilt mechanism which causes a reduction in transmitter output during rapid stimulation. Preston & Van Maanen (1953) found an increase in the paralysing effect of all neuromuscular blocking agents when investigating their effects at increasing frequencys of stimulation. This would suggest that any blocking agent which reduces the e.p.p., pre or postsynaptically, will cause any naturally occurring decline to be more obvious.

A presynaptic effect of curare in many cases will be of no great importance, the effect simply adding to the postsynaptic block which is the required result. In the present context however, if the decline in e.p.p. is an artifact of curare application then an effect on this decline by potassium free solution would have no relevance outside the conditions of the curare block.

Few authors have considered how the presynaptic action of curare may be mediated. The most usual working hypothesis is that

the curare in some manner prevents release by a direct action. The molecule, it is known, combines with the postsynaptic membrane and competes with the transmitter for receptor sites. If it is assumed that the quaternary groups in both choline and curare confer this specificity, then it is logical to suppose that it could similarly compete at the presynaptic membrane for a choline re-uptake site. This may be coupled with the knowledge that HC3 is a similar molecule and does compete with choline for a presynaptic re-uptake site, and also has similar postsynaptic effects at higher concentrations. There does therefore, seem to be good reason to consider a presynaptic action. This would act through a decrease of choline uptake, reduction of transmitter synthesis, and thus a reduction in available store. This would fit the observation that in a previously non-blocked preparation, curare causes Wedensky inhibition of e.p.p. amplitudes, and that this is frequency dependent. Two predictions may be made from this hypothesis. The effect of curare on the transmitter output will be small when the turnover is small, i.e. in a magnesium blocked preparation. In a preparation where an anticholinesterase is used to enable assay of released acetylcholine, there may be no effect of curare at all if the system for transporting choline does not transport acetylcholine. Thus, experiments using these methods to investigate presynaptic action of curare are suspect. The effect of curare, if this hypothesis is accepted, will only be evident under conditions of rapid turnover of transmitter and favourable conditions for resynthesis. The results obtained with the cyclic stimulation method are in agreement with the hypothesis. The effect of curare and HC3 appear to cause the same effect at low doses, when HC3 is known to be acting presynaptically.

It could be postulated that the effect of potassium reduction is to antagonise the effect of curare on the presynaptic membrane.

However, a simple antagonism of the effect of curare could not account for the observed effects of potassium free solution during the experiments using cyclic stimulation and non-blocked preparations. It is considered therefore that the results could support an hypothesis linking the effect of curare and potassium free solution. Thus, the low potassium may facilitate the presynaptic uptake of choline and curare may antagonise it.

Chapter XIV

Final hypothesis and concluding observations

Consideration of the points raised in the preceding chapters leads to the conclusion that no simple answer may be given in explanation for the original observations. Because of the diverse effects of potassium free solution on the system under investigation, many changes occur in the different processes which together enable the nerve action potential to excite the muscle fibres. Two main facts emerge as being important. Firstly, there is a measurable increase in the electrical potential across the membrane in which the postsynaptic receptors for the transmitter are situated. Secondly, there appears to be an increase in available transmitter within the presynaptic nerve terminal.

Improvement in the maintenance of the postsynaptic resting potential during activity will probably improve the transfer of impulses from the nerve to the muscle as the system begins to fail during prolonged activity, as in tetanic contraction. Reduction of transmitter stores resulting in reduced release, combined with a lowered membrane potential caused by increased extracellular potassium, will cause the e.p.p. to be reduced in amplitude. When this amplitude falls below the threshold level for activation of the muscle, no transfer of impulse can take place and thus the contraction begins to fail. This however cannot be the total answer. Krnjevic and Miledi (1959) in a paper dealing specifically with presynaptic propagation failure, suggested that the failure of contraction during tetanus stimulation may be attributed to block of the fine terminals of the motor nerve. This condition was aggravated by anoxia. It is thus probable that this type of block is brought about by an increase in internal sodium, a decrease in internal potassium and an increase in

the external potassium surrounding these fine structures. All of these effects resulting in a reduction of excitability at the nerve endings and thus a block in transmission. If this type of block was occurring in the experiments reported herein, when using the cyclic stimulation technique, then potassium free solution has some effect on it. Thus, by lowering the extracellular potassium concentration surrounding the nerve endings, the excitability of the fine parts of the terminal could have been better maintained and normal transmission preserved.

Superfusing with potassium free solution results in a reduction in fatigue of some part of the preparation leading to a better maintained tetanic contraction. It was demonstrated that an opposite effect could be achieved by use of HC3, d-Tubocurarine and calcium ions, all of which are known to affect the amplitude of the e.p.p. It was also shown that addition of choline chloride to the bathing fluid resulted in a similar effect to that of potassium free solution. The conclusion reached therefore, must be that the failure of tetanic contraction observed in these experiments is more complex than can be accounted for by a conduction block of the presynaptic nerve terminal. The results considered together are better explained by an action of potassium free solution on some property of transmission. The decline in tension observed is possibly linked with a reduction in transmitter output, although possibly associated with some block of nerve conduction as described by Krnjevic and Miledi (1959).

Evidence has been presented to justify a theory involving increase of transmitter in the nerve terminal. How this increase is achieved is not certain. It is postulated that the change in potassium distribution affects the nerve terminal and facilitates synthesis of

transmitter which will add to the releasable pool. It would achieve this by increasing the supply of choline from the synaptic cleft to the synthesis point, presumably within the cell, and possibly also by stimulating the production of acetylcholine directly, acting through a change in intracellular sodium concentration. Although it must be stated that this is a tentative hypothesis it forms a useful basis for further investigations into the mechanism of ionic control of transmitter turnover.

To enable a distinction to be made between the actions described, one on the postsynaptic structures and the other on the presynaptic structures, a more accurate estimate of the effect of the membrane potential on the e.p.p. under potassium free conditions must be obtained. It would be important to have a knowledge of the transmitter equilibrium potential at the resting potentials obtained, and experimentally determined under conditions of very low extracellular potassium. Thus a correction could be applied and the increase in transmitter output could be calculated accurately on an amplitude basis.

A number of experiments could be performed, the results of which may provide more information about the validity of the hypothesis involving presynaptic actions of potassium free solution. If curare is used as a neuromuscular blocking agent, more knowledge of its actions on choline uptake must be obtained. A comparison of curare and HC3 could be made using trains of stimuli with and without choline added to the bathing solution. If it is shown that curare has the same effect as HC3 under these conditions then care must be exercised when using it as a blocking agent in experiments where transmitter recycling is important.

If the quantal content of each e.p.p. in a train of stimuli in a curare blocked preparation can be calculated, a more accurate

estimate of the immediately available store could be obtained. Thus, the effect of HC3 and its antagonism by choline and low potassium solution could be investigated. If the action of low extracellular potassium is to increase synthesis, some increase in quantal size is also to be expected.

Experimental determination of quantal content and quantal size changes using magnesium block may prove difficult, as the preliminary experiments using this procedure showed in this investigation. Experiments where the quantal content is depressed are quite different in terms of transmitter turnover from those in which the neuromuscular block is postsynaptic. Thus, if there is no reduction in store size during release it is difficult to show an improvement in maintenance unless it is possible to increase it above normal. This may not be possible. Less effect of the potassium free solution on magnesium blocked e.p.p.s follows from the hypothesis because only a few quanta are released. The probability of release is reduced so far below normal that changes in the population may be very small. If there is a change, not in the number of quanta making up the store, but in the size of the quanta then this should be measurable, even with low outputs, if choline is added and the potassium free solution facilitates the transport as hypothesised.

Another prediction which may be made from the hypothesis is that the use of inhibitors of acetylcholine esterase should markedly reduce the enhancing effect of the potassium free solution. By preventing breakdown of the transmitter, free choline will not be formed and possibly no transport will take place.

The transmitter supply in the nerve ending can be exhausted by deprivation of glucose. To investigate effects on synthesis rate, recovery could be followed using potassium free solutions and the same

solution with added choline in comparison with normal Krebs solution. If the potassium free solution facilitates choline uptake, an improvement in recovery should be demonstrable. In such an experiment, no blocking agent should be needed at first as the e.p.p.s will be initially very small.

The uptake mechanism for choline if sensitive to changes in either resting potential or potassium distribution could be expected to be linked to the ionic pumps within the membrane known to exist for sodium, potassium and calcium. To investigate such a link, the blocking action of cardiac glycosides, or other ionic transport blocking agents, could be used in a preliminary procedure designed to test this hypothesis. Pairs of pulses could then be used to estimate changes in available store size. This technique would require few pulses and thus be suitable in such a situation where prolonged stimulation in the presence of blocking agents may lead to rapid depolarisation of the nerve terminal. This procedure would also provide an important comparison with the effect of potassium free solutions on block of the membrane sodium pumping activity.

It is interesting to note that during the course of this work a possible link was shown between changes in potassium distribution and dystrophic diseases. It was demonstrated by Howland (1974) that there is an abnormally large potassium conductance in the membrane of erythrocytes, liver mitochondria and brain mitochondria from dystrophic mice. This he suggested could be a basis for membrane disfunction in both genetic and nutritional types of dystrophy. It is also known that in certain types of dystrophy and in the associated disease of Myasthenia Gravis, there is a decrease in transmitter output. (Thesleff 1960, Elmqvist et al 1964, Duchen & Stephani 1971). Thus,

the very speculative postulate could be proposed that if an increase in potassium gradient can lead to increased transmitter in the nerve terminal, a decrease, caused by leaky membranes, could lead to less transmitter, even if the conductance were not so great as to cause block of action potentials in the nerve or muscle.

SECTION V
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REFERENCES

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Appendix

During the course of the work reported in this thesis the two abstracts appended were accepted by the Physiology Society for publication.

HALL. A.E., HILTON. Elizabeth L. & WEST. D.C.
(1972). J. Physiol 226, 95-96P.
Neuromuscular transmission in potassium free Krebs solution.

HALL. A.E., HILTON. Elizabeth L. & WEST. D.C.
(1975). J. Physiol 256, 78-79P.
A simple and sensitive method for displaying effects of agents affecting neuromuscular properties.

These two publications are submitted with the thesis as part of the work.

All experimentation and specialised equipment design for the reported work, both in this thesis and these previous publications, was carried out by the author.

[*From the Proceedings of the Physiological Society, July* 1972]
Journal of Physiology, **226**, 95–96*P*

Neuromuscular transmission in potassium-free Krebs solution

By A. E. Hall, Elizabeth L. Hilton and D. C. West. *Department of Physiology, Bedford College, London NW1 4NS*

We have observed that the mechanical response during a tetanus of the rat diaphragm stimulated via the phrenic nerve is enhanced in potassium-free Krebs solution. The effect is unexpected since Goffart & Ritchie (1952) showed that K^+ ions increased the active state of muscle. However, since hyperpolarization which may result from a changed K^+ ion distribution has been reported to be associated with increased end-plate potentials (Takeuchi & Takeuchi, 1961) we have investigated the effect of K^+-free solution electrophysiologically.

Initially we checked that there was no demonstrable change in refractory period in the fine nerve terminals on transfer to K^+-free media which might have enabled the preparation to give an enhanced response under repetitive stimulation.

The possibility that there was a change in transmitter output was therefore investigated. The end-plate potential and the frequency and amplitude of miniature end-plate potentials were used as indices of transmitter output. Micro-electrode studies of the muscle end-plate showed that in K^+-free media the resting potential was hyperpolarized by *ca.* 30 mV. This is similar to the observations of Akiyama & Grundfest (1971) in frog sartorius but contrasts with those of Blackman, Ginsborg & Ray (1963) who found no change in the resting potentials in sympathetic ganglia when in K^+-free media. There was no consistent effect on the frequency or amplitudes of miniature end-plate potentials. The end-plate potential in junctions depressed by tubocurarine were approximately doubled in K^+-free solutions. This finding is comparable with the observations of Hubbard & Willis (1962) who found larger end-plate potentials after electrical hyperpolarization.

The mechanical effects of K^+-free media are similar to the action of adrenaline. In a comparison of the two effects we have confirmed Krjnević & Miledi's (1958) observations that with adrenaline the frequency of miniature end-plate potentials is increased but there is no change in the resting potential at the end-plate region. It would appear therefore that although the mechanical responses are similar the electrophysiological basis is different.

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[*From the Proceedings of the Physiological Society*, 18–19 *December* 1975
Journal of Physiology, **256**, 78–79*P*]

A simple and sensitive method for displaying effects of agents affecting neuromuscular properties

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Many workers have studied various aspects of neuromuscular function using mechanical recording. Such studies may involve twitch amplitude, tetanus amplitude, twitch-tetanus ratio, post-tetanic potentiation or depression, time to peak of the tetanus or tension decline during a tetanus. By using a simple method of cyclic stimulation we have found it possible to display these properties together on the same record.

Using the rat diaphragm–phrenic nerve preparation twitch responses are obtained by stimulating the nerve at 1 Hz for 55 sec followed by a 5 sec tetanus at 50 Hz. This cycle of stimulation is repeated throughout an experiment. These frequencies have been found to be most suitable but other frequencies have similar effects. Under control conditions stable responses are obtained for long periods.

One property of particular sensitivity is the decline of tetanic tension which may be defined as 'tetanic droop'. We find that this property is affected by a variety of agents at concentrations which are so low as to have negligible effects on the twitch amplitude. This method conveniently displays the reduction in transmission caused by fatigue during tetanic stimulation (Krnjević & Miledi, 1958).

Records obtained using isometric recording and cyclic stimulation (and displayed on a Devices hot pen chart recorder) show distinctly different over-all patterns with agents known to affect neuromuscular junctions in different ways. The effects of low K^+ solutions for instance, as described by Hall, Hilton & West (1972), are clearly demonstrated by a characteristic response pattern.

To elicit a tetanus after a series of twitches in a repetitive and reproducible manner it is important to provide that the first tetanic pulse occurs at the same interval following the preceding twitch. A suitable twitch/tetanus stimulator has been designed which delivers the first tetanic pulse when the first omitted twitch pulse would have been due. The tetanic train of pulses can be independently adjusted to have frequencies in the range 30–200 Hz. The twitch frequency, the cycle repeating time and tetanus duration, may also be varied.

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