BLOCKADE OF THE NEUROMUSCULAR JUNCTION BY TETRACYCLINE ANTIBIOTICS

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ABSTRACT

The general scope of blockade of the neuromuscular junction by antibiotics is outlined and the literature relevant to the tetracyclines is reviewed. The original observations by BEZZI & GESSA (1960) that tetracyclines can enhance the degree of blockade in the partially curarised rat diaphragm preparation are confirmed and further extended. A similar effect is exerted in the frog sciatic nerve sartorius muscle preparation where the ability of tetracyclines to produce neuromuscular blockade even in the absence of tubocurarine is demonstrated, the muscle remaining responsive to direct stimulation.

Tetracyclines are known to be chelating agents, forming complexes with divalent cations. The formation of tetracycline calcium complexes is investigated by means of a potentiometric titration method. It is shown that the formation of such tetracycline calcium complexes does not however bring about neuromuscular blockade solely by means of reducing the external calcium ion concentration surrounding the muscle fibre.

Intracellular microelectrode recording techniques are used to demonstrate that tetracyclines bring about blockade of the neuromuscular junction by means of both a postsynaptic action and a decrease in the quantal content of neurotransmitter release.

I wish to thank the Department of Pharmacology for the use of facilities and to express my gratitude to Professor B.L. Ginsborg and my supervisor Dr. J. Walker for their encouragement.

In accordance with the requirements of Regulation 2.4.15.

I declare that this thesis has been composed by Nathaniel Rough Muir and that the work presented herein is my own.

Signed:

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SYNOPSIS

Not all antibiotics have neuromuscular blocking properties, however various members of unrelated groups of antibiotics are known to exert such a neuromuscular blocking action; included in these groups are the tetracyclines.

The tetracyclines are all very closely related structurally; the antimicrobial properties of these antibiotics are similar to the extent that when resistance arises to one of the compounds there is cross resistance with the others. Chlortetracycline was the first of these compounds discovered and was introduced in 1948. It was soon recognised that the tetracyclines were chelating agents, forming complexes with divalent cations. Although the neuromuscular blocking effect of tetracycline HCl was first reported in 1959 very few reports have subsequently been published.

In chapter 1 the general scope of blockade of the neuromuscular junction by antibiotics is outlined and the literature relevant to the tetracyclines is reviewed. In chapter 2 the original observations that tetracyclines can enhance the degree of neuromuscular blockade in the already partially blocked phrenic nerve hemidiaphragm preparation of the rat are confirmed. In chapter 3 it is established that tetracyclines exert a similar effect in the partially curarised sciatic nerve sartorius muscle preparation of the frog, a preparation which offers a number of experimental advantages over the rat diaphragm phrenic nerve preparation. In chapter 4 the ability of tetracyclines to produce neuromuscular blockade in the absence of a blocking agent such as

tubocurarine whilst the muscle remains responsive to direct stimulation is demonstrated.

Tetracyclines form complexes with divalent cations, and since the calcium ion plays such an essential role in the release of neurotransmitter from the presynaptic terminal, it was considered necessary to establish just how low the calcium ion concentration in the Ringer solution can be before there is any perceptible effect on twitch tension; this is done in chapter 5. However reduction of the calcium ion concentration by the formation of chelation complexes with tetracyclines is not the main mechanism whereby these antibiotics achieve their neuromuscular blocking action.

Electrophysiological techniques are used in chapters 6 to 13. Various factors influencing the accuracy of estimates of the number of quanta released per impulse are discussed in chapter 6; the electrophysiological recording methods employed are described in chapter 7. The dissociation of tetracyclines in Ringer solution results in a substantial decrease in the pH of the solution consequently it was necessary to select a suitable buffering agent; following tests reported in chapter 8 the buffering agent HEPES was selected. In chapter 9 evidence for both pre and postsynaptic actions of tetracyclines is presented. In chapter 10 it is established that EPP amplitude continues to decrease even when the calcium concentration of the Ringer is increased. Further evidence for both pre and postsynaptic actions of tetracyclines is presented in chapter 11. In chapter 12 it is estab-

lished that blocking concentrations of tetracyclines have no significant effect on membrane constants. Chapter 13 investigates the effects of tetracyclines on muscle membrane potential.

The metal complexation of tetracyclines and calcium is examined in chapter 14. General conclusions are discussed in the final chapter.

CHAPTER 1

INTRODUCTION

ANTIBIOTIC BLOCKADE OF THE NEUROMUSCULAR JUNCTION

INTRODUCTION

ANTIBIOTIC BLOCKADE OF THE NEUROMUSCULAR JUNCTION

The neuromuscular blocking properties of various antibiotics have been known for some time. Since the first cases of prolonged respiratory depression from neomycin-ether combinations were reported by PRIDGEN (1956) antibiotics of the streptomycin group, the polymyxins and the tetracyclines have all been reported to cause muscle paralysis under clinical conditions when administered into the peritoneal cavity. The majority of reports of adverse clinical effects have occurred in patients under treatment with antibiotics who in addition received either a competitive neuromuscular blocking drug (FOGDALL and MILLER 1974) or ether anaesthesia (CORRADO et al 1959). Episodes of muscle paralysis have also followed systemic administration in a few unanaesthetised patients, those afflicted with myasthenia gravis being particularly vulnerable (HOKKANEN 1964). Streptomycin-induced weakness has also been observed in unanaesthetised subjects (LODER and WALKER 1959). The clinical incidence of antibiotic induced paralysis and the scope of antibiotic involvement in the blockade of neuromuscular function has been reviewed by PITTINGER et al 1970, 1972.

Not all antibiotics display neuromuscular blocking properties. Among those which are reported to be devoid of neuromuscular blocking activity are: cephalothin, chloramphenicol, erythromycin, hamycin, novobiocin, oleandomycin, penicillins, rifamycin, ristocetin, tyrothricin and vancomycin.

Following the observation that some patients receiving antibiotic treatment were more sensitive to the muscular relaxant action of tubocurarine BEZZI & GESSA (1959) investigated the effects of streptomycin, tetracycline, chloramphenicol and penicillin injected into the marginal vein of the ear in rabbits in which a slight paralysis due to tubocurarine had been produced. Streptomycin and tetracycline increased the paralysis but chloramphenicol and penicillin did not. This was the first report concerning a possible neuromuscular blocking action exerted by tetracycline to appear in the literature.

A number of antibiotics have been the subject of experimental studies utilising in vivo and in vitro preparations and the neuromuscular blocking activity of 18 antibiotics demonstrated (PITTINGER 1972). Of these, of direct relevance to the present study are oxytetracycline, methylpyrrolidine tetracycline, rolitetracycline, and tetracycline. However much more of the work reported in the literature concerns antibiotics of the streptomycin-neomycin type rather than the tetracyclines, and it is in relation to the neuromuscular blocking action of the former group that what have been termed by PITTINGER (1972) as the chelation and the competitive hypotheses have been formulated. The relevant features of neuromuscular block by these antibiotics will be described in terms of these hypotheses for it is in relation to them that the mode of action of the tetracyclines will be considered and tested experimentally.

Certain features of the paralytic activity of streptomycin and neomycin are consistent with the effects of competitive neuromuscular blocking agents in that they produce a progressive, flaccid

paralysis of striated muscle without evidence of an initial excitatory effect, contracture is not evident and the affected muscle still responds to direct stimulation. However certain other features of the block produced by the antibiotic are similar to those produced by magnesium suggesting an additional presynaptic involvement.

Analysis of the mode of action of antibiotics of the streptomycin type in animal experiments, in vivo and in vitro, has shown that they produce a flaccid paralysis and a reduction in the response to acetylcholine administered close-arterially. VITAL BRAZIL and CORRADO (1957) also showed that high doses of streptomycin produced respiratory arrest, due to a neuromuscular block similar to that produced by magnesium ions, and similarly reversed by calcium. The chemical structures of streptomycin and neomycin are closely related, and the neuromuscular block caused by these two antibiotics is similar. The neuromuscular effect of neomycin is also reversed by neostigmine and calcium (CORRADO and RAMOS 1959) however reversal of the block by neostigmine is both slow in onset and short in duration in contrast to the rapid and complete reversal following the administration of calcium chloride.

Using microelectrodes ELMQVIST and JOSEFSSON (1962) obtained electrophysiological evidence on the nature of the blocking action of neomycin in the isolated rat phrenic-diaphragm nerve-muscle preparation. They showed that both pre and postsynaptic sites of action are involved. The block is due to a reduction in the amplitude of the endplate potential and this reduction is partly caused by a neomycin induced decrease in the sensitivity of the

postjunctional endplate membrane to the depolarising action of acetylcholine. It was shown that neomycin antagonised endplate depolarisation in the frog sartorius caused by acetylcholine and also acetylcholine contractures of the denervated rat diaphragm. Neomycin did not significantly alter the spontaneous prejunctional activity, recorded as MEPP's since neither their frequency nor time-course were significantly affected; however, a presynaptic involvement was shown by the prevention of a potassium induced increase in MEPP frequency (LILEY 1956) in the presence of neomycin. Under these conditions (potassium ion concentration 30 mM; neomycin 0.3 mg ml^{-1}) raising the calcium ion concentration from 2.0 to 8.0 mM restored the response.

Although ELMQVIST and JOSEFSSON (1962) reported that during neomycin block the amplitudes of successive EPP's fluctuated at random they did not attempt to analyse these fluctuations in terms of the quantal hypothesis of acetylcholine release. They excluded a calcium binding effect of neomycin and postulated that its prejunctional action is such that neomycin like the magnesium ion competes or interacts with the calcium ion at some step in the prejunctional process which leads to transmitter release. If this were the case, neomcyin should reduce the amount of acetylcholine released in response to stimulation of the nerve. No such reduction in the output of acetylcholine, collected over a period of 30 minutes from a neomycin blocked frog gastrocnemius muscle subjected to interrupted tetanic stimulation via the sciatic nerve, was detected by DRETCHEN et al (1972) who assayed the acetylcholine content of the bathing medium using the superfused guinia pig ileum

(GADDUM, 1953). Two preparations were obtained from each animal, one was used to obtain a measure of the acetylcholine released in the absence of antibiotics, thus serving as a control for the other antibiotic treated preparation.

DRETCHEN et al found no statistically significant change in acetylcholine release in the presence of neomycin, streptomycin, kanamycin and polymyxin B; acetylcholine release was significantly reduced when gentamicin was present. It should be noted that complete neuromuscular block did not develop since these authors chose two concentrations of each of the antibiotics tested, such that one concentration produced a 50% and the other a 70% decrease in the tension developed in response to interrupted tetanic stimulation.

This evidence of DRETCHEN et al is not supported by that of VITAL BRAZIL & PRADO-FRANCESCHI (1969) who investigated quantitatively the effect of neomycin on the release of acetylcholine resulting from phrenic nerve stimulation in the rat. Organ bath fluids were assayed for acetylcholine by their effect on the cat blood pressure. It was found that the amount of acetylcholine collected from a non stimulated preparation over a fixed period of time was indistinguishable from that collected from a stimulated preparation in the presence of a blocking dose of neomycin. However when both neomycin and calcium chloride were present the amount of acetylcholine collected during a period of indirect stimulation was some 80% of that collected over a similar period of time in the absence of the antibiotic. Similar results were obtained with gentamicin. These observations demonstrate that the effect of neomycin and gentamicin is to depress the

release of acetylcholine prejunctionally. This supports the indirect evidence of ELMQVIST & JOSEFSSON regarding neomycin, and was interpreted as evidence that the neuromuscular block produced by these antibiotics is mainly due to a presynaptic effect.

The effects of neomycin and gentamicin upon acetylcholine induced contractions of the isolated and chronically denervated hemidiaphragm of the rat were also investigated by VITAL BRAZIL & PRADO-FRANCESCHI. They reported that the two antibiotics caused a parallel shift of the dose-response curves, gentamicin being a much stronger antagonist than streptomycin.

The in vivo effects of streptomycin, viomycin and kanamycin on the development of twitch tension in the sciatic-gastrocnemius preparation of the cat were studied by KUBIKOWSKI & SZRENIAWSKI (1963). They observed that in a preparation in which the development of twitch tension in response to electrical stimulation of the sciatic nerve had been completely blocked by a dose of streptomycin injected into the ipsilateral femoral vein, it was rapidly restored following an intravenous injection of calcium gluconate and similar results were obtained in both viomycin and kanamycin blocked preparations. Anticholinesterases, with the exception of ambenonium chloride were much less effective in relieving the block produced by streptomycin; recovery proceeded slowly and to a value lower than that attained as a result of the injection of calcium. However ambenonium chloride produced a more rapid and complete recovery than either acetylcholine or calcium gluconate.

In comparing the relative effectiveness of anticholinesterases and calcium in restoring neuromuscular transmission in the presence

of either streptomycin or neomycin it should be noted that anticholinesterases, with the exception of ambenonium chloride, are usually effective only when partial blockade prevails. However calcium is highly effective in restoring neuromuscular transmission after complete block due to either magnesium, streptomycin or neomycin. This contrasts with its relative effectiveness in overcoming a neuromuscular block due to tubocurarine; in that case anticholinesterases are highly effective whereas calcium is only partially effective in restoring neuromuscular transmission (PITTINGER 1966).

What has come to be known as "the competitive hypotheses" was postulated by VITAL BRAZIL & PRADO-FRANCESCHI (1969) in a report on the nature of the neuromuscular block produced by neomycin and gentamicin. While recognising a decrease in the sensitivity of the postjunctional membrane to the depolarising action of acetylcholine they stressed that the primary cause of the neuromuscular blockade produced by these antibiotics was the prejunctional inhibition of the release of acetylcholine from the nerve terminal. Their assertion was that antibiotics of the streptomycin - neomycin group, like magnesium ions, blocked acetylcholine release by competing with calcium ions for specific sites (X) at the prejunctional membrane. At these sites (X) complexes formed with either magnesium ions (MgX) or antibiotics (AX) are ineffective, unlike the complex formed with calcium ions (CaX) which is fully effective in leading to the release of acetylcholine from the nerve terminal in response to neurol stimulation. The work of del CASTILLO & KATZ (1954) and del CASTILLO & ENGBAEK (1954) suggested that the amount of acetylcholine released in response to a nerve stimulus was a function of the relative concentra-

tions of calcium and magnesium ions. The competitive hypotheses evolved from considerations of the actions of antibiotics in relation to the effects of magnesium ions at the neuromuscular junction. The competitive aspect of the hypotheses relates to the inhibition of the release of acetylcholine as a consequence of the antibiotic competing with calcium ions for the sites (X) at the prejunctional membrane.

In what has been designated by PITTINGER "the chelation hypotheses" CORRADO (1963) attributed the mechanism of neuromuscular blockade by streptomycin, neomycin and kanamycin to their ability to reduce the level of calcium in the blood. He supported this assertion by drawing from clinical evidence concerning the therapeutic effectiveness of calcium administration in relieving antibiotic induced neuromuscular blockade, pointing out that the adverse effect of the antibiotic is potentiated when the patient has a low calcium level, such as may occur when large amounts of citrated blood are administered (CORRADO, RAMOS & ESCOBAR 1959). On the other hand vitamin D administration, which leads to a higher blood calcium level, abolishes the nephrotoxic and ototoxic effects of prolonged antibiotic administration. However he did not offer any experimental evidence of his own in support of his assertion that the calcium level is lowered by antibiotics, referring to the work of HAVA, SOBEK & MIKULASKOVA (1961) who claim that the ionised calcium level is lowered by neomycin, while total calcium was unchanged. This evidence is at variance with that obtained by ELMQVIST & JOSEFSSON (1962) who, using the murexide technique of SCHWARZENBACH (1949), claim that the calcium ion activity in solutions containing sodium, potassium and calcium ions in the same amounts as in the bathing

fluid of their rat diaphragm preparations was not altered by neomycin.

In attributing the major effect of the streptomycin group of antibiotics to the formation of complexes with calcium ions and consequent reduction in endplate potential CORRADO stressed a postsynaptic action but did not explicitly state nor exclude a prejunctional magnesium like effect.

Certainly in the case of the streptomycin neomycin group of antibiotics the available experimental evidence favours the competitive hypotheses of VITAL BRAZIL & PRADO-FRANCESCHI which rejects the concept of diminished calcium ionisation and stresses a prejunctional effect suggesting that antibiotics, like magnesium, compete with calcium ions for sites on the nerve terminal. However, in the case of neuromuscular blockade due to tetracyclines, which are known to have an affinity for divalent cations (ALBERT 1956), CORRADO'S concept may, have some validity in that these antibiotics may act by reducing the level of ionised calcium. However, unlike CORRADO, who stressed a postsynaptic action, one would envisage such a reduction in the level of ionised calcium as giving rise to neuromuscular block by a presynaptic action, specifically a reduction in the quantity of acetylcholine released in response to neural stimulation.

Following their initial observation that certain antibiotics could influence the level of paralysis produced by tubocurarine, in vivo in the rabbit (BEZZI & GESSA 1959) they reported their experimental observations in detail (BEZZI & GESSA 1960). GESSA (1960) demonstrated the neuromuscular blocking action of tetracycline, in vivo, in the sciatic nerve-tibialis anterior muscle of

the rabbit and, in vitro, in the isolated phrenic nerve-diaphragm preparation of both the rat and the guinea pig. In 1961 BEZZI & GESSA further demonstrated, in vivo, in the rabbit the potentiating effect of streptomycin, tetracycline, oxytetracycline and kanamycin on a slight paralysis in rabbits due to tubocurarine when given in doses which themselves had no apparent effect. In some instances a partial paralysis could be induced, in animals not previously treated with tubocurarine, by the intravenous injection of the antibiotic alone at sufficiently high doses. Tetracycline hydrochloride at a dose of 50 mg Kg^{-1} was most effective, producing paralysis in 4 out of 8 animals compared with 3 out of 18 animals at a dose of 25 mg Kg^{-1}. In the absence of pretreatment with tubocurarine, oxytetracycline was ineffective, producing no observable paralysis in 5 animals. In vitro experiments on the rat diaphragm preparation showed that kanamycin and tetracycline hydrochlordie caused a blockade of neuromuscular transmission. That tetracycline and kanamycin affect neuromuscular transmission through different mechanisms was shown by the observation that neostigmine antagonises the blockade induced by kanamycin but not that caused by tetracycline. This difference is emphasised by the anti-acetylcholine effect on the frog rectus abdominis exerted by kanamycin but not by tetracycline. BEZZI & GESSA (1961) reported that during the antibiotic induced neuromuscular blockade direct stimulation of the muscle remained fully effective.

The in vivo neuromuscular blocking action of oxytetracycline (OTC), methylpyrrolidine tetracycline (PMT) and streptomycin was investigated in the cat by KUBIKOWSKI & SZRENIAWSKI (1963). They

found the following an intravenous injection of PMT (50 mg Kg^{-1}) the development of twitch tension in the indirectly stimulated tibialis anterior muscle was completely blocked within three minutes. The response was restored to 53% of its preantibiotic treatment level seven minutes after the injection of calcium gluconate. In a similarily blocked preparation neostigmine produced a much smaller recovery, the response reaching a maximum of 13% of its initial level within one minute. The figures quoted are the average values obtained from four animals. KUBIKOWSKI & SZRENIAWSKI demonstrated that the compound action potential, recorded from the tibialis anterior muscle in response to stimulation of the peroneal nerve, decreased in amplitude as the neuromuscular block due to the injection of antibiotic into the ipsilateral femoral vein developed and then recovered with the injection of calcium gluconate. Within two minutes of the administration of oxytetracycline hydrochloride (50 mg Kg^{-1}) the compound action potential amplitude decreased to 30% of its initial value. Within one minute, following the injection of 200 µg of ambenonium chloride the response was fully restored. Intravenous injections of acetylcholine chloride (50 mg Kg^{-1}) while producing a 25% recovery in the streptomycin blocked preparation were completely without effect on the blockade produced by either OTC or PMT. Another difference between streptomycin and the two tetracyclines was that while in all three cases calcium gluconate restored the response to at least 50% of its preantibiotic treatment value, the time taken to reach maximum recovery was five minutes in the case of the OTC and PMT compared with only one minute in the case of streptomycin.

Whilst these experiments using extracellular recording of the muscle action potential make little contribution to determining whether the blockade is due to a pre or postsynaptic action of the antibiotic they illustrate the similarities and also the differences between the neuromuscular block produced by streptomycin and the two tetracyclines OTC and PMT. KUBIKOWSKI & SZRENIAWSKI suggested that since not only tetracyclines but several other antibiotics possess chelating properties (FOYE 1961) the competitive or depolarising action of neurotoxic antibiotics might depend on the size of the ion complexes thus formed. However they observed no neuromuscular blockade when they injected EDTA into their preparations. They were of the opinion that the same antibiotic can produce neuromuscular blockade by different mechanisms of action depending on its duration and concentration in the vicinity of the endplate. They also suggested that postantibiotic paralysis of the endplate is biphasic, a short initial phase of competitive blockade being followed by a prolonged period of depolarisation combined with calcium ion depletion.

The influence of high magnesium and low calcium on the neuromuscular blocking action of oxytetracycline in the horse was investigated by BOWEN & McMULLAN (1975) in a study primarily concerned with determining if the neuromuscular blocking activity of oxytetracycline was responsible for adverse reactions in some horses following intravenous injection of this antibiotic. The neurally evoked contractile response of the upper lip muscles of horses anaethetised with halothane were used to evaluate the in vivo neuromuscular blocking action of oxytetracycline. No reduction in the

contractile response occurred with doses of OTC of 28 mg Kg^{-1}. However when a partial neuromuscular blockade, causing a 50% reduction in the initial response, was induced by the infusion of magnesium sulphate the antibiotic then caused a further decrease in the twitch tension. In these experiments the serum calcium concentration could also be reduced by the infusion of 2% sodium oxalate. BOWEN & McMULLAN concluded that calcium binding contributed little to the neuromuscular blocking effect of oxytetracycline and that such a neuromuscular blocking action does not play a significant role in the accute type of adverse reaction to this antibiotic in the horse.

From the experimental evidence available from these early studies concerning the neuromuscular blocking action of tetracyclines no definite conclusions can be drawn concerning whether the site of action is primarily pre or postsynaptic. CORRADO'S concept concerns a reduction in the calcium ion concentration and VITAL BRAZIL'S competitive hypotheses likens the neuromuscular blocking effect of the streptomycin - neomycin group of antibiotics to that caused by magnesium therefore it is appropriate to consider the role of calcium ions in neuromuscular transmission and its antagonism by magnesium ions; particularly in view of the known affinity of tetracycline for divalent cations.

The current view of the events leading to transmitter release at the frog neuromuscular junction may be summarised as follows; when an action potential invades the axon terminal, the resultant depolarisation causes channels which are selectively permeable to calcium ions to open for a brief period. A concentration gradient exists between the intraterminal free calcium level of approximately 5 x

10^{-7} M (BAKER 1972) and the 2 x 10^{-3} M level of calcium found in frog plasma (BOYLE & CONWAY 1941). Therefore an increased calcium ion permeability causes calcium ions to enter the terminal to produce an increase in intraaxonal calcium concentration. During the period that the calcium concentration is significantly increased, calcium ions activate the neurotransmitter release mechanism. One view of the mechanism of release envisages it as taking place when synaptic vesicles, filled with acetylcholine, fuse with the axon membrane and release their contents by exocytosis into the synaptic cleft.

Calcium has long been known to be involved in neuromuscular transmission. JENKINSON (1957) and later DODGE & RAHAMIMOFF (1967) showed that the amount of acetylcholine released by a nerve impulse depends on extracellular calcium concentration. However that calcium need not be present during the period of acetylcholine release was shown in experiments performed by KATZ & MILEDI (1967) in which calcium ions were applied to a nerve terminal maintained in a Ringer solution containing tetrodotoxin and a low concentration of calcium. They found that when depolarising pulses were applied to the nerve calcium must be present in the bathing solution around the terminal at a very specific time nearly coincident with the terminal depolarisation but before the actual release of quanta.

Evidence supporting the view that it is the intraterminal free calcium level that leads to transmitter release comes from experiments by KITA & VAN DER KLOOT (1974). By treating frog neuromuscular preparations with the ionophore X-537A, a compound which is known to carry divalent cations across lipid membranes, they showed that an increase in miniature endplate potential frequency was obtained only

when calcium was present in the bathing medium, strongly suggesting that the increased spontaneous release results from an increased intraterminal concentration. Also in the giant fibre system of the squid where it is possible to penetrate both the pre and postsynaptic fibres MILEDI (1973) showed that injection of calcium into the presynaptic axon causes transmitter release.

The neuromuscular blocking properties of several antibiotics were investigated by WRIGHT & COLLIER. In 1977 they demonstrated that both neomycin and streptomycin act presynaptically; reducing the amount of ACh released from the rat diaphragm preparation during phrenic nerve stimulation. Evidence that neomycin blocks ACh release by blocking the influx of Ca^{++} into the presynaptic endings of the isolated ganglion preparation of the rat during stimulation was obtained from experiments in which paired superior cervical ganglia were bathed in low Ca Krebs solution. One ganglion was stimulated and a measure of the Ca uptake due to stimulation was derived by subtracting the Ca uptake in the nonstimulated from the uptake in the stimulated ganglion. Preganglionic stimulation increased the accumulation of ^{45}Ca by 50 to 100%. A similar % increase in uptake was measured in the presence of a blocking concentration of d-tubocurarine (2×10^{-4} M) however in the presence of a concentration of neomycin (2×10^{-3} M), which in low Ca Krebs solution (0.5 mM) completely blocked ganglionic transmission, the increased accumulation of ^{45}Ca induced by preganglionic stimulation was abolished.

WRIGHT & COLLIER (1977) measured the ionised calcium concentration in Krebs solution containing neomycin B by means of a calcium

ion electrode. They demonstrated that neomycin B (2.0 mM) had no significant effect on the ionised calcium concentration. They concluded that since neuromuscular blockade ocurrs at concentrations of 2.0 mM or less, calcium chelation by neomycin B does not contribute to the presynaptic blocking effect. They also demonstrated that at an ionised calcium ion concentration of 1.6 mM the neuromuscular block produced by neomycin had a greater presynaptic component than the neuromuscular block produced by streptomycin and that neomycin is at least twice as potent as streptomycin in inhibiting ACh release from the neuromuscular junction. However streptomycin is more potent than neomycin in inhibiting the response to injected ACh.

WRIGHT & COLLIER place great emphasis on the presynaptic action of both neomycin and streptomycin and, although they do not refute the existence of a postsynaptic component of the neuromuscular block produced by these antibiotics, they did not investigate the postsynaptic action of either neomycin or streptomycin. They mention that the ionised calcium concentration plays an important role in determining whether the pre or postsynaptic effect is predominant. The presynaptic component of the neuromuscular block will be markedly potentiated by a decrease in the ionised calcium concentration. Whereas an increase in the ionised calcium ion concentration will decrease the presynaptic component of the block; the postsynaptic component will remain unaffected. However, if the ionised calcium concentration is markedly increased, the postsynaptic component of the block may possibly be increased (ELMQVIST & JOSEFSSON 1962; NASTUK & LIU 1966).

Further evidence supporting the view that streptomycin acts

primarily by a calcium reversible prejunctional mechanism is supported by the work of SINGH, HARVEY & MARSHALL (1979) who used intracellular recording techniques to investigate the neuromuscular blocking properties of streptomycin, lincomycin, polymyxin B and clindamycin in the mouse phrenic nerve hemidiaphragm preparation. They found that at the concentrations tested none of these antibiotics altered the membrane resting potential. A postsynaptic component of the block by streptomycin (1.23 mM) was shown by a reduction in MEPP amplitude (56% $\pm$ 8% lower than the control value). The quantal content of evoked release in the presence of 1.23 mM streptomycin was estimated at 9 $\pm$ 0.8 compared to a control quantal content of 152 $\pm$ 17 (estimated from EPP variance, in a cut fibre preparation with EPP amplitude corrected for non linear summation and referred to a standard membrane potential of -40 mV).

WRIGHT & COLLIER (1976b) demonstrated that clindamycin (3.6×10^{-3} M) had a local anaesthetic effect on a frog desheathed nerve preparation whereas lincomycin (1.5×10^{-2} M) was devoid of such an effect. Both clindamycin (8×10^{-4} M) and lincomycin (4×10^{-3} M) depressed the response of the rat diaphragm to stimulation via the nerve and also to a similar extent to direct stimulation of the muscle, evidence which WRIGHT & COLLIER considered as indicating that the predominant neuromuscular blocking effect of clindamycin is due to an effect on the muscle whereas lincomycin is considered to act primarily by depressing neuromuscular transmission. FIEKERS, MARSHALL & PARSONS (1979) report that both of these antibiotics exert a considerable influence on endplate channel behaviour. Clindamycin shortened the time course of miniature endplate current (m.e.p.c.)

decay, reduced m.e.p.c. amplitude and reduced its voltage sensitivity without altering its exponential nature. The effect of lincomycin on m.e.p.c. decay rate was both voltage and concentration dependent. Lincomycin converted the decay of m.e.p.c's into a exponential function consisting of a fast and a slow component. The time constant of m.e.p.c. decay is regarded as being a measure of the mean open time of endplate channels in twitch fibres. FIEKERS et al consider that the effects of clindamycin and lincomycin on m.e.p.c. decay time constants are consistent with the view that these antibiotics interact with the open state of the receptor channel complex, creating a channel with little or no conductance.

Experimental studies with rolitetracycline, tetracycline and oxytetracycline have demonstrated their neuromuscular blocking properties (BEZZI & GESSA 1961), KUBIKOWSKI & SZRENIAWSKI 1963), however, the site of action of these drugs at the neuromuscular junction has not been determined. WRIGHT & COLLIER (1976a) investigated the site of action of polymyxin B and rolitetracycline. Rolitetracycline (3.6×10^{-3} M) produced a decrease in action potential amplitude but no change in the rate of rise of the action potential in the desheathed frog sciatic nerve preparation. Polymyxin B (1.8×10^{-4} M) caused a decrease in both the amplitude and rate of rise of the action potential. In a manner similar to local anaesthetic agents such as lidocaine this local anaesthetic like action of polymyxin B is dependent upon the charged form of the molecule since at a pH of 9.2 these effects were virtually abolished. Evidence suggesting that rolitetracycline produces neuromuscular block by an effect at the neuromuscular junction rather than by a direct effect upon the muscle was demonstrated in

experiments using the rat diaphragm phrenic nerve preparation. Rolitetracycline at a concentration which produced a 95% inhibition of twitch tension in response to stimulation via the nerve. Rolitetracycline (concentration range 8×10^{-4} to 1.5×10^{-3} M) brought about an 18% reduction in the twitch tension developed in response to direct stimulation of the muscle, d-tubocurarine blocked the response to direct stimulation of the muscle to a similar extent (a 20% reduction).

The effect of the two antibiotics on postsynaptic events was assessed by determining, in the phrenic nerve diaphragm preparation of the rat, the log dose response plot for nerve stimulation and the log dose response plot for ACh injection into the thoracic inferior vena cava. Rolitetracycline, at a concentration that blocked the response to nerve stimulation by 50%, inhibited the response to injected ACh by 80%. A similar response to injected ACh was obtained when a control preparation was treated with d-tubocurarine. Polymyxin B, however, was relatively more effective than d-tubocurarine; concentrations that blocked the response to stimulation by 50% completely abolished the response to the muscle to injected ACh.

Using a radioenzyme assay technique WRIGHT & COLLIER (1976a) demonstrated that rolitetracycline (7×10^{-4} M) did not significantly effect either spontaneous release or evoked release of ACh in the rat phrenic nerve diaphragm preparation.

The reversal of antibiotic induced muscle paralysis by 3, 4-diaminopyridine in the isolated phrenic nerve hemidiaphragm of the mouse was investigated by SINGH, HARVEY & MARSHALL (1978a). They found that tetracycline (18.7 mM) and oxytetracycline (11.9 mM) both selectively blocked responses to nerve stimulation; the block produced by tetra-

cycline was not reversed by 3, 4-diaminopyridine. 3, 4-diaminopyridine initially completely reversed the neuromuscular block produced by oxytetracycline, however complete block was restored within 20 to 25 minutes in the continuing presence of 3, 4-diaminopyridine. The 3, 4-diaminopyridine induced reversals of blockade induced by polymixin B, lincomycin and clindamycin were well maintained.

SINGH et al considered that the reversal of antibiotic induced neuromuscular blockade by 3, 4-diaminopyridine is probably due to an increase in transmitter output since aminopyridines are known to prolong action potential duration and the resultant increase in the influx of calcium into the presynaptic nerve terminals producing an increase in evoked ACh release (LUNDH, LEANDER & THESLEFF, 1977). Since both calcium and 3, 4-diaminopyridine reverse, to some extent, the effects of oxytetracycline whereas both agents are ineffective in reversing the blockade established by tetracycline was regarded by SINGH et al as evidence supporting their view that the action of 3, 4-diaminopyridine involves calcium ions.

Rolitetracycline and oxytetracycline both potentiate neuromuscular blockade produced by d-tubocurarine and Mg^{++} but their effects are not consistently reversed by anticholinesterases or by calcium (KUBIKOWSKI & SZRENIAWSKI 1963; BEZZI & GESSA 1961). These earlier observations were further supported by the findings of SINGH, HARVEY & MARSHALL (1978b), who investigated the neuromuscular blocking properties of some 16 antibiotics and the effects of neostigmine and increased calcium concentration upon the antibiotic induced paralysis of the phrenic nerve hemidiaphragm preparation of the mouse. Dose-response curves, for each of the antibiotics acting

for 5 minutes, were obtained and the concentration of antibiotic necessary to produce an 80 to 90% decrease in twitch tension determined. For tetracycline hydrochloride and oxytetracycline hydrochloride, the appropriate concentrations were 20.5 $\pm$ 0.3 mM and 10.4 mM respectively.

Neuromuscular paralysis induced by oxytetracycline was completely reversed by 5 mM calcium; neostigmine induced reversals, however, only ranged from 5 to 20% of the control twitch amplitude. 5 mM calcium was completely ineffective in overcoming the block due to 20.5 mM tetracycline hydrochloride, however, when the calcium concentration was increased to 10 mM a 44% recovery occurred. Neostigmine had no effect on tetracycline hydrochloride blocked preparations. An increase in calcium concentration had little effect on preparations that had been exposed for 20 to 40 minutes to neuromuscular blocking concentrations of oxytetracycline or tetracycline hydrochloride.

SINGH et al (1978b) further reported that both oxytetracycline and tetracycline hydrochlorides at high concentrations, or during long exposure to these antibiotics, affect the contractility of the muscle. Tetracycline hydrochloride and oxytetracycline (12.1 mM) initially augmented the twitch amplitude, followed by a slowly developing blockade of direct muscle stimulation which was often accompanied by contracture of the muscle. This augmentation of directly elicited responses was greater than the more transient augmentation of indirectly elicited twitches. At concentrations similar to those required to abolish twitches elicited by indirect stimulation, blockade of directly stimulated muscles by tetracycline and oxytetracycline ranged from 0 to 20%. WRIGHT & COLLIER (1976)

reported a similar reduction (18%) in the twitch tension developed by direct stimulation of the muscle of the rat diaphragm in the presence of concentrations of rolitetracycline (8×10^{-4} to 1.5×10^{-3} M) which brought about a 95% reduction of the twitch tension developed in response to stimulation via the nerve.

SINGH et al (1978b) concluded that although their experiments demonstrated that tetracycline and oxytetracycline appear to inhibit neuromuscular transmission and that, under certain circumstances this neuromuscular paralysis can be partially reversed by calcium, the precise site of action for these drugs remains to be elucidated.

From investigations of the quantitative relationship between the amplitude of the evoked endplate potential and the external calcium and magnesium ion concentrations the general finding is that transmitter release rises steeply with increasing calcium and varies with magnesium concentration in a manner suggesting simple competition between these two divalent cations.

Whether the neuromuscular block produced by the tetracycline group of antibiotics can be explained simply on the basis of a reduction in the concentration of ionised calcium alone, due to the formation of chelation complexes with the antibiotic, or whether there is an additional involvement, namely, that tetracyclines, like magnesium ions, compete with calcium ions for specific sites on the motor nerve terminal forms the basis of the experimental investigations presented in this thesis.

N.B. The expression "% reduction" will appear recurrently throughout the various sections of this thesis; therefore a % reduction in any specified parameter will be defined as

$$100\left(1 - \frac{\text{the magnitude of that parameter during the experiment}}{\text{the control magnitude of that parameter}}\right)$$

Similarly the expression "tetracycline" will be used as a generic term to indicate that it is the tetracycline group of antibiotics as a whole that are being considered. When a particular member of the tetracycline group of antibiotics is being discussed it will be specifically named; thus the term "tetracycline HCl" identifies the particular antibiotic tetracycline hydrochloride.

CHAPTER 2

THE EFFECTS OF TETRACYCLINES ON TWITCH TENSION IN THE ISOLATED HEMIDIAPHRAGM OF THE RAT

THE EFFECTS OF TETRACYCLINES ON TWITCH TENSION IN THE ISOLATED HEMIDIAPHRAGM OF THE RAT

INTRODUCTION :- In 1960 GESSA showed that tetracycline HCl at a concentration of approximately one millimolar, produced an almost complete neuromuscular blockade of the isolated hemidiaphragm of the rat. BEZZI & GESSA (1960, 1961) also demonstrated, in vivo in the rabbit, the enhancement of a slight paralysis due to tubocurarine, by tetracycline HCl given in doses which when administered by themselves had no apparent effect.

In the following series of experiments both the ability of tetracycline HCl, oxytetracycline HCl and minocycline HCl to produce almost a complete neuromuscular blockade and also to enhance a partial blockade due to tubocurarine were investigated in the isolated hemidiaphragm of the rat.

METHODS :- Rat phrenic nerve hemidiaphragm preparations, as described by BULBRING (1946), were suspended in an organ bath of 25 ml volume. The rib end of the preparation was firmly attached to a fixed stainless steel rod, the apex of the diaphragm was attached by a short piece of suture thread to a light, noncompliant stainless steel rod attached to an isometric tension transducer (GRASS FT 101). The electrical output from the transducer was displayed on a chart recorder (DEVICES). The phrenic nerve was stimulated supramaximally via a suction electrode at a rate of one stimulus every fifteen seconds. Typical stimulus parameters were a rectangular pulse of 0.5 msec. duration and 4.0 volts amplitude.

The tissues of both male or female albino rats (Wistar strain) weighing between 120 and 150 gm were used. The Krebs solution used had the following composition (millimoles per litre);

Na^+	141.0	Cl^-	104.8
K^+	5.9	$H_2PO_4^-$	2.2
Ca^{++}	2.6	HCO_3^-	24.9
Mg^{++}	1.2	SO_4^{--}	1.2
GLUCOSE	11.0		

The temperature of the preparation and of all the solutions to be added was maintained at 37° C by an electrically heated water bath and thermostat. A mixture of 95% O_2 and 5% CO_2 was bubbled over the surface of the preparation. The organ bath fluid volume was maintained constant at 25ml by means of an overflow. Fresh solutions were introduced into the lower part of the organ bath; the displaced fluid leaving via the overflow.

For each preparation tubocurarine at a concentration in the region of 2.0×10^{-6} M was added to the Krebs solution in order to produce a stable level of partial neuromuscular blockade in which the twitch tension developed in response to maximal stimulation of the phrenic nerve decreased to a value between 30% and 60% of that attained before the tubocurarine was present.

Curare-Krebs solution containing a particular concentration of antibiotic was introduced into the organ bath and allowed to act for a period of 15 minutes before being washed out with curare-Krebs.

At least 30 minutes of washing out was allowed before a further dose of the antibiotic was administered. Concentrations of the antibiotic were selected to produce responses in the region of from a 20% to 80% decrease in twitch tension. Once a series of responses to three or more increasing concentrations of antibiotic in curare-Krebs solution were obtained the preparation was washed in curare free Krebs for an extended period until the twitch tension returned to and remained stable at its original pre curarised value. A further set of responses to three or more increasing concentrations of the antibiotic in curare free Krebs were then recorded.

RESULTS:- A percentage reduction in twitch tension is defined as

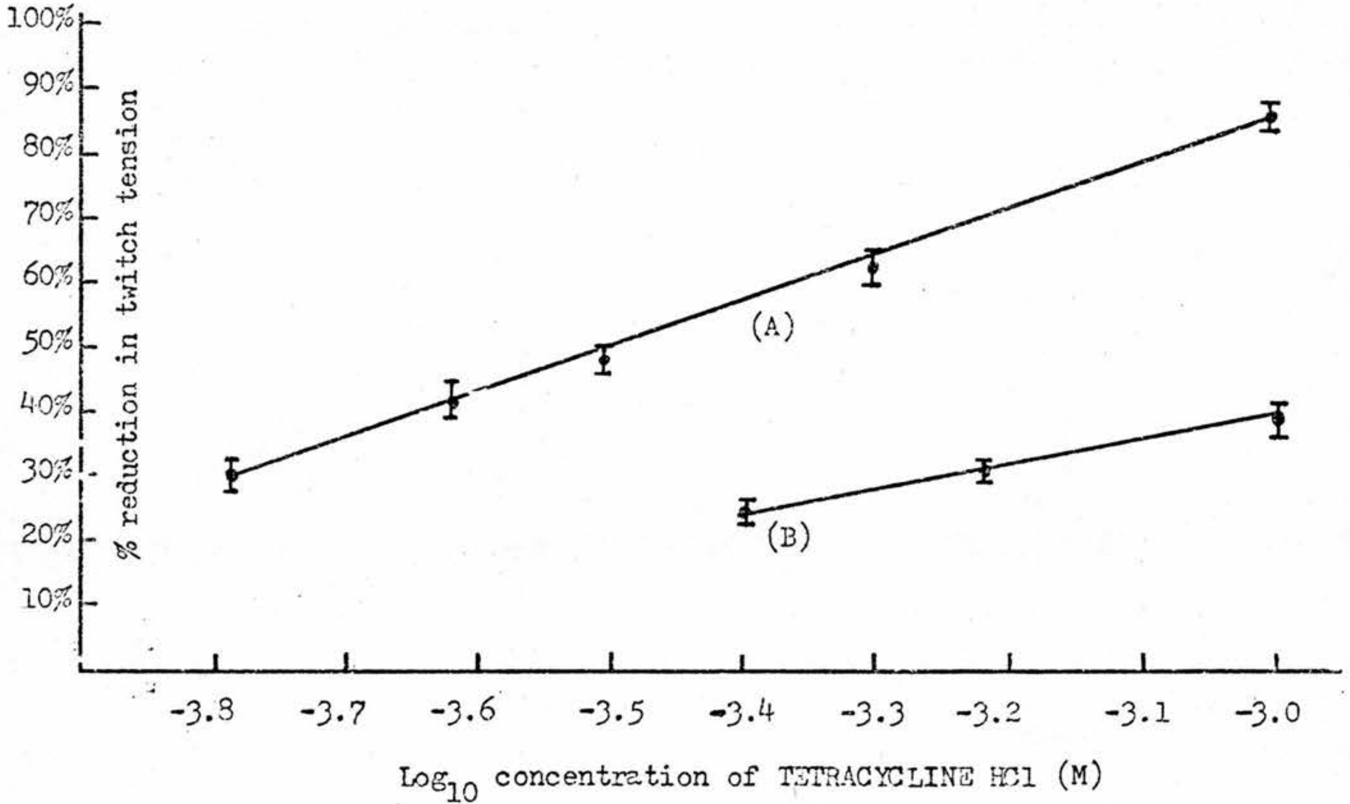
$$100\left(1-\frac{\text{twitch tension amplitude after 15 minutes in the antibiotic}}{\text{twitch tension amplitude before the antibiotic was present}}\right)$$

In figure 1 the results from four preparations are presented. The antibiotic used was tetracycline HCl; in (A) five concentrations of the antibiotic in the range 1.6×10^{-4} to 1.0×10^{-3} M made up in Krebs solution containing tubocurarine were applied. Following a 60 minute period of washing out in curare free Krebs solution, during which time the twitch tension fully recovered to its control level prior to the addition of tubocurarine, three doses of tetracycline HCl in the range 4.0×10^{-4} M to 1.0×10^{-3} M were added in turn and the results from the four experiments presented in (B). The error bars on the graph represent the standard error of the mean, N = 4.

FIGURE 1.

THE REDUCTION IN TWITCH TENSION DUE TO TETRACYCLINE HCl

(4 PREPARATIONS)



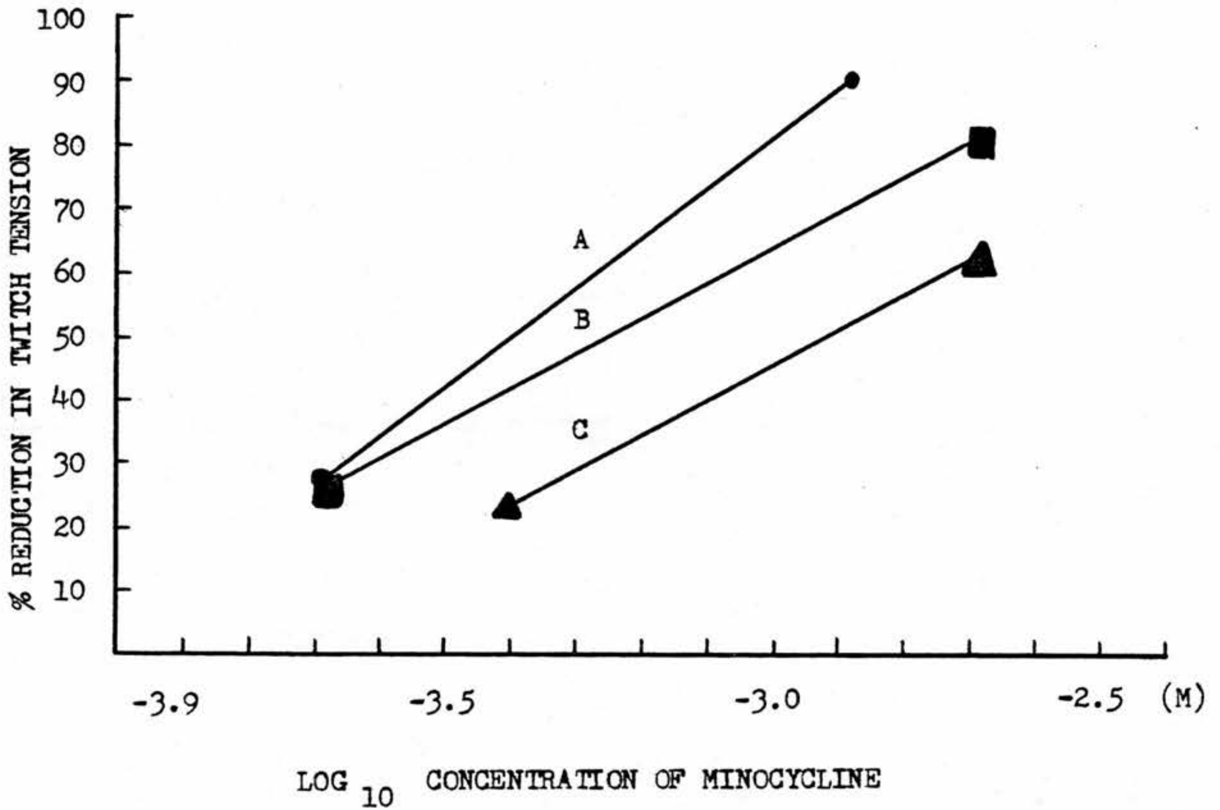
(A) TETRACYCLINE HCl in Krebs solution containing 2.0×10^{-6}M tubocurarine.

(B) TETRACYCLINE HCl in Krebs solution containing no tubocurarine.

In both cases the Krebs solution contained Calcium 2.6 mM and Magnesium 1.2 mM.

FIGURE 2

THE EFFECT OF MINOCYCLINE ON TWITCH TENSION



LOG $_{10}$ CONCENTRATION OF MINOCYCLINE

A MINOCYCLINE IN TUBOCURARINE 2.0 x 10^{-6}M.

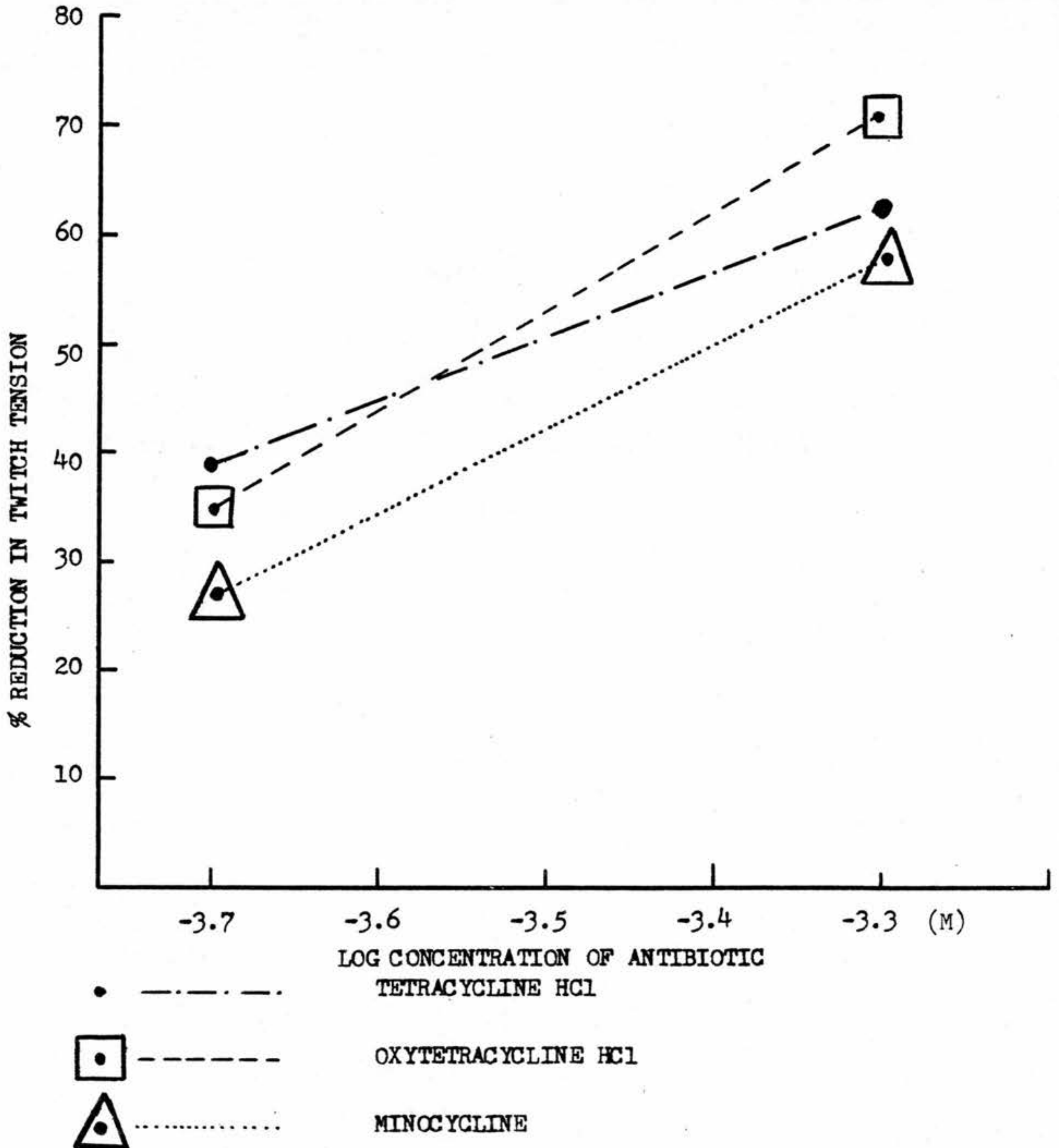
B MINOCYCLINE IN Mg^{2+} 12.0 mM.

C MINOCYCLINE ONLY

FIGURE 3

THE RELATIVE EFFECTIVENESS OF 3 ANTIBIOTICS IN REDUCING TWITCH TENSION IN A RAT DIAPHRAGM PREPARATION PARTLY BLOCKED BY TUBOCURARINE.

(2 CONCENTRATIONS OF EACH ANTIBIOTIC TESTED ON ONE PREPARATION)



In figure 2 the results from four preparations are presented. The antibiotic used was minocycline; in (A) three concentrations of the antibiotic in the range 2.0×10^{-4}M to 1.0×10^{-3}M made up in Krebs solution containing tubocurarine were applied. Following a 60 minute period of washing out in curare free Krebs solution, during which time the twitch tension fully recovered to its precurare treatment level, three doses of minocycline in the range 2.0×10^{-4}M to 2.0×10^{-3}M were added and the results from these four preparations presented in (B). Following a further period of washing out, during which the preparations fully recovered, a stable level of partial neuromuscular blockade was produced by raising the magnesium ion concentration of the Krebs solution. Three doses of minocycline in the range 2.0×10^{-4}M to 2.0×10^{-3}M made up in the Krebs solution containing the increased magnesium ion concentration and the results from four preparations presented in (C).

In figure 3 the results from one preparation are shown in which the relative effectiveness of tetracycline HCl, oxytetracycline HCl and minocycline in enhancing a partial neuromuscular blockade due to tubocurarine was investigated. The antibiotics were made up in Krebs solution containing tubocurarine; only two concentrations of the antibiotics were used:- 2.0×10^{-4}M & 5.0×10^{-4}M. Under these conditions minocycline is less effective than either of the other two antibiotics. At the higher concentration oxytetracycline HCl produces a larger decrease in twitch tension than does tetracycline HCl at the same concentration.

CONCLUSION:-The results of these in vitro experiments on the isolated hemidiaphragm of the rat are in agreement with the observations made by GESSA (1960) and BEZZI & GESSA (1961).

CHAPTER 3

THE EFFECTS OF TETRACYCLINES ON TWITCH TENSION IN THE PARTIALLY CURARISED FROG SARTORIUS PREPARATION

THE EFFECTS OF TETRACYCLINES ON TWITCH TENSION IN THE PARTIALLY CURARISED FROG SARTORIUS PREPARATION

INTRODUCTION :- Virtually all of the earlier experimental studies concerning the neuromuscular blocking properties of the tetracycline group of antibiotics were carried out on small mammals, primarily by means of in vivo techniques.

The original observations concerning the neuromuscular blocking properties of tetracycline HCl were made, in the rabbit, BEZZI & GESSA (1959, 1960); in 1961 they extended their observations to include oxytetracycline HCl and also to further demonstrate the ability of tetracycline HCl to decrease the twitch tension developed in response to stimulation of the phrenic nerve in the rat diaphragm preparation first reported by GESSA in 1960. Similarly in 1963 KUBIKOWSKI & SZRENIAWSKI demonstrated the neuromuscular blocking action of both oxytetracycline and methylpyrrolidine tetracycline in the cat sciatic nerve gastrocnemius muscle preparation.

A substantial part of the current body of knowledge concerning the various mechanisms involved in the process of neuromuscular transmission in voluntary muscle stems from experimental observations in which the sciatic nerve sartorius muscle preparation of the frog was used. Also it was considered that numerous practical advantages were to be gained by using the frog sartorius preparation as opposed to the rat diaphragm preparation. Therefore a preliminary series of experiments were conducted in order to establish that the tetracycline group of antibiotics also exert a neuromuscular blocking action in the frog sciatic nerve sartorius muscle preparation.

METHODS :- Frog sciatic nerve sartorius muscle preparations (Rana pipiens) were suspended in an organ bath of 25 ml volume. The muscle, which was dissected out still attached to the acetabulum, was secured to a rigid "Perspex" framework by means of a nylon screw located in the socket of the acetabulum. The tendon of the muscle was secured by a short length of suture thread to a noncompliant, stainless steel rod connected to an isometric tension transducer (GRASS INSTRUMENTS FT 101), the output of which was displayed on a hot wire strip chart recorder (DEVICES). The nerve was stimulated supramaximally at a rate of one stimulus per minute via a suction electrode. The stimulus parameters producing maximum twitch tension were carefully determined for each preparation and the amplitude of the applied stimuli were then set at a value 2.5 X that required to produce maximal twitch tension at the start of each experiment.

Provision was made for the rapid change of solutions by introducing new solution into the bottom of the chamber and displacing the solution already present. A constant level was maintained by allowing displaced fluid to overflow. All the experiments were conducted at room temperature (18 to 20° C).

In order to produce a stable level of partial neuromuscular block the preparation was curarised in two stages (NASTUK, W.L. & ALVING, B.O. 1959). The preparation was bathed for 10 minutes in HEPES buffered Ringer solution containing tubocurarine at a concentration of 1.4×10^{-6} M. The tubocurarine concentration was then decreased to 0.7×10^{-6} M; over a period of approximately 30 minutes a stable level of partial neuromuscular blockade was attained, the resultant decrease in twitch tension being from 25% to 60% of the value before the tubo-

curarine was present; antibiotics were made up in this solution.

Ringer solution has no inherent pH buffering capability, it was therefore necessary to include a buffering system which could maintain the pH at a more physiologically acceptable level than the acidic values of pH produced as a result of the dissociation of the antibiotic in solution.

The buffering agent HEPES (British Drug House) was selected as fulfilling the necessary requirements (see chapter 7, methods section and also chapter 8 "The Effects of HEPES Buffered Ringer On Quantal Content").

The Ringer solution was buffered at pH 7.0 rather than the correct physiological value of 7.4 for the following reasons:-

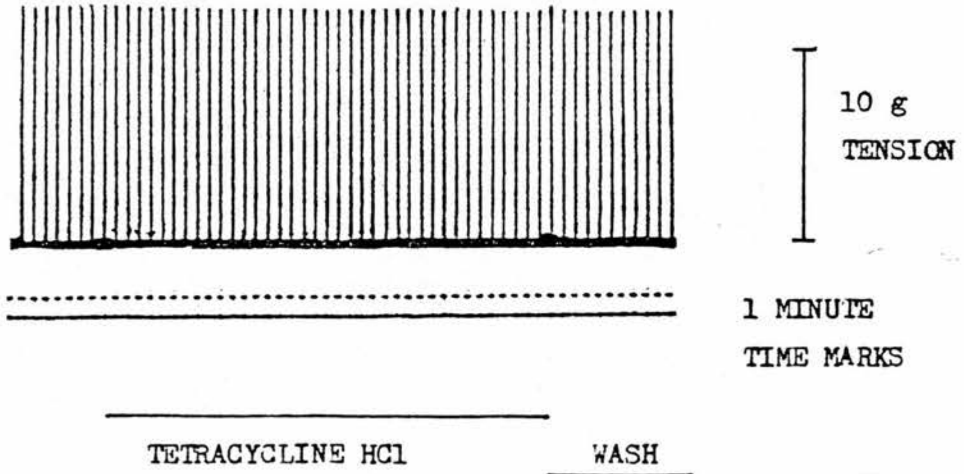
(A) tetracycline and its derivatives are slowly destroyed in solutions at pH 7.0 and higher; in general the more alkaline the pH the more rapidly the antibiotics are destroyed.

(B) to buffer the solution pH at 7.0 requires the addition of approximately one third the quantity of NaOH necessary to stabilise the pH at 7.4 with consequently less disturbance of the ionic composition of the Ringer solution.

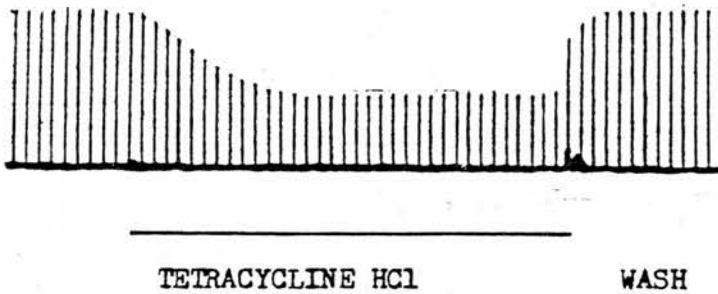
Preparations were bathed in HEPES buffered Ringer solution of the following composition (mM); sodium 112.4; potassium 2.5; calcium 1.8; chloride 117.1; HEPES 3.0. Dextrose (1.0 g L^{-1}) was added to the solution and 100% oxygen was bubbled gently over the surface of the sartorius muscle which was maintained at 20° C.

FIGURE 4

THE EFFECT OF TETRACYCLINE (HCl 3.0×10^{-5}M) ON TWITCH TENSION.



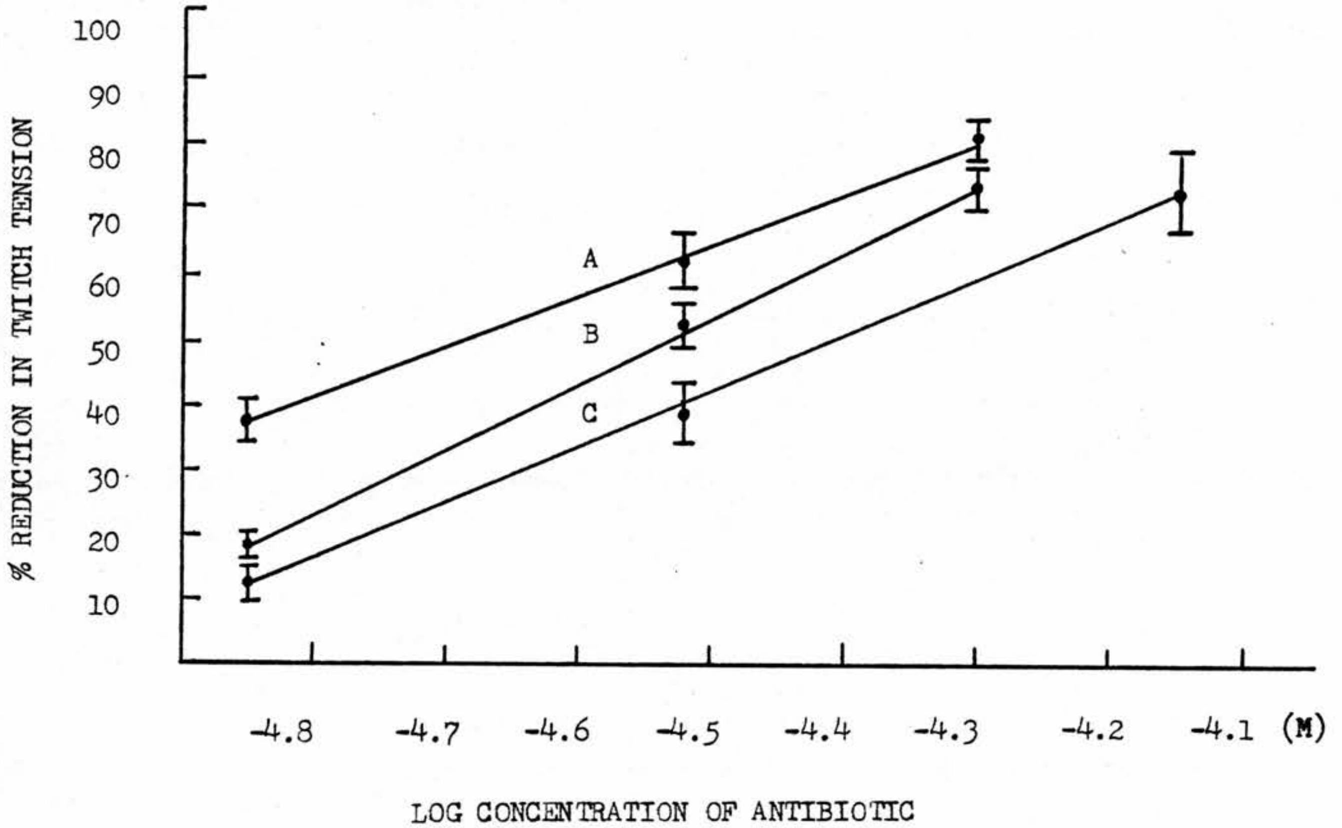
(A) IN THE ABSENCE OF A PARTIAL NEUROMUSCULAR BLOCK DUE TO TUBOCURARINE.



(B) IN THE PRESENCE OF A PARTIAL NEUROMUSCULAR BLOCK DUE TO TUBOCURARINE (0.7×10^{-6}M).

FIGURE 5

THE EFFECT OF TETRACYCLINE HCl, CHLORTETRACYCLINE HCl & MINOCYCLINE ON TWITCH TENSION OF THE PARTIALLY CURARISED FROG SARTORIUS PREPARATION



LOG CONCENTRATION OF ANTIBIOTIC

A = CHLORTETRACYCLINE HCl

B = TETRACYCLINE HCl

C = MINOCYCLINE

The antibiotics were made up in HEPES buffered Ringer solution (pH = 7.0) containing tubocurarine at a concentration of 7.0×10^{-7} M.

RESULTS :- Figure 4(A) shows that tetracycline HCl at a concentration of 3.0×10^{-5} M has no perceptible effect on the twitch tension in the indirectly stimulated sciatic nerve sartorius muscle preparation of the frog; however when the same concentration of the antibiotic is applied to a similar preparation in which a stable level of partial neuromuscular blockade by tubocurarine has been established, a 50% decrease in twitch tension occurrs. The two preparations used in this experiment were from the same frog.

The results from a number of experiments similar to that described above are presented in graphical form in figure 5; the three antibiotics used were chlortetracycline HCl, tetracycline HCl and minocycline. The error bars shown on the graphs represent the standard error of the mean of four results obtained from four preparations. Three concentrations of a particular antibiotic are each allowed to act for a period of 35 minutes. An extended period of washing out in normal Ringer solution containing tubocurarine, during which the twitch tension amplitude fully recovered, was allowed between each application of the antibiotic in tubocurarine containing Ringer. Each of the antibiotics was tested on four preparations, a total of twelve preparations were used.

CONCLUSION :- The results obtained using the frog sciatic nerve sartorius muscle preparation are similar to those obtained using the rat hemidiaphragm preparation as described in the previous section. The results illustrate the ability of these antibiotics (at concentrations which by themselves exert no detectable decrease in twitch tension) to enhance the degree of neuromuscular blockade in preparations in which partial neuromuscular blockade by tubocurarine had been established.

CHAPTER 4

THE EFFECTS OF TETRACYCLINES ON TWITCH TENSION

THE EFFECTS OF TETRACYCLINES ON TWITCH TENSION

INTRODUCTION :- The ability of tetracyclines to produce complete neuromuscular blockade in the absence of tubocurarine was investigated. In some experiments provision was made for applying stimulation directly to one entire side of the sartorius muscle through an electrode assembly specifically designed for this purpose (figure 6). In these experiments direct and indirect stimulation was applied alternately.

METHODS :- The preparation was mounted as described on page 28 and illustrated in figure 6. In the experiments where direct and indirect stimulation was applied alternately care was taken to ensure that supramaximal stimulation was applied throughout the course of the experiment. The stimulus parameters producing maximum twitch tension were carefully determined for each preparation and each mode of stimulation, the amplitudes of the applied stimuli were then set at a value 2.5 X that required to produce maximal twitch tension at the start of the experiment.

Typical stimulus parameters used were:-

Indirect stimulus parameters amplitude 5.0 volts, 0.2 ms duration.

Direct " " " 10.0 " 10.0 " " .

FIGURE 6

ORGAN BATH AND STIMULATION ARRANGEMENTS

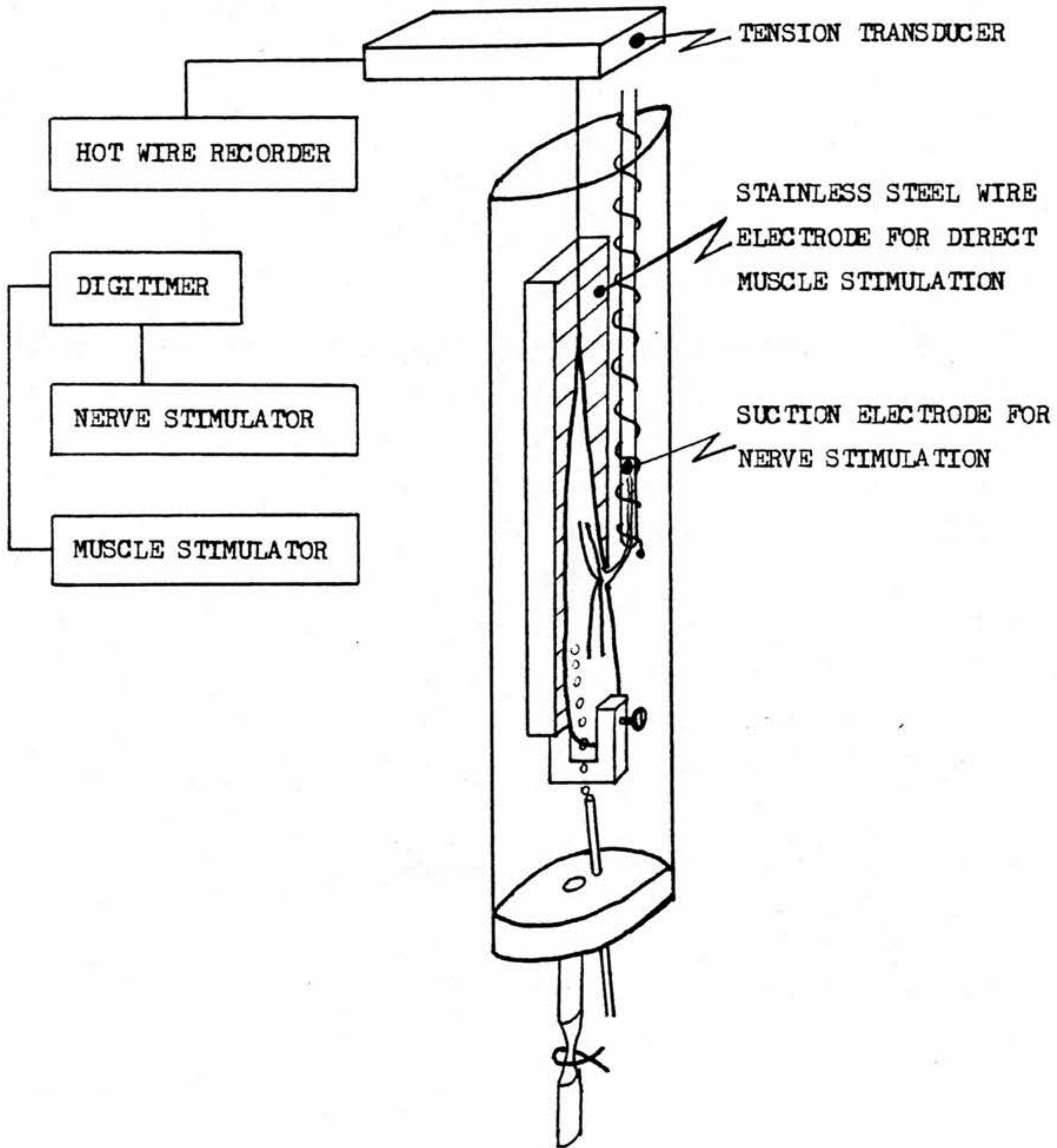
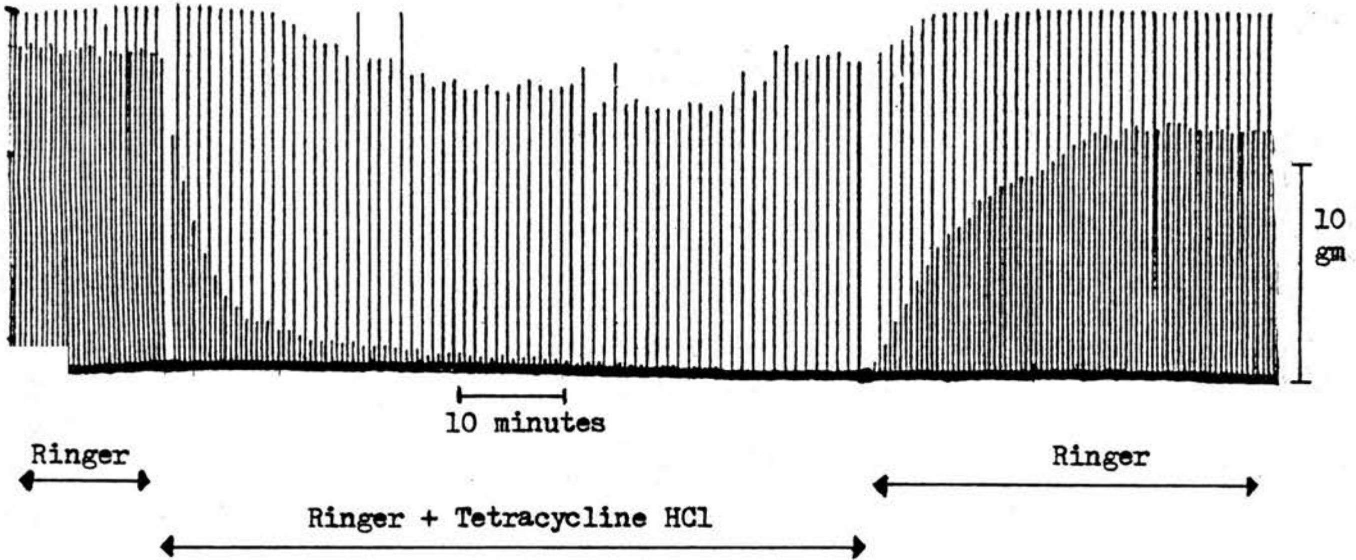


FIGURE 7

The effect of Tetracycline HCl (2.0×10^{-3}M) on twitch tension in the presence of HEPES buffered Ringer solution (pH = 7.0) containing an initial calcium ion concentration of 1.8×10^{-3}M.



Supramaximal stimulation was applied to the frog sciatic nerve - sartorius muscle preparation; alternate stimuli being applied, indirectly via the sciatic nerve and then directly to the sartorius muscle.

The initial twitch tension of 14.8 gm developed in response to the indirect stimulation via the sciatic nerve decreased by 50% within 3.0 minutes following the introduction of HEPES buffered Ringer solution of initial calcium ion concentration of 1.8 mM containing 2.0×10^{-3} M Tetracycline HCl; the indirectly developed twitch tension becoming imperceptible after 57 minutes. During this period the twitch tension developed in response to direct stimulation of the muscle was little affected, giving a clear indication that the tetracycline was exerting its effect on twitch tension at the neuromuscular junction rather than directly upon the contractile mechanism of the muscle.

TABLE 1

THE EFFECT OF TETRACYCLINE HCl ON TWITCH TENSION

TETRACYCLINE HCl concentration = 2.0×10^{-3}M.

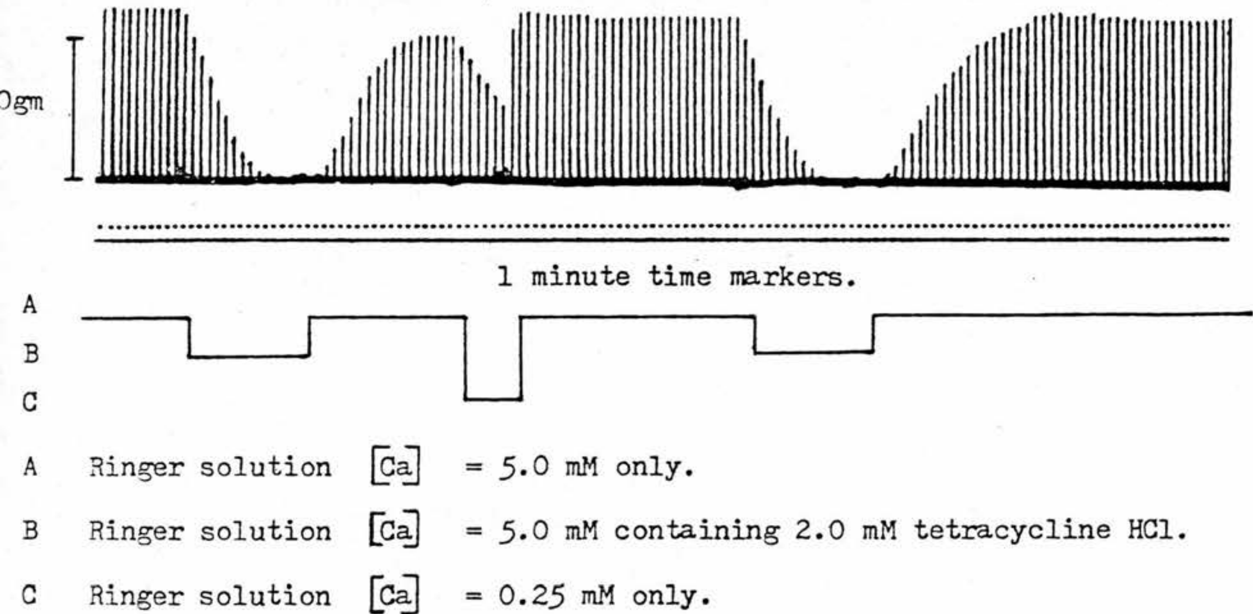
Initial calcium ion concentration = 1.8×10^{-3}M.

HEPES buffered Ringer solution pH = 7.0.

PREPARATION No	Time to 50% inhibition (minutes)	Time to 90% inhibition (minutes	Time to 100% inhibition (minutes)	% Recovery
1	3	11	57	76
2	2.6	10	52	66
3	10	31	40	53
4	12	32	block incomplete	100
5	6.5	21	41	70
6	6	10	52	66
AVERAGE	6.68	19.25	45.4	71.5
S	3.74	10.33	8.62	15.90
S_y	1.53	4.22	3.85	6.49

FIGURE 8

The effect of tetracycline HCl ($2.0x10^{-3}M$) on twitch tension in the presence of HEPES buffered Ringer solution (pH = 7.0) containing an increased concentration of calcium ($5.0x10^{-3}M$ cf. $1.8x10^{-3}M$).



The frog sciatic nerve sartorius preparation was bathed in HEPES buffered Ringer solution (pH = 7.0) containing calcium ions at a concentration of 5.0 mM. Tetracycline HCl at a concentration of 2.0 mM was made up in this high calcium concentration Ringer. Once a stable level of twitch tension of 12.1gm was established in the presence of the high calcium Ringer solution (A) the twitch tension decreased to an imperceptible level within 12 minutes of the introduction of the tetracycline HCl containing Ringer solution (B). Following a period of wash-out with the high calcium Ringer, Ringer solution with a calcium ion concentration of only 0.25 mM was introduced (C). Note that the rate of decrease in twitch tension was less rapid in the low calcium Ringer solution than in the presence of the high calcium Ringer containing the antibiotic.

TABLE 2

THE EFFECT OF TETRACYCLINE HCl ON TWITCH TENSION IN HIGH Ca^{2+} RINGER

TETRACYCLINE HCl concentration 2.0×10^{-3}M.

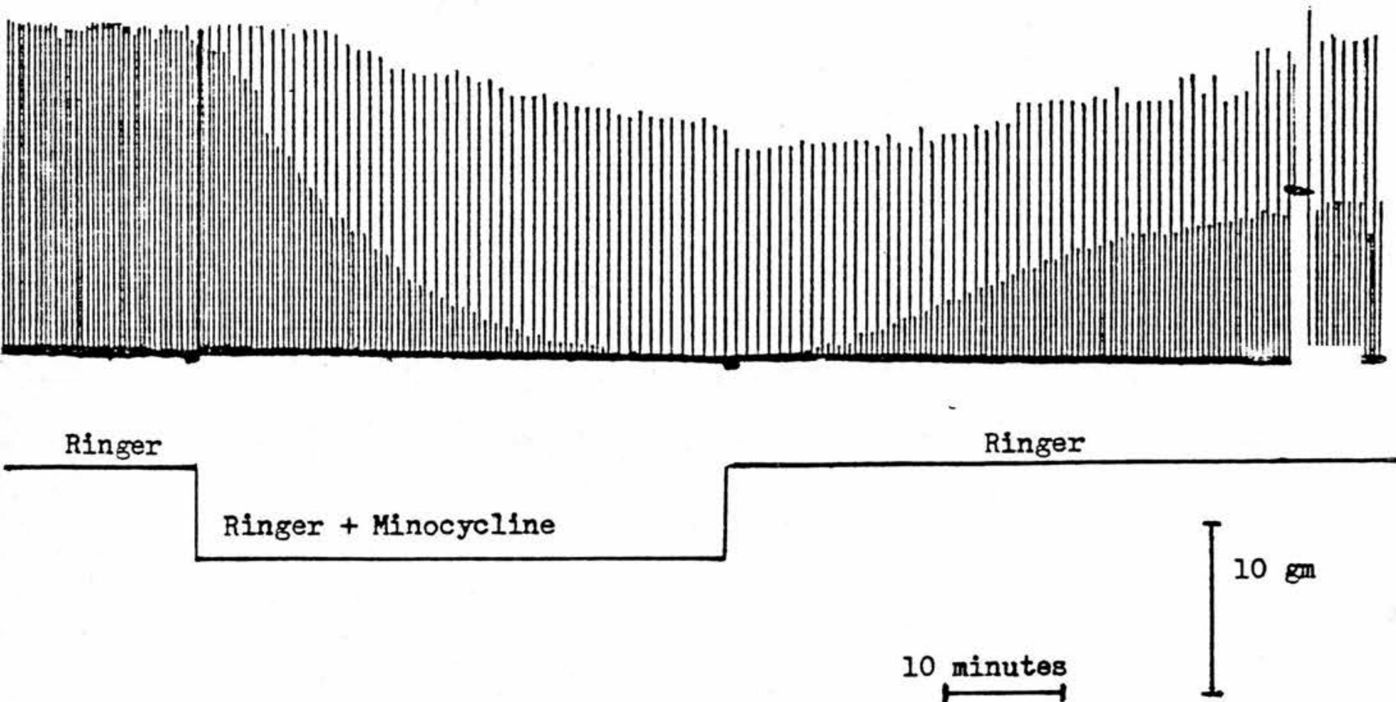
Initial calcium ion concentration 5.0×10^{-3}M.

HEPES buffered Ringer solution pH = 7.0.

PREPARATION No	Time to 50% inhibition (minutes)	Time to 90% inhibition (minutes	Time to 100% inhibition (minutes)	% Recovery
1	3	10	18	60
2	6	10.5	27	64
3	3.5	9.5	13	68
4	5	10	15	100
5	4	10	13	100
6	4	11	17	85
AVERAGE	4.25	10.17	17.17	79.50
S	1.08	0.52	5.23	18.02
S_y	0.44	0.21	2.14	7.36

FIGURE 9

The effect of MINOCYCLINE (2.0×10^{-3}M) on twitch tension in the presence of HEPES buffered Ringer solution (pH = 7.0) containing an initial calcium ion concentration 5.0×10^{-3}M.



Supramaximal stimulation was applied to the frog sciatic nerve sartorius muscle preparation; alternate stimuli being applied, indirectly via the sciatic nerve and then directly to the muscle.

The initial twitch tension of 19.1 gm developed in response to the indirect stimulation via the sciatic nerve decreased by 50% within 11 minutes following the introduction of HEPES buffered Ringer solution of initial calcium ion concentration of 5.0×10^{-3}M containing 2.0×10^{-3}M Minocycline; the indirectly developed twitch tension becoming imperceptible after 40 minutes.

TABLE 3

THE EFFECT OF MINOCYCLINE ON TWITCH TENSION.

MINOCYCLINE HCl concentration 2.0 x 10^{-3}M.

Initial calcium ion concentration 1.8 x 10^{-3}M.

HEPES buffered Ringer solution pH = 7.0.

PREPARATION No	Time to 50% inhibition (minutes)	Time to 90% inhibition (minutes)	Time to 100% inhibition (minutes)	% Recovery
1	37	106	168	22
2	41	99	block incomplete	70
3	52	90	block incomplete	73
4	18	80	105	60
5	15	67	150	46
6	18	63	130	29
AVERAGE	30.17	84.17	113.25	50
S	15.28	17.27	35.34	21.31
S_y	6.24	7.05	17.67	8.70

TABLE 4

THE EFFECT OF MINOCYCLINE ON TWITCH TENSION

MINOCYCLINE HCl concentration $= 2.0 \times 10^{-3}$M.

Initial calcium ion concentration $= 5.0 \times 10^{-3}$M.

HEPES buffered Ringer solution pH = 7.0.

PREPARATION No	Time to 50% inhibition (minutes)	Time to 90% inhibition (minutes)	Time to 100% inhibition (minutes)	% Recovery
1	9	18	30	45
2	7	25	45	56
3	3.5	15	23	50
4	4	16	27	48
5	15	37	67	62
6	11	34	58	67
AVERAGE	8.25	24.17	41.67	54.67
S	4.38	9.50	18.00	8.57
S_y	3.88	7.35	7.35	3.50

CONCLUSION:- From the results presented in tabular form and from the sample traces of twitch tension recordings it can be seen that both tetracycline HCl and minocycline at a concentration of 2.0×10^{-3} M produce a substantial loss of twitch tension in the indirectly stimulated sciatic nerve sartorius preparation of the frog. When the initial calcium concentration was 1.8 mM complete neuromuscular blockade was not achieved in one of the six preparations exposed to tetracycline HCl. Similarily, complete blockade was achieved in only 4 of the 6 preparations exposed to minocycline. When the initial calcium concentration was raised to 5.0 mM and the antibiotic concentration maintained at 2.0 mM, complete neuromuscular blockade developed in all six of the preparations exposed to each of the antibiotics.

In order to establish whether these antibiotics act at the neuromuscular junction, or directly upon the excitation contraction coupling mechanism of the muscle fibres, several experiments were conducted in which supramaximal stimuli were delivered alternately via the nerve and directly to the muscle fibres. Figure 7 shows a trace recorded during one such experiment demonstrating clearly that while tetracycline HCl (2.0×10^{-3} M) causes the twitch tension developed in response to stimulation of the sciatic nerve to decrease to a level where it can no longer be detected; the twitch tension developed in response to direct stimulation of the muscle remains substantially unimpaired. The downward trend in the directly stimulated twitch tension record may arise as a consequence of one or more of the following:-

(A) an experimental artefact such as the alteration of the position of the sartorius muscle relative to the stimulating electrode resulting in the loss of optimum contact and the stimulation becoming less than maximal.

(B) HODGKIN & HOROWICZ (1960) demonstrated that the initial twitch tension developed in response to direct muscle stimulation is related to the muscle membrane potential, the decrease in the directly stimulated twitch tension may be due to the decrease in the muscle fibre membrane potential which occurs when the antibiotic is present in the Ringer solution (see chapter 13).

(C) activation of the muscle is brought about by depolarisation of the surface membrane resulting in the release of calcium ions from intracellular stores in the sarcoplasmic reticulum. Tetracyclines form chelation complexes with calcium and assuming that the contractile force developed by a muscle fibre reflects the quantity of calcium released from the sarcoplasmic reticulum it is possible that the reduction in the free calcium ion concentration brought about by the tetracycline might lead to a decrease in the force developed in response to direct stimulation of the muscle fibres.

However it is clear that the tetracycline group of antibiotics bring about a reduction in twitch tension in the indirectly stimulated nerve muscle preparation by virtue of their action at the neuromuscular junction rather than by any effect of the antibiotic on the excitation contraction coupling mechanism of the muscle fibres themselves.

In order to compensate for the reduction of the available concentration of calcium ions resulting from chelation by the tetracycline HCl (2.0×10^{-3} M), the calcium concentration of the Ringer solution was increased from 1.8 to 5.0 mM. Figure 8 shows a trace from one such experiment; in which it is clearly demonstrated that the tetracycline still brings about a complete reduction in twitch tension irrespective of the available calcium ion concentration being greatly

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increased. Consequently the tetracycline group of antibiotics must exert their effect on the neuromuscular junction by some mechanism other than by simply reducing the available calcium ion concentration.

CHAPTER 5

THE EFFECT OF CALCIUM CONCENTRATION ON THE TWITCH TENSION OF THE INDIRECTLY STIMULATED FROG SCIATIC NERVE SARTORIUS PREPARATION

THE EFFECT OF CALCIUM CONCENTRATION ON THE TWITCH TENSION OF THE INDIRECTLY STIMULATED FROG SCIATIC NERVE SARTORIUS PREPARATION

INTRODUCTION :- The dependence of the development of twitch tension on the concentration of the calcium present in the bathing medium was investigated and an estimate obtained of how low that calcium concentration could be before a significant decrease occurs in the recorded twitch tension from the indirectly stimulated sciatic nerve sartorius muscle preparation of the frog.

METHODS :- Frog sciatic nerve sartorius muscle preparations (Rana pipiens) were suspended in an organ bath of 25 ml volume (figure 6) as previously described on page 28. Ringer solutions were made up containing various concentrations of calcium, ranging from no added calcium to the normal frog Ringer calcium concentration of 1.8 mM.

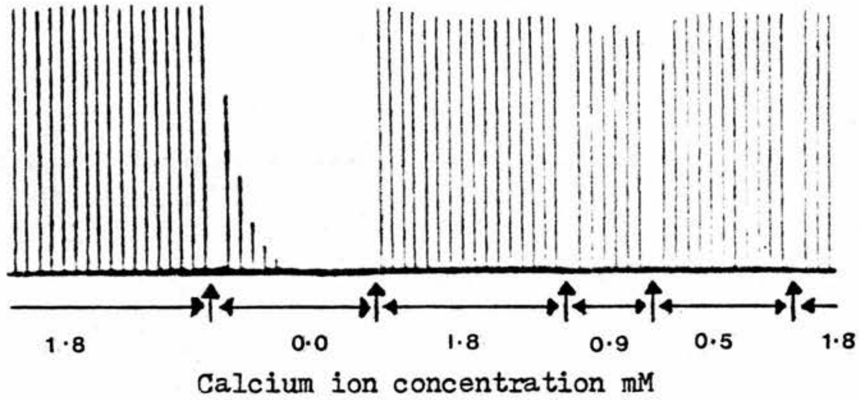
RESULTS :- From the results shown in figure 10 it can be seen that at the low stimulation rates employed in these experiments reducing the calcium ion concentration present in the Ringer solution from

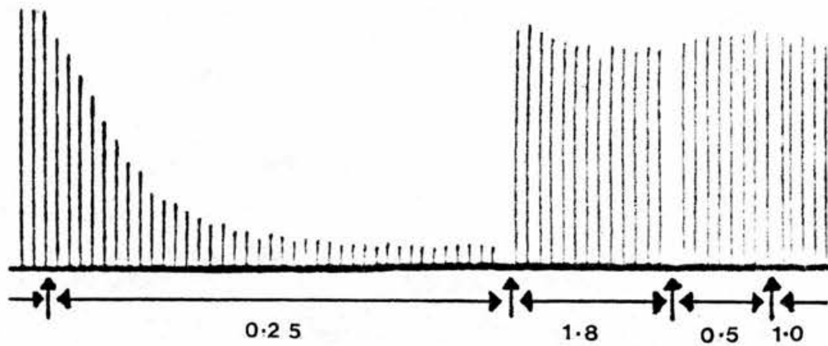
the normal 1.8 mM to 0.5 mM produces no apparent reduction in twitch tension in the indirectly stimulated frog sartorius preparation at 20° C. However reducing the calcium ion from 1.8 mM to 0.25 mM does cause a reduction in twitch tension of 90%. This 90% reduction in twitch tension being achieved within 7 minutes of the calcium concentration being reduced to 0.25 mM.

CONCLUSIONS :- In order to explain the reduction in twitch tension brought about by tetracyclines solely in terms of their chelating effect on the calcium concentration of 1.8 mM normally present in frog Ringer solution it would be necessary to show that the efficacy of this chelation is such that it can bring about a reduction in the free calcium ion concentration to a value less than 0.5 mM. However in order to produce a neuromuscular block in excess of a 90% reduction in twitch tension it would be necessary for the calcium concentration to be reduced to below 0.25 mM by chelation with the tetracycline.

FIGURE 10

THE EFFECT OF CALCIUM CONCENTRATION ON THE TWITCH TENSION OF THE INDIRECTLY STIMULATED FROG SCIATIC NERVE SARTORIUS PREPARATION





10 gm
twitch
tension

Time marks at 30 second intervals

Vertical arrows indicate when bath contents were altered.

CHAPTER 6

ESTIMATION OF THE NUMBER OF QUANTA RELEASED PER NERVE IMPULSE

ESTIMATION OF THE NUMBER OF QUANTA RELEASED PER NERVE IMPULSE

The prevailing concept of the process of neuromuscular transmission holds that the transmitter is stored in the presynaptic terminal in the form of a large number (n) of preformed packets, or quanta, each of which has a probability (p) of being released in response to a nerve stimulus. The mean number of quanta released per stimulus (m) is given by

$$m = n.p \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Assuming that at the frog neuromuscular junction the average amplitude of the spontaneously occurring miniature endplate potentials (MEPP's) corresponds to the unit potential generated by the release of a single quantum of transmitter (del CASTILLO & KATZ, 1954, BOYD & MARTIN, 1956, ELMQVIST & QUASTEL, 1965) a direct estimate of the average of the average quantal content of a series of synaptic potentials is given by the ratio of the mean amplitude of evoked EPP's V to the mean amplitude of the spontaneously released MEPP's v_1 thus

$$m_1 = \frac{V}{v_1} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Before applying this method due attention must be given to two main sources from which errors in the estimation of m could arise.

Since at any one junction MEPP amplitude has a coefficient of variation of from 20% to 25% a quite accurate estimate of average quantum size is obtainable from measurements of between 10 and 20 MEPP's.* It is essential, however, that the MEPP's are clearly identifiable above the background noise level of the recording, since if an unknown number of MEPP's are not identified because their amplitude falls below the noise level no reliable estimate of average MEPP amplitude can be determined. * (B.L. GINSBORG, personal communication).

The direct method of estimating quantal content imposes certain restrictions on the experimental design and due consideration must be given to a number of possible sources of error which can arise.

One such source of error can be attributed to the non-linearity of the postsynaptic response. To illustrate how this non-linearity arises refer to figure 11. In this model, which is an adaptation of that proposed by MARTIN, 1955, the resting potential of the muscle fibre is represented by E and the transverse conductance between the inside and the outside of the fibre by G. Each MEPP arises from the momentary closure of any one of the switches SW1 to SWm, this momentarily shunts the fibre membrane locally with a conductance g in series with a small voltage e. The result is flow of depolarising current i, and a small reduction of the potential difference between the inside and the outside. If only a few of the switches are closed, corresponding to a low quantal content, the increment of conductance will be very small compared to the transverse conductance G and consequently the small potentials that result from the depolarising currents flowing through each of the shunt conductances g will summate linearly. However as more and more switches close, corresponding to

an increase in quantal content, although the closure of each switch still brings into circuit a fixed conductance g the resultant depolarisations do not add in a linear manner. A correction factor to compensate for this non-linear summation of unit potentials was devised by MARTIN (1955). Using this correction factor the corrected amplitude V^1 for each EPP can be calculated from the relationship:-

$$V^1 = \frac{E}{E - V} \cdot V \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

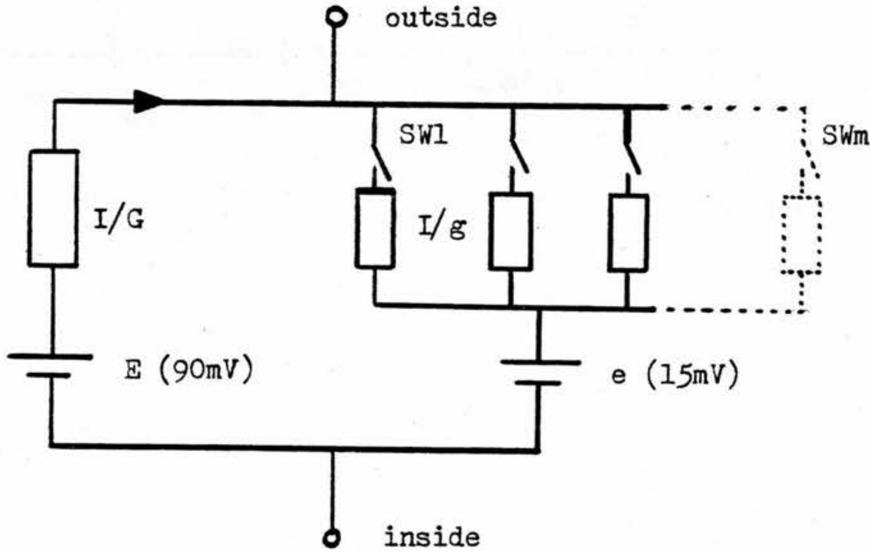
Where E is the difference between the resting membrane potential and the acetylcholine equilibrium, or reversal potential (-15 mV; TAKEUCHI & TAKEUCHI, 1960). V is the measured EPP amplitude.

One criticism levelled at MARTIN'S correction is that the model from which it was derived does not take into account the postsynaptic membrane capacitance which will tend to attenuate the recorded EPP amplitude. In the view of WERNIG, 1975; BENNETT, FLORIN & PITTIGREW, 1976, MARTIN'S correction over compensates for the non-linear summation of unit potentials by some 50%, consequently the correction factor applied in the following series of experiments was 50% of MARTIN'S correction.

Although most of the MEPP amplitudes measured at the frog neuromuscular junction fit a normal distribution curve, there are a number whose amplitude is several times that of the modal amplitude. However such 'giant' MEPP's were not included in the analysis since they have been shown by MENRATH & BLACKMAN, (1970) not to contribute as quantal components of evoked EPP's.

FIGURE 11

DIAGRAM ILLUSTRATING AN ELECTRIC CIRCUIT EQUIVALENT OF MEPP's



The resting potential of the muscle fibre is represented by E, and the characteristic input impedance of the fibre by I/G (approximately 100,000 ohms). Each MEPP arises from the temporary closure of one switch, which shunts the fibre membrane with a resistance I/g (approximately 10 megohms) in series with a small voltage e (15 mV, negative inside). The result is flow of depolarising current i, and a small reduction of the p.d. between inside and outside. Closure of additional switches produces larger depolarisation, but the increments become smaller the larger the number of closed switches (m), and the depolarisation cannot exceed the difference between the resting membrane potential and the acetylcholine reversal potential i.e. 75 mV.

Depressing the sensitivity of the postsynaptic response by the use of tubocurarine is not a suitable technique for the estimation of quantal content by the direct method since MEPP amplitudes are also depressed and cannot be readily identified above the noise level.

There are indirect methods of estimating quantal content and these arise as a consequence of the statistical properties of the release mechanism. Thus a summary of the hypotheses is, the transmitter is held available in the presynaptic terminal in the form of a large number (n) of quanta, each of which has a very small but finite probability (p) of being released in response to a nerve stimulus. It follows from the hypotheses that during a series of trials the number of quanta in each individual endplate potentials should fluctuate in a manner predicted by the binomial distribution. If p is small, as is likely during block by Mg^{++} or low Ca^{++}, this will be approximated by the Poisson distribution.

The Poisson distribution makes it possible to predict, for any series of EPP's, the number of responses containing 0,1,2,3,4 or more quanta. That is, the number of responses containing x quanta (n_x) is given by

$$n_x = \frac{Ne^{-m} m^x}{x} \qquad \text{.... (4)}$$

where N is the total number of trials and x can have values of 0,1, 2,3 etc.

If p is small, as is likely during block by either Mg^{++} or by low Ca^{++}, there will be occasions when no quanta at all will

be released in response to a nerve stimulus. Such failures to produce an EPP provide a method whereby an estimate of quantal content can be made which is quite independent of a knowledge of both the transmitter reversal potential and the cell membrane potential and is also quite unaffected by any considerations of the non-linearity of the postsynaptic response. The primary requirement being that the signal to noise ratio is such that a unit response can be clearly identified and cannot be misjudged to be a failure.

The Poisson distribution predicts that the number of failures should be given by

$$n_0 = Ne^{-m} \qquad \text{.... (5)}$$

from which an estimate m_0 can be obtained

$$m_0 = \ln (N)/(Nf) \qquad \text{.... (6)}$$

Where N = the total number of nerve stimuli applied during the trial period and Nf = the number of failures occurring during the trial period.

The failure method is only applicable to situations where the quantal content is not more than 3. If the proportion of failures is low, large sampling errors can occur. It can be shown that the optimal proportion of failures lie between 0.1 and 0.3; also that in order to reduce the standard error of m to about 10% some 170 responses would have to be observed.

A unique property of the Poisson distribution is that both the mean and the variance are numerically equal. This property allows a third method of estimating quantal content to be used. The variance method provides a means of estimating quantal content in the presence of blocking agents which cause a decrease in the recorded MEPP amplitude; however it is necessary to apply the method to quite long sequences of EPP's in order to attain an acceptable degree of accuracy, the standard error of m is typically 20% when estimated from the amplitudes of EPP's. As the method will be applied primarily to experimental situations where the quantal content per stimulus is large, it is essential to calculate the variance from the amplitudes of the EPP's only after they have been individually corrected for the effects of the non-linear summation of unit responses. The signal to noise ratio was such that it was considered to be unnecessary to subtract the variance of the noise from the variance calculated from the corrected EPP amplitudes.

$$m_2 = \frac{\bar{v}^2}{V} \qquad \text{.................(7)}$$

where v^2 = square of corrected average EPP amplitudes.

The variance of the individually corrected EPP amplitudes is given by:-

$$V = \frac{\sum (v - \bar{v})^2}{N - 1} \qquad \text{......................(8)}$$

In the headings of the various tables presented in the results sections of the following chapters mnemonics are used in place of the appropriate equations.

QUANTAL CONTENT AS ESTIMATED FROM	MNEMONIC
m_0	$\ln \frac{NEPP}{NF}$
m_1	$\frac{AVEPP}{AVMIN}$
m_2	$\frac{AVEPP^2}{VAREPP}$

An estimate of the normal quantal content released per nerve stimulus in the frog sartorius muscle preparation is available from the work of MARTIN, (1955). From measurements of the coefficient of variation of EPP amplitudes recorded from six curarised muscle fibres in Ringer solution of normal ionic composition MARTIN reported values of m for the six fibres as follows:- 95 ± 10 (standard error of the mean), 240 ± 15, 107 ± 64 ± 8, 80 ± 9 and 106 ± 10. Another estimate of between 90 and 150 was arrived at from the ratio of the endplate current to the miniature endplate current in the voltage clamped frog sartorius fibres by TAKEUCHI & TAKEUCHI, (1960).

CHAPTER 7

ELECTROPHYSIOLOGICAL RECORDING TECHNIQUES

ELECTROPHYSIOLOGICAL RECORDING TECHNIQUES

PREPARATION:-

The sartorius muscle of the frog (Rana pipiens) together with a suitable length of the sciatic nerve was used. The preparation was mounted, with the inner surface uppermost, in a "perspex" bath and was secured at one end by two threads attached to the tendon at the tibial end of the muscle. The other end of the muscle was secured by two additional threads attached to the connective tissue which had previously secured the pelvic end of the sartorius muscle to the acetabulum. Care was taken to ensure that no muscle fibres were damaged either during the dissection or when securing the attachment threads. All experiments were carried out within an ambient temperature range of 19° to 22° centigrade.

RECORDING EQUIPMENT:-

Conventional intracellular recording arrangements were used (FATT & KATZ, 1951), however the impedance matching function of the "cathode follower" used by these authors was achieved using operational amplifier techniques. The necessary very high input impedance of approximately 500 megohms was achieved using an operational amplifier (Computing Techniques type E78) equipped with field-effect input transistors. This operational amplifier was connected in a source follower configuration and was analagous in function and effect to the cathode follower employed by FATT & KATZ. The input current drawn by the source follower was determined by measuring the voltage drop due to this current flowing through a screened, close tolerance resistance, of 500 megohms connected between the device

input and the ground return. The input current measured was 1.3 x 10^{-14} amperes.

Endplate potentials recorded intracellularly have a rapid rising phase, with a rise time from onset to peak of less than a millisecond, consequently there will be a number of components of their frequency spectrum extending up to approximately ten kilohertz. Therefore in order to avoid distortions of the wave shape these higher frequency components of the input signals must be preserved. The resistance of the microelectrodes used was in the range 10 to 14 megohms, this resistance in conjunction with the input capacitance of the source follower and the stray capacitance of the microelectrode to ground form, in effect, a low-pass filter. This low-pass filter, which is situated between the signal source and the input of the source follower, will attenuate the higher frequency components of any signal recorded by the microelectrode. In order to ensure that the -3 db point of this low-pass filter is kept as high as possible so as to avoid introducing distortions into the recorded endplate potentials the input capacitance must be kept as low as possible. This is achieved in two main ways:-

(1) By the technique of "input guarding" whereby a metal shield surrounding the body of the microelectrode is connected to a proportion of the in-phase signal derived from the low impedance output of the source follower, a technique introduced by NASTUK & HODGKIN in 1950.

(2) By avoiding inserting the shank of the microelectrode to a depth of more than 2 or 3 mm below the surface of the Ringer solution. Although virtually all of the resistive component of the microelectrode

is located within 1 mm of its tip; the capacitance to ground depends largely on just how far into the Ringer solution the microelectrode tip is inserted.

The bath electrode was a chloride coated silver wire and was connected via a DC voltage calibrator to the ground return point of the dual source follower, the output of which was displayed, directly coupled, on an oscilloscope (Tektronix 5000 series D12 display unit with 5A22N vertical amplifier & 5B12 time base). The membrane potential was measured by inserting a "backing off" potential from the DC calibrator connected in series with the bath electrode, to return the base line displayed on the CRT to a chosen reference point. EPP traces were photographed from the CRT with a 35 mm oscilloscope camera (Cossor). MEPP's were recorded on moving 35 mm film from another oscilloscope (Tektronix 502) whose time base was free running. The signal to noise ratio of the recording system was optimised by limiting the bandwidth of the amplifier, the high frequency filter being set so that the response was 3 db down at 10 KhZ.

STIMULATION:-

Preparations were stimulated electrically by applying depolarising rectangular pulses (0.05 Milliseconds duration & 4.0 volts amplitude) to the sciatic nerve via a capillary electrode (FURSHPAN & POTTER, 1959) from an isolated stimulator triggered from a DEVICES DIGITIMER. In experiments where the quantal release of transmitter was deliberately reduced by the use of magnesium the preparation was stimulated at a rate of one stimulus per 2.5 seconds, equivalent to a stimulus frequency of 0.4 HZ. However in curare blocked preparations, where the quantal content per stimulus is very large, a

much lower stimulation rate of one stimulus per 10 seconds was adopted in order to avoid the effects of fatigue (B.L. GINSBORG, Personal communication).

PERFUSION SYSTEM:-

The preparation was mounted in a recording chamber, of internal dimensions:- length 7.0 cm, width 0.9 cm and 1.2 cm deep, milled out in a perspex block. A bridge, in the form of an outer segment cut from a cylindrical section of perspex 0.9 cm thick and 100 cm in diameter, was free to move 1.0 cm along the length of the chamber and this was positioned beneath the preparation directly under the recording site. In this manner the immersed length of the electrode tip was kept to less than 3.0 mm thereby minimising the capacitance of the electrode tip region to ground.

Solutions entered the recording chamber at the end at which the tibial region of the muscle was mounted and after flowing smoothly over the muscle were then drawn off from the surface by means of a curved glass tube connected to a vacuum pump. The preparation was screened from both the fluid inlet and outlet by means of nylon filters (obtained from disposable blood-transfusion sets); these served the dual function of smoothing the flow of perfusate across the preparation and preventing the possible disturbance or dislodgement of the electrode by bubbles of air which had become trapped in the perfusing tube circuit during the course of changing solutions. Ringer solutions were perfused through the chamber at a constant rate of flow of 2.0 ml per minute by means of a Watson Marlow roller pump. The effective volume of the recording chamber was 4.0 ml.

In order to minimise interference from the 50 HZ AC mains supply it was necessary to electrically isolate the recording chamber fluid

from both the inlet and outlet. Interruption of the conductive path provided by the ions of the Ringer solution was achieved by inserting a 4 cm air gap in both inlet and outlet connections (figure 14).

MICROELECTRODES:-

Microelectrodes were pulled vertically on an apparatus similar to that described by FRANK & FOURTES (1955). The Pyrex glass tubing used contained a glass microcapillary bonded to the wall of the lumen of the tube which formed the barrel of the microelectrode (2.0 mm o.d. & 1.0 mm i.d. supplied by Clark Electromedical). Microelectrodes used for recording were filled with 3 molar KCl introduced into the shank of the electrode by means of a long thin hypodermic needle and syringe. Trapped air bubbles were removed by vigorous tapping, the electrode tip filling by capillary action aided by the integral glass microfilament. Microelectrodes used for passing current were similarly filled, however 3 molar potassium acetate was used in preference to KCl.

Before use the electrical resistance of the microelectrode was measured, the method used in illustrated schematically in figure 13. Voltage measurements were made using an electrometer with a very high input impedance (Vibron model 33 B-2). The tip of the electrode was immersed in normal saline; a source of EMF of 100 mV was then connected in series and the potential registered on the electrometer was recorded and designated V1. A shunt resistance RC of 10 megohms was brought into circuit by closing SW1 to form, in conjunction with the microelectrode resistance RE, a potential divider across the 100 mV source E. The voltage thus measured at the junction of RE and RC being designated V2. The microelectrode resistance RE was then

FIGURE 12

SCHEMATIC DIAGRAM OF THE MICROELECTRODE RECORDING ARRANGEMENTS

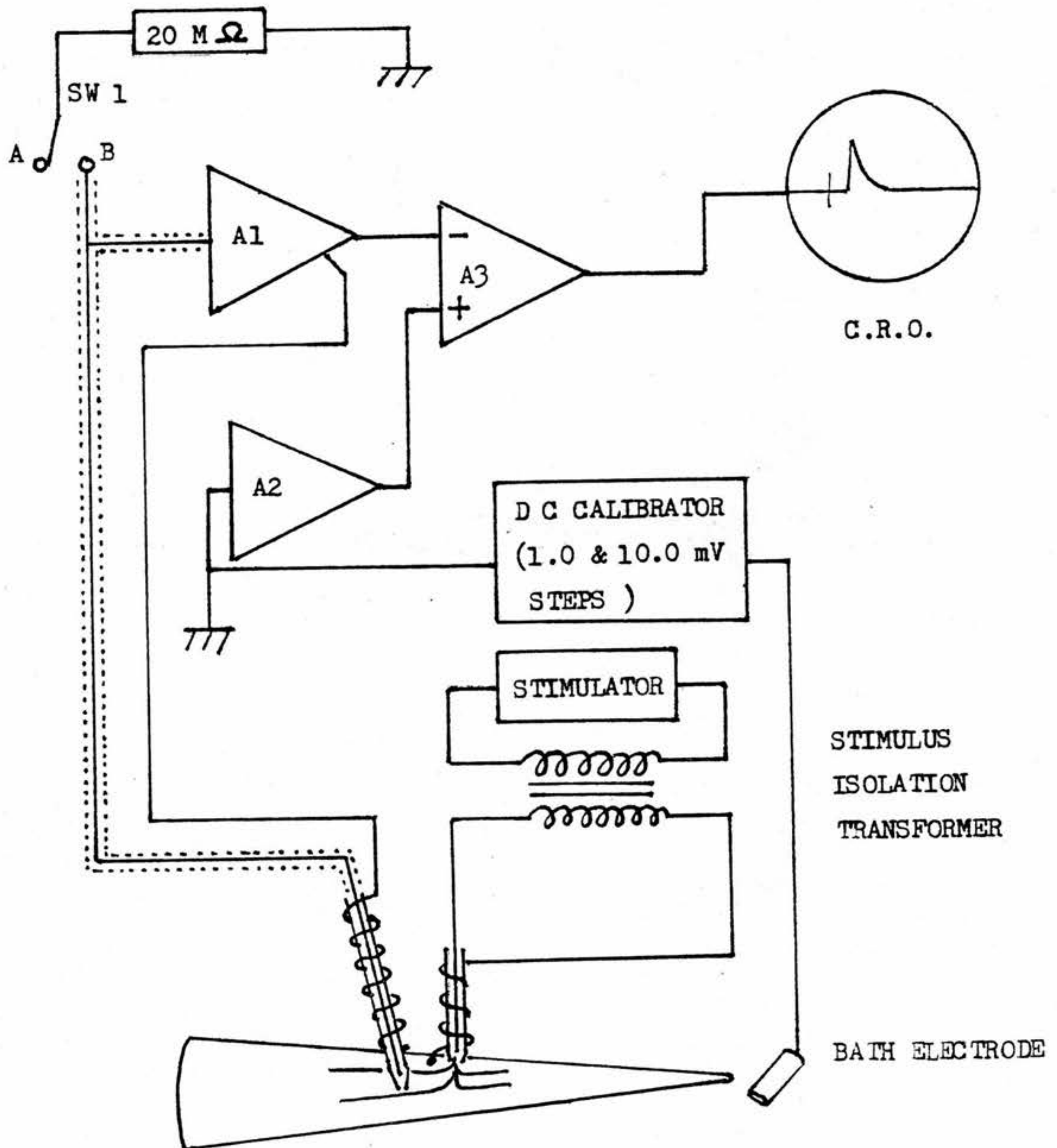


FIGURE 13

SCHEMATIC DIAGRAM OF THE MICROELECTRODE RESISTANCE TEST CONFIGURATION

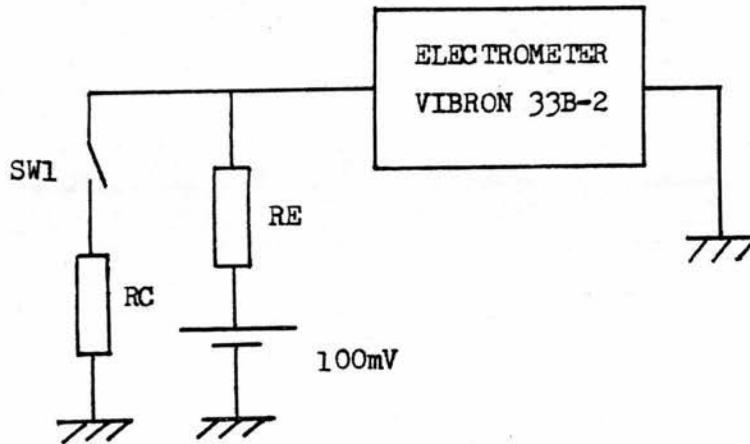
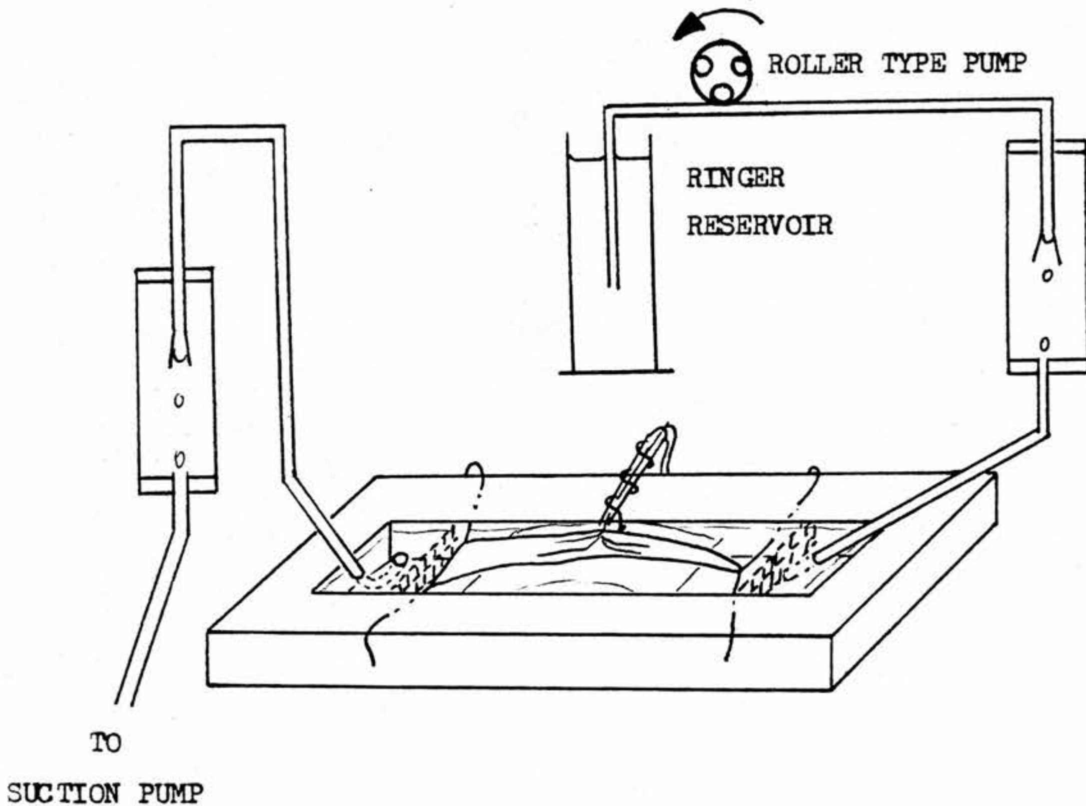


FIGURE 14

PERFUSION SYSTEM AND DETAIL OF THE METHOD OF DC ISOLATION OF THE BATH SOLUTION



calculated from the relationship:-

$$RE = 10 \times \frac{V1 - V2}{V2} \text{ megohms}$$

In a similar manner the microelectrode resistance in situ with a cell could be determined by applying a square-wave voltage pulse between the bath electrode and earth potential and observing the change in potential V1 before, and V2 after the input to the source follower was shunted to earth potential via a 20 megohm resistance.

For recording purposes microelectrodes with resistances between 9 & 12 megohms were used. Microelectrodes with resistances within the range 8 to 10 megohms were used to pass current through the cell membrane (see chapter 12).

SOLUTIONS:-

Even when low concentrations of tetracyclines were present they caused a reduction in the pH of unbuffered Ringer solutions. Tetracycline hydrochloride at a concentration of 10^{-5} M caused the pH to decrease to approximately 6.0 whilst a concentration of 5×10^{-4} M caused the pH to decrease to approximately 4.0. Consequently it was necessary to use a buffering agent to hold the pH constant at some selected value within the physiological range. In selecting a suitable buffering agent it was necessary to reject any such agent which by itself may have some effect either on the quantal content of evoked transmitter release or on the concentration of ionised calcium present in the Ringer solution. The use of an isotonic phosphate buffer was rejected on the grounds that chelation with calcium can

occur to produce calcium phosphate and thus reducing the amount of free calcium available in the Ringer solution. Similarly the use of TRIS buffer was rejected on the grounds that TRIS has been suspected of exerting a slight curare like effect (B.L. GINSBORG, personal communication). The buffering agent HEPES (British Drug Houses) was selected following a series of experiments as described in the next chapter.

CHAPTER 8

THE EFFECTS OF HEPES BUFFERED RINGER ON QUANTAL CONTENT

THE EFFECTS OF HEPES BUFFERED RINGER ON QUANTAL CONTENT

INTRODUCTION :- It was found that even with quite low concentrations of tetracyclines the pH of the Ringer solution decreased, showing that these antibiotics produced a marked increase in acidity when introduced into unbuffered Ringer solution. Consequently it was necessary to use a buffer system in order to maintain the pH of the solution within the physiological range. It was essential to ensure that the buffering agent used did not produce a reduction in quantal content nor affect the calcium ion concentration.

METHODS :- The normal methods of electrophysiological recording were used as described in detail in the previous section. A series of experiments were conducted in which the quantal content was estimated at a particular endplate, firstly in unbuffered Ringer solution containing calcium ions (0.9 mM) and magnesium ions at a specific concentration within the range 8.0 to 14.0 mM. Then, with the microelectrode still in situ in the fibre, the bathing solution was changed to a Ringer solution otherwise identical to the previous solution but now containing HEPES buffer at a concentration of 3.0 mM, the pH of the solution was adjusted to 7.0 with N / 10 NaOH. After an equilibrating period of 20 minutes a second series of recordings of EPP's and MEPP's were made and from these an estimate of the quantal content of evoked release in the presence of the buffering agent HEPES was obtained.

TABLE 5

THE EFFECTS OF HEPES BUFFERED RINGER ON QUANTAL CONTENT

EXPT.	AVEPP / AVMIN	NEPP	$AVEPP^2$ / VAREPP	FMIN (HZ)	AVEPP (MV)	AVMIN (MV)	RP (MV)
1 R	2.76	2.73	2.82	2.4	1.85	0.67	93
1 R+H	2.76	2.72	2.74	2.5	1.87	0.68	91
2 R	1.92	1.88	1.07	1.2	1.03	0.54	87
2 R+H	1.91	1.85	1.41	1.1	1.02	0.53	85
3 R	1.90	1.80	2.49	3.5	0.50	0.26	96
3 R+H	1.91	1.73	2.27	3.2	0.51	0.27	94
4 R	2.08	2.11	2.24	0.8	0.75	0.36	89
4 R+H	2.07	2.39	2.36	1.0	0.76	0.37	90
5 R	4.40		4.44	4.3	1.41	0.32	91
5 R+H	4.13		4.56	4.5	1.24	0.30	89
6 R	4.36		4.23	1.9	2.09	0.48	94
6 R+H	4.32		4.03	1.7	2.11	0.49	93

R = Unbuffered Ringer solution Ca = 0.9 mM, Mg in the range 8.0 to 14.0 mM.

R+H = HEPES buffered Ringer solution of identical calcium and magnesium ion concentrations to the unbuffered Ringer.

In all six experiments only one fibre in each of the six separate preparations was used. In each case the calcium ion concentration was maintained steady at 0.9 mM, however in order to obtain a range of values of quantal content the magnesium ion concentration for each of the individual experiments was selected from the range 8.0 to 14.0 mM. A series of approximately 160 EPP's were recorded from each endplate before the introduction of the HEPES buffered Ringer when following an equilibrating period of 20 minutes a further 160 EPP's were recorded and the results presented in table 5.

STATISTICS :- Student's t test was used to analyse data. P values < 0.05 were considered to be statistically significant.

RESULTS :- Spontaneous transmitter release was unaffected by the presence of HEPES buffer in the Ringer solution. In each of the six preparations tested, no statistically significant differences were found between the average MEPP amplitude recorded in unbuffered Ringer when compared with the average MEPP amplitude recorded from the same endplate in the presence of HEPES buffered Ringer. Similarly MEPP frequency was unaffected. There was no significant change in the membrane potential under similar conditions.

CONCLUSION :- There are no reports in the literature regarding the binding of calcium ions or magnesium ions by HEPES. From the results of the control experiments it was concluded that HEPES fulfilled the necessary prerequisites of a buffering agent suitable for use in experiments where changes in quantal content are the subject of the investigation.

CHAPTER 9

THE EFFECT OF TETRACYCLINES UPON QUANTAL CONTENT IN THE MAGNESIUM BLOCKED NEUROMUSCULAR JUNCTION OF THE FROG SARTORIUS PREPARATION

THE EFFECT OF TETRACYCLINES UPON QUANTAL CONTENT IN THE MAGNESIUM BLOCKED NEUROMUSCULAR JUNCTION OF THE FROG SARTORIUS PREPARATION.

INTRODUCTION:- The effects of tetracyclines upon miniature endplate potential amplitude and frequency and on the quantal content of evoked release in the magnesium blocked neuromuscular junction of the frog sciatic nerve sartorius muscle preparation were investigated.

METHODS:- Frog sciatic nerve sartorius muscle preparations (Rana pipiens) were set up for electrophysiological recording as previously described in the section dealing with electrophysiological recording techniques. HEPES buffered Ringer solution (pH = 7.0) containing calcium ions at a concentration of either 0.9 mM or 1.8 mM and also magnesium ions at a specific concentration within the range 5.0 mM to 14.0 mM was perfused through the recording chamber. An endplate was located and a series of 120 stimuli were applied at a rate of one pulse per five seconds. If the MEPP frequency appeared to be low a further series of sweeps were recorded at a lower time-base velocity to ensure that an adequate sample size of MEPP's was obtained. Immediately following this initial series of recordings Ringer solution of the same initial composition as that previously used but now containing one of the tetracycline group of antibiotics at a concentration of 5.0×10^{-4} M (pH = 7.0) was perfused through the recording chamber. Further series of recordings were taken over 10 minute intervals commencing at 10 and 30 minutes following the introduction of the antibiotic into the chamber.

TABLE 6

TETRACYCLINE HCl $5x10^{-4}$M, Ca = 0.9mM, Mg = 5.0mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.69	5.90	8.56		9.04	96	4.3
2	0.35	1.35	3.86		3.90	88	4.5
3	0.33	1.26	3.82		3.93	83	4.2
4	0.56	2.39	4.27		4.44	81	4.4

1 = Recordings taken over a 10 minute period prior to the introduction of tetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of tetracycline HCl.

3 = Recordings taken from 30 to 40 minutes after the introduction of tetracycline HCl.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the tetracycline HCl had commenced.

TABLE 7

TETRACYCLINE HCl $5x10^{-4}$M, Ca = 0.9mM, Mg = 5.0mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.42	3.43	8.08		8.29	92	1.7
2	0.23	0.85	3.74	3.57	3.73	86	1.6
3	0.21	0.75	3.59	3.62	3.68	78	1.7
4	0.34	1.43	4.21		4.47	76	1.9

1 = Recordings taken over a 10 minute period prior to the introduction of tetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of tetracycline HCl.

3 = Recordings taken from 30 to 40 minutes after the introduction of tetracycline HCl.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the tetracycline HCl had commenced.

TABLE 8

TETRACYCLINE HCl 5×10^{-4}M, Ca =1.8mM, Mg =10^{-3}M, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.34	5.58	16.41		17.28	96	9.5
2	0.23	2.69	11.69		12.14	90	9.8
3	0.22	2.50	11.36		12.07	88	9.3
4	0.23	2.62	11.39		12.56	85	9.8

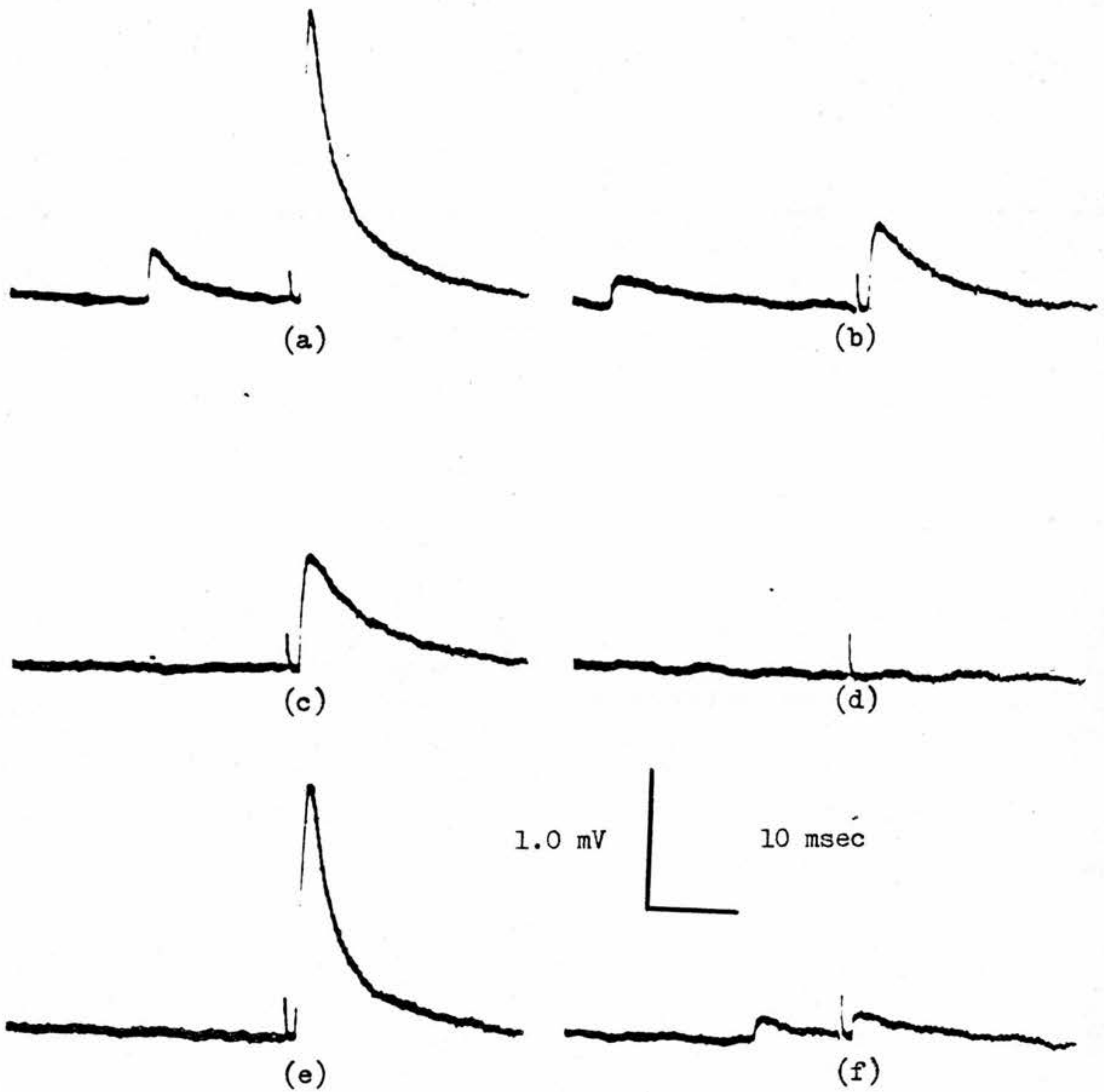
1 = Recordings taken over a 10 minute period prior to the introduction of tetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of tetracycline HCl.

3 = Recordings taken from 30 to 40 minutes after the introduction of tetracycline HCl.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the tetracycline HCl had commenced.

FIGURE 15



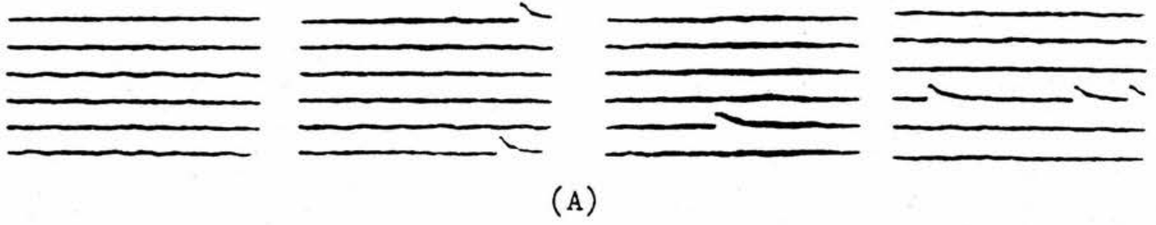
TETRACYCLINE HCl $5x10^{-4}M$, $[Ca^{2+}]$ = 0.9mM, $[Mg^{2+}]$ = 5.0mM, pH = 7.0

(a) & (b) **EPP's** and **MEPP's** recorded in Ringer solution prior to the introduction of TETRACYCLINE HCl

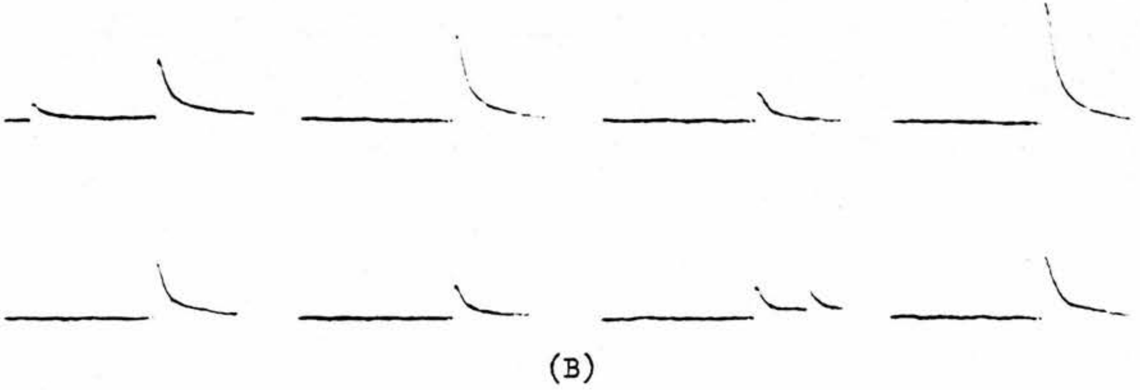
(c) & (d) 15 minutes in Ringer solution containing TETRACYCLINE

(e) & (f) 30 " " " " "

FIGURE 16

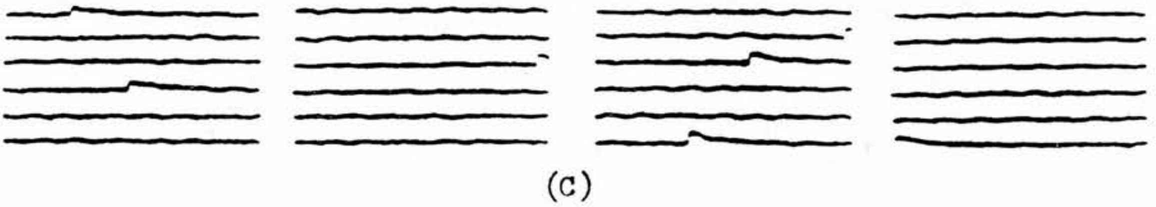


(A)

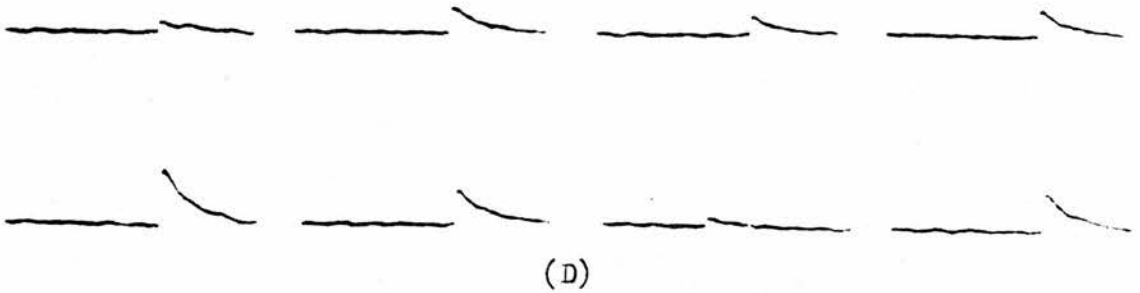


(B)

I = 1.0 millivolt calibration. —— 10 msec.



(C)



(D)

(A) & (B) Controls recorded in HEPES buffered Ringer $[Ca^{2+}] = 1.8$mM; $[Mg^{2+}] = 12.0$mM.

(C) & (D) **EPP's** & **MEPP's** Recorded after a period of 30 minutes in TETRACYCLINE HCl 5×10^{-4}M.

TABLE 9

MINOCYCLINE $5x10^{-4}$M, $[Ca^{++}]$ = 1.8mM, $[Mg^{++}]$ = 12.0mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.55	0.66	1.19	1.10	1.22	90	2.8
2	0.27	0.28	1.04	1.10	1.09	84	7.4
3	0.13	0.10	0.80	0.83	0.83	79	16.4
4	0.51	0.49	0.97	0.99	1.02	85	6.9

1 = Recordings taken over a 10 minute period prior to the introduction of minocycline.

2 = Recordings taken from 10 to 20 minutes after the introduction of minocycline.

3 = Recordings taken from 30 to 40 minutes after the introduction of minocycline.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the minocycline had commenced.

TABLE 10

MINOCYCLINE $5x10^{-4}M$, $[Ca^{++}] = 1.8mM$, $[Mg^{++}] = 12.0mM$, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.58	3.28	5.62		5.80	92	7.1
2	0.21	0.39	1.86	1.85	1.93	82	20.9
3	0.17	0.30	1.80	1.76	1.88	80	28.4
4	0.48	1.92	3.97		4.63	89	13.5

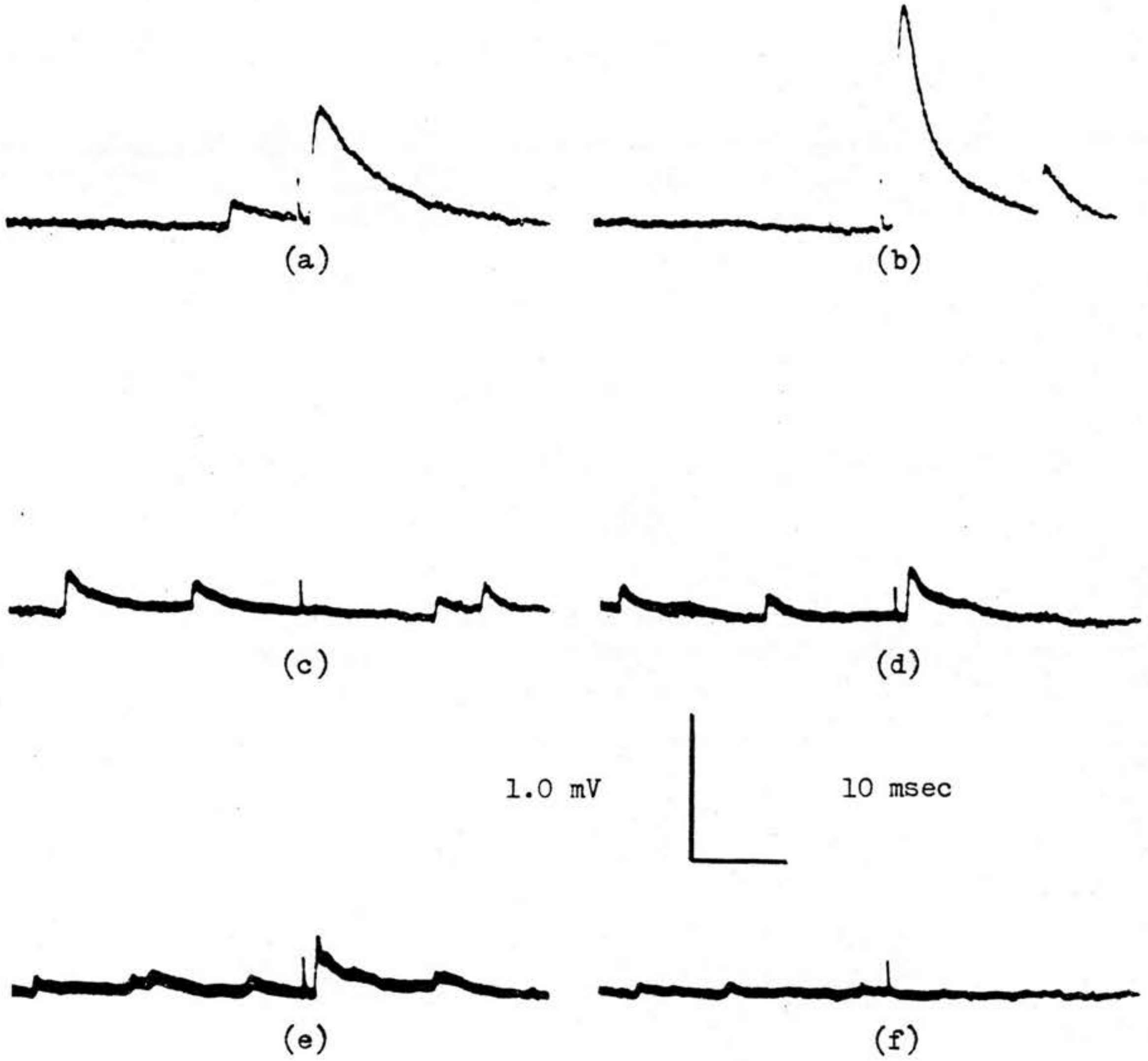
1 = Recordings taken over a 10 minute period prior to the introduction of minocycline.

2 = Recordings taken from 10 to 20 minutes after the introduction of minocycline.

3 = Recordings taken from 30 to 40 minutes after the introduction of minocycline.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the minocycline had commenced.

FIGURE 17



MINOCYCLINE HCl 5×10^{-4}M, $[Ca^{2+}]$ = 1.8mM, $[Mg^{2+}]$ = 12.0mM, pH = 7.0

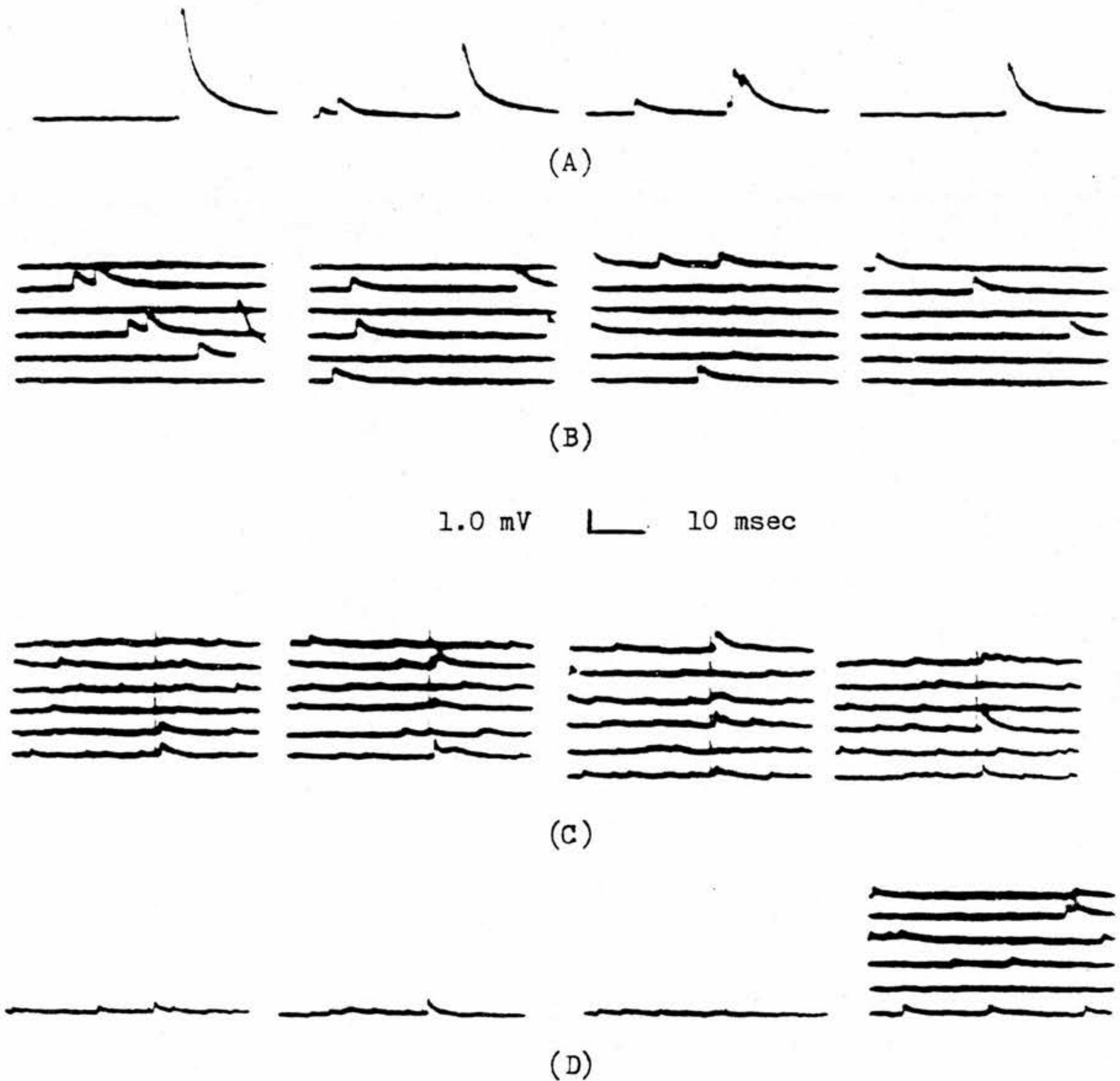
(a) & (b) EPP's and MEPP's recorded in Ringer solution prior to the introduction of MINOCYCLINE

(c) & (d) 15 minutes in Ringer solution containing MINOCYCLINE

(e) & (f) 30 " " " " "

FIGURE 18

MINOCYCLINE



(A) & (B) Controls recorded in HEPES buffered Ringer $[Ca^{2+}] = 1.8mM$; $[Mg^{2+}] = 12.0mM$.

(C) & (D) EPP's & MEPP's Recorded after a period of 30 minutes in MINOCYCLINE $5x10^{-4}M$.

N.B. The amplifier gain remained unaltered at 1.0 millivolts between successive sweeps on the composite traces.

TABLE 11

CHLORTETRACYCLINE HCl 5×10^{-4}M, Ca = 1.8mM, Mg = 12.0mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.46	5.70	12.28		14.12	94	2.8
2	0.28	2.62	9.42		10.31	90	2.6
3	0.20	1.43	7.31		8.63	87	2.3
4	0.12	0.19	1.63	1.66	1.66	85	2.5

1 = Recordings taken over a 10 minute period prior to the introduction of chlortetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of chlortetracycline HCl.

3 = Recordings taken from 30 to 40 minutes after the introduction of chlortetracycline HCl.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the chlortetracycline HCl had commenced.

TABLE 12

CHLORTETRACYCLINE HCl $5x10^{-4}$M, Ca = 1.8mM, Mg = 12.0mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.44	7.07	16.07		16.00	90	3.6
2	0.34	2.33	6.85		6.78	82	3.7
3	0.24	0.46	1.92	1.93	1.96	73	3.6
4	0.38	2.49	6.56		6.88	72	3.9

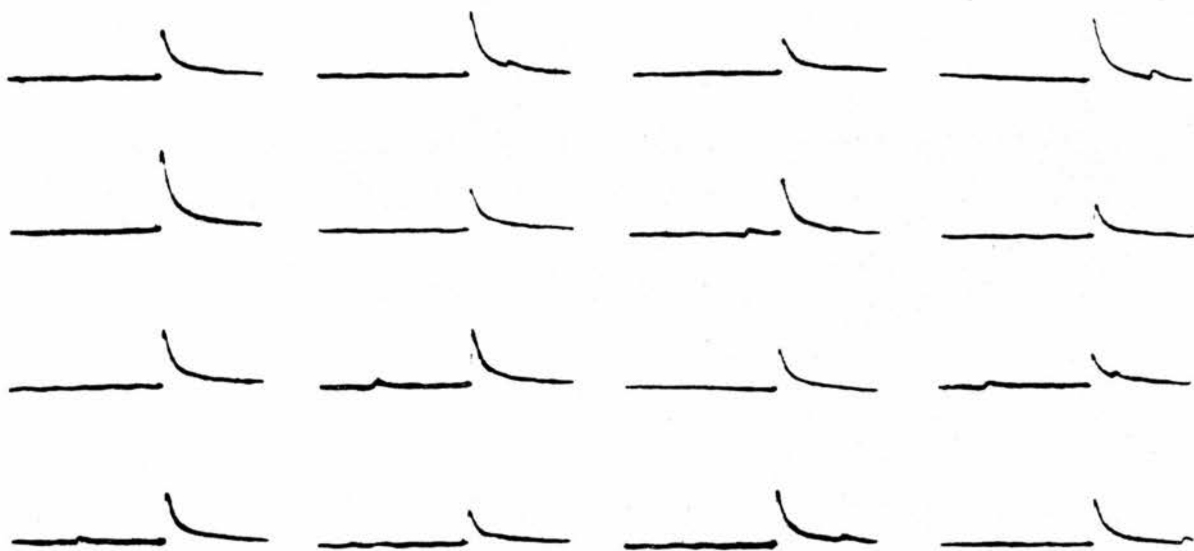
1 = Recordings taken over a 10 minute period prior to the introduction of chlortetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of chlortetracycline.

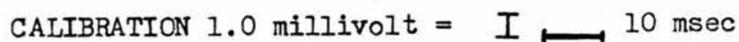
3 = Rrecordings taken from 30 to 40 minutes after the introduction of chlortetracycline HCl.

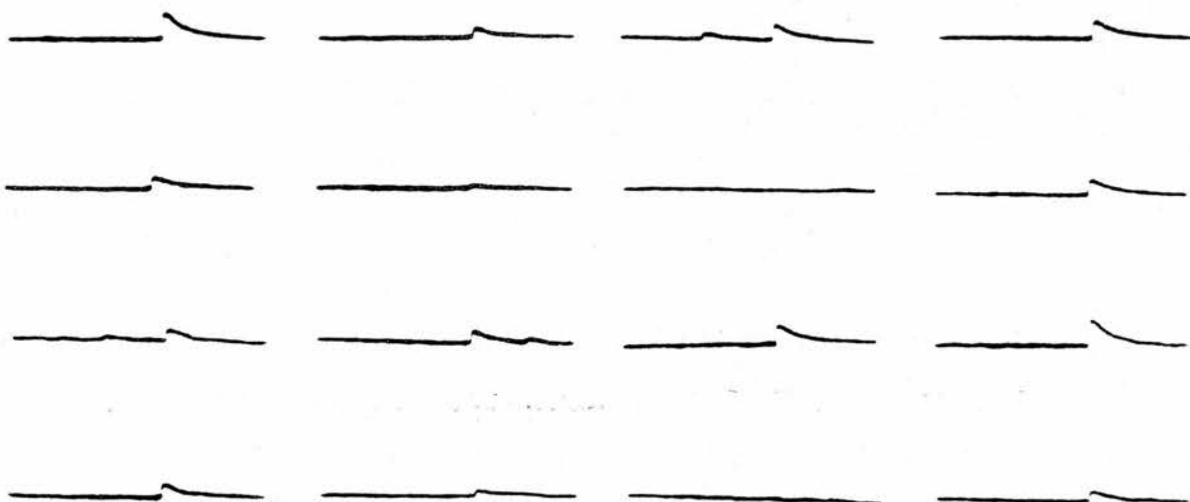
4 = Recordings taken from 30 to 40 minutes after the wash-out of the chlortetracycline HCl had commenced.

FIGURE 19



EPP's & MEPP's Recorded in HEPES buffered Ringer
Ca^{2+} = 1.8mM; Mg^{2+} = 12.0mM; pH = 7.0.





EPP's & MEPP's Recorded after a period of 30
minutes in HEPES Ringer & CHLORTETRACYCLINE HCL $5x10^{-4}$M.

TABLE 13

OXYTETRACYCLINE HCl 5×10^{-4}M, Ca = 1.8 mM, Mg = 12.0 mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln\frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.47	6.03	12.83		13.50	92	4.2
2	0.33	2.97	9.00		9.48	85	4.0
3	0.30	2.30	7.67		7.73	81	4.2
4	0.43	4.87	11.32		11.52	84	4.4

1 = Recordings taken over a 10 minute period prior to the introduction of oxytetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of Oxytetracycline.

3 = Recordings taken from 30 to 40 minutes after the introduction of oxytetracycline HCl.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the oxytetracycline HCl had commenced.

TABLE 14

OXYTETRACYCLINE HCl $5x10^{-4}$M, Ca = 0.9 mM, Mg = 5.0 mM, pH = 7.0

X	AVMIN (mV)	AVEPP (mV)	$\frac{AVEPP}{AVMIN}$	$\ln \frac{NEPP}{NF}$	$\frac{(AVEPP)^2}{VAREPP}$	RP (mV)	MEPP F (HZ)
1	0.51	4.28	8.39		8.62	90	3.6
2	0.37	2.29	6.20		6.31	84	3.0
3	0.33	1.88	5.70		5.90	80	3.2
4	0.45	3.47	7.72		8.02	83	3.2

1 = Recordings taken over a 10 minute period prior to the introduction of oxytetracycline HCl.

2 = Recordings taken from 10 to 20 minutes after the introduction of oxytetracycline.

3 = Recordings taken from 30 to 40 minutes after the introduction of oxytetracycline HCl.

4 = Recordings taken from 30 to 40 minutes after the wash-out of the oxytetracycline HCl had commenced.

DISCUSSION:- From the sample traces of EPP and MEPP recordings shown and data presented in tabular form it can be seen that tetracycline HCl, chlortetracycline HCl and oxytetracycline HCl do not significantly alter MEPP frequency. Minocycline, however, produces a large increase in MEPP frequency. All of these antibiotics produce both a decrease in MEPP amplitude and quantal content.

FATT & KATZ (1952) described the principal features of spontaneous release of acetylcholine as observed experimentally at the frog neuromuscular junction. They concluded that factors which alter MEPP frequency relate to the nerve terminal and the mechanisms involved in the release of acetylcholine; whereas MEPP amplitude is determined by postsynaptic factors. However it has subsequently been shown that MEPP amplitude can be decreased through a presynaptic mechanism by agents such as hemicholinium No. 3 (HC-3) and triethylcholine. HC-3 inhibits the transport of choline across the presynaptic nerve terminal membrane and, following a period of high frequency stimulation sufficient to exhaust the preformed transmitter store, results in a decrease in MEPP amplitude. MacINTOSH & COLLIER (1976) further consider that HC-3 may also exert an intracellular action by blocking the uptake of acetylcholine into vesicles after being transported across the membrane into the axoplasm of the presynaptic terminal.

MEPP frequency is controlled by the membrane potential of the presynaptic nerve endings. MEPP frequency can be increased by substances such as the calcium ionophore X-537A (KITA & van der KLOOT, 1974), cardiac glycosides (BIRKS & COHEN, 1968a,b), and certain components of venoms such as β-bungarotoxin from the venom of the Taiwan banded krait (CHANG & HUANG, 1974). Other factors which can influence the rate of spontaneous release include alterations in temperature, osmotic pressure and the degree of

stretch to which the preparation is subject. A delayed increase in MEPP frequency follows an increase in external potassium concentration; however none of these other factors are particularly relevant to the present experimental conditions and can therefore be discounted as being the reason for the substantial increase in MEPP frequency which occurs following the introduction of minocycline into the Ringer solution.

CONCLUSIONS:- From the results tabulated it can be seen that tetracycline HCl, chlortetracycline HCl, oxytetracycline HCl and minocycline all exert both a presynaptic and a postsynaptic effect. The postsynaptic effect is demonstrated by the reduction in MEPP amplitude. A presynaptic hemicholinium like mechanism affecting MEPP amplitude can be discounted on the grounds that with the quantal content of evoked release already substantially reduced by Mg^{++} ions, the acetylcholine released, either spontaneously or by evoked release, will be derived from the preformed releasable store of acetylcholine already available within the nerve terminals. The presynaptic effect of these antibiotics is demonstrated by the reduction in the quantal content of the evoked release and also in the case of minocycline the large increase in MEPP frequency.

CHAPTER 10

DETERMINING THE TIME-COURSE OF THE REDUCTION OF EPP AMPLITUDE DUE TO THE PRESENCE OF TETRACYCLINES IN THE PARTIALLY CURARISED FROG SARTORIUS PREPARATION IN RINGER SOLUTION OF INITIAL CALCIUM ION CONCENTRATIONS OF 1.8 mM OR GREATER

DETERMINING THE TIME-COURSE OF THE REDUCTION OF EPP AMPLITUDE DUE TO THE PRESENCE OF TETRACYCLINES IN THE PARTIALLY CURARISED FROG SARTORIUS PREPARATION IN RINGER SOLUTION OF INITIAL CALCIUM ION CONCENTRATIONS OF 1.8 mM OR GREATER

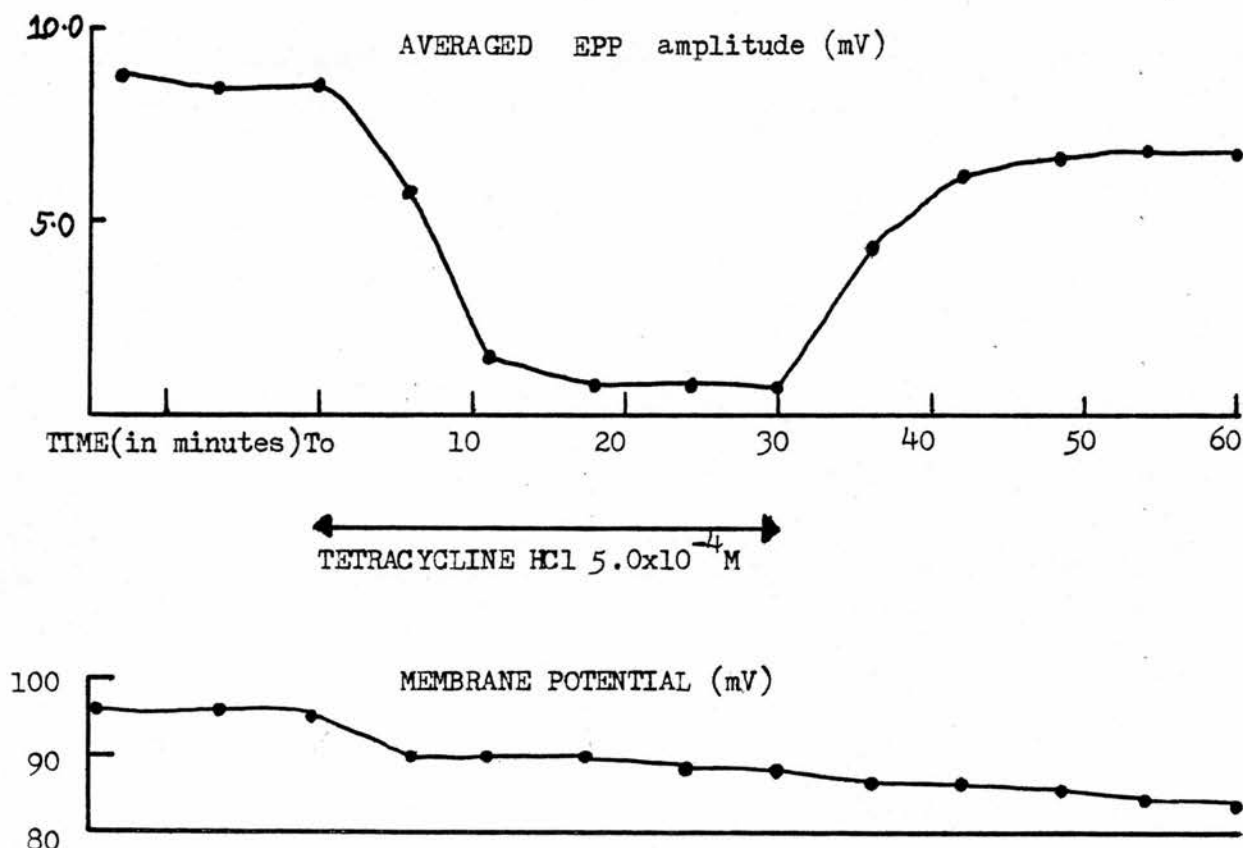
INTRODUCTION:- In order to avoid depletion of the store of acetylcholine available for release in the presynaptic terminal it is necessary to limit the rate of neural stimulation; consequently in these experiments a rate of one stimulus every ten seconds was adopted. This slow rate of stimulation, however, imposes certain limitations with regard to following the time-course of the neuromuscular blocking action of the tetracycline group of antibiotics.

Since tubocurarine was used to reduce the amplitude of the EPP's to a level such that the sartorius muscle will not twitch in response to stimulation of the sciatic nerve, MEPP's will not be visible. Similarly since no magnesium has been included in the Ringer solution, no "failures" will occur. Consequently the only remaining technique available whereby quantal content can be estimated is the "variance" method. In order to attain an acceptable degree of accuracy from the variance method it is necessary to apply it to quite long sequences of contiguous recordings of EPP's (the standard error of the mean of m being typically 20% when estimated from the amplitudes of only 50 MEPP's). In order to obtain accurate estimates of the reduction in EPP amplitude following the introduction of tetracycline into the Ringer solution 150 or more EPP's would have to be recorded. Since the stimulation rate has to be kept low, for the reasons previously given, a series of 150 recordings would take some 25 minutes to acquire, which is too long to be of any value in following the time-course of

the events under investigation. The method chosen therefore was to employ electronic signal averaging techniques to record, at six minute intervals, the average of 32 successive EPP's throughout the experiment. In this way the time-course of the reduction of EPP amplitudes can be monitored, however, for the reasons previously stated, the effect upon the quantal content of evoked release cannot be obtained directly.

METHODS:- Frog sciatic nerve sartorius muscle preparations were set up in the conventional manner for electrophysiological recording as described in a previous section. A stable level of partial neuromuscular blockade by tubocurarine was attained by ensuring that the preparation was curarised in two stages (NASTUK AND ALVING, 1959) in HEPES buffered Ringer solution (112.4 mM Na, 2.5 mM K^+, 117 mM Cl^-, 1.8 mM Ca^{2+}, 3.0 mM HEPES buffer, pH 7.0 d-tubocurarine 2.0 μM). After a period of 60 minutes in the above solution an endplate was located and a series of EPP's recorded. The criterion adopted for deciding on the adequacy of the location of the endplate was to accept recordings from a particular location only if the rise time of the EPP's was less than 2.0 milliseconds. In addition to being displayed on an oscilloscope the output of the source follower was connected to the input of an electronic signal averager (Biomac 2000), from the output of which the computed average of 32 successive EPP's were recorded on a hot wire chart recorder (Devices) at six minute intervals. In order to calibrate the signal averager traces a pulse of 1.0 milliseconds duration and either 1.0 or 5.0 millivolts amplitude, as appropriate, was added to each EPP sweep by introducing this pulse across a 10 ohm resistor inserted in the ground return of the voltage calibrator circuit.

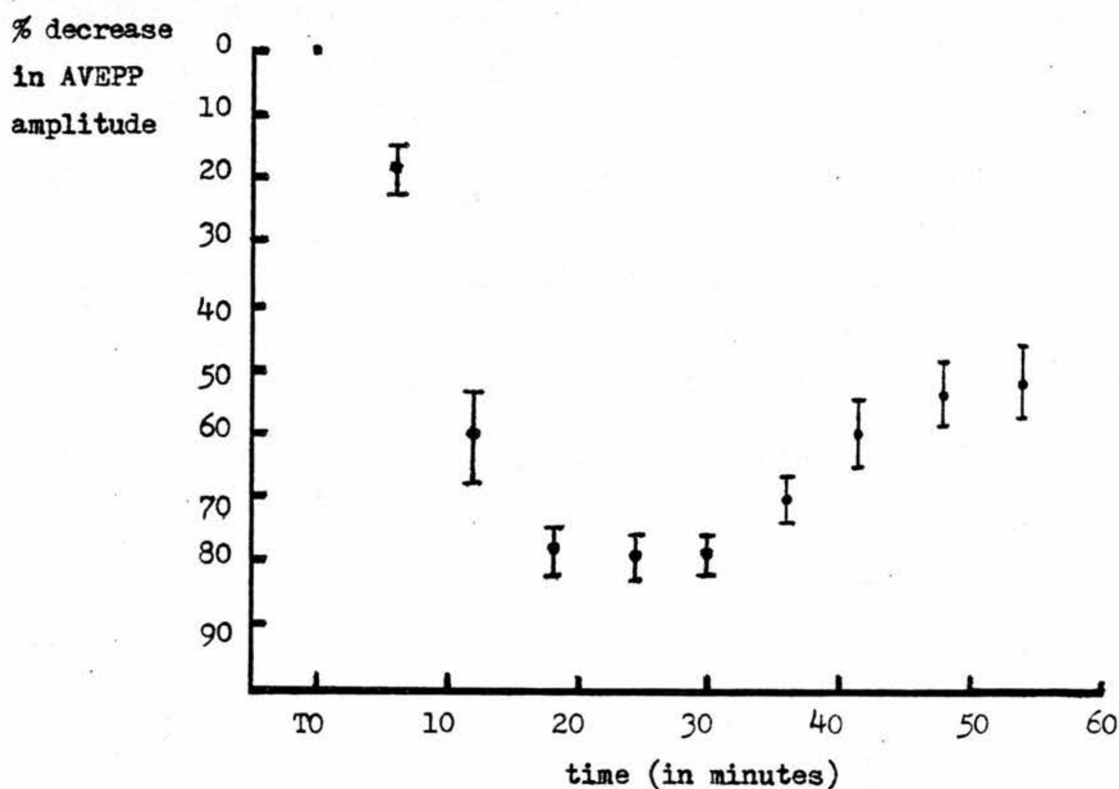
FIGURE 20



One individual experiment in the series in which the average amplitude of 32 successive EPP's was established at 6 minute intervals. Prior to To the preparation was bathed in HEPES buffered Ringer solution (pH = 7.0) containing calcium at a concentration of 1.8mM and tubocurarine at a concentration of 2.0×10^{-6}M, for the 30 minutes following To the Ringer solution contained Tetracycline HCl at a concentration of 1.0×10^{-4}M in addition to the tubocurarine; after 30 minutes the bathing solution composition was changed back to that used prior to To.

FIGURE 21

THE TIME-COURSE OF THE REDUCTION OF EPP AMPLITUDE DUE TO THE PRESENCE OF TETRACYCLINE HCl($5.0x10^{-4}$M) IN RINGER SOLUTION CONTAINING TUBOCURARINE .



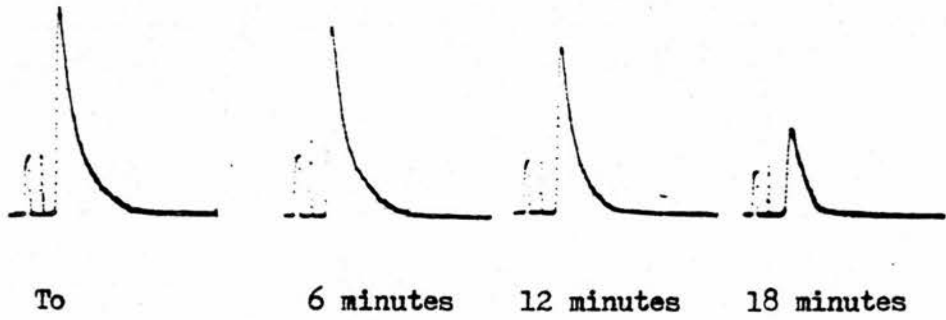
Ringer & tubocurarine

TETRACYCLINE HCl 5.0×10^{-4}M

The results of 5 individual experiments are shown, the error bars show the standard error of the mean (N=5). HEPES buffered Ringer solution (pH = 7.0), calcium ion concentration = 1.8mM, tubocurarine concentration = $2.0x10^{-6}$M.

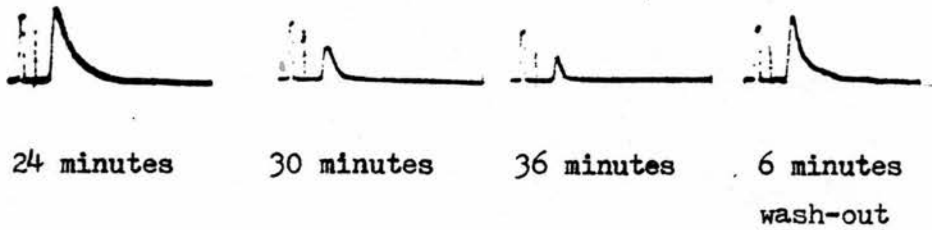
FIGURE 22

CHLORTETRACYCLINE HCl (5.0×10^{-4}M), $[Ca^{++}] = 1.8 \times 10^{-3}$M.



To | 6 minutes | 12 minutes | 18 minutes

5.0 mV calibration pulse — 5 msec.

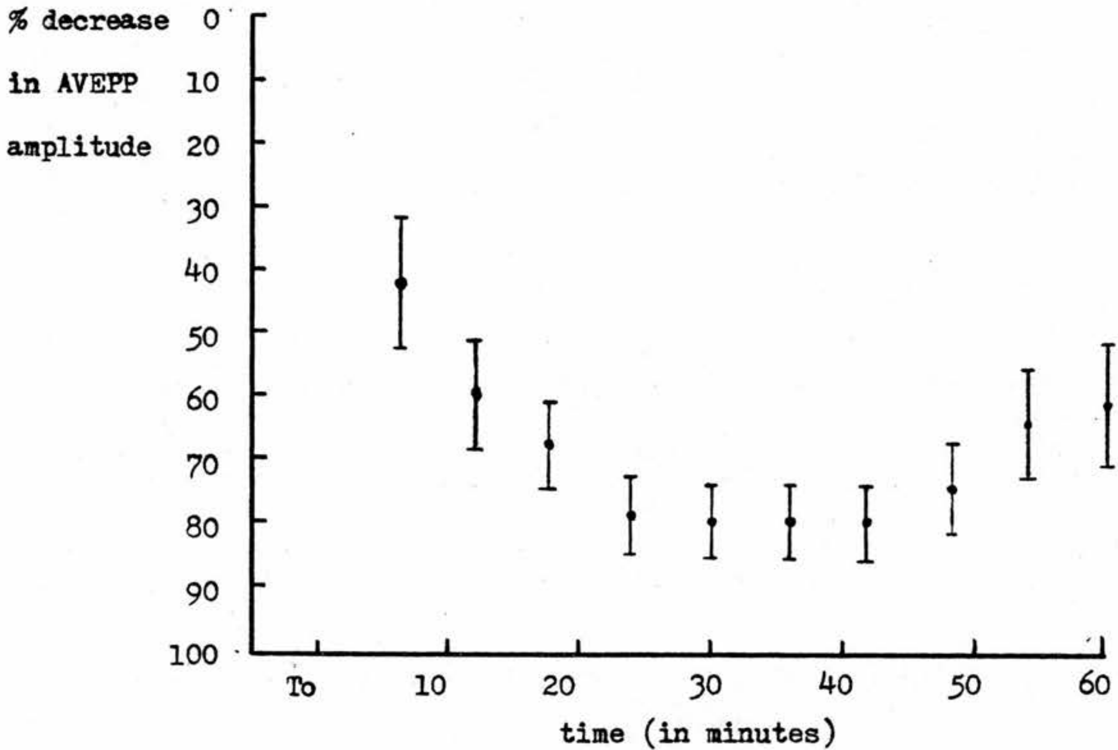


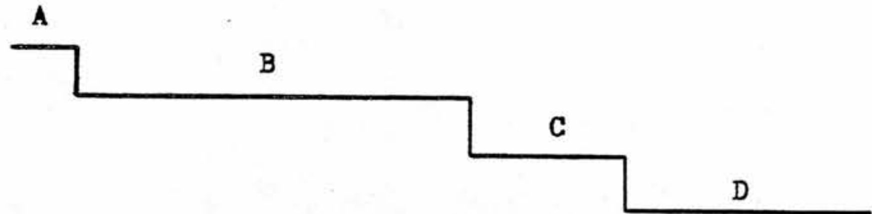
24 minutes | 30 minutes | 36 minutes | 6 minutes wash-out

TIME (minutes)	% decrease in average EPP amplitude.
To	
6	10.9
12	21.2
18	57.1
24	68.0
30	84.0
36	89.1

FIGURE 23

THE TIME-COURSE OF THE REDUCTION OF EPP AMPLITUDE DUE TO THE PRESENCE OF CHLORTETRACYCLINE HCl ($5.0x10^{-4}$M) IN RINGER SOLUTION CONTAINING TUBOCURARINE AND INCREASED CALCIUM CONCENTRATION.





A & D RINGER SOLUTION & TUBOCURARINE $[Ca^{++}] = 1.8x10^{-3}$M

B " " " " "

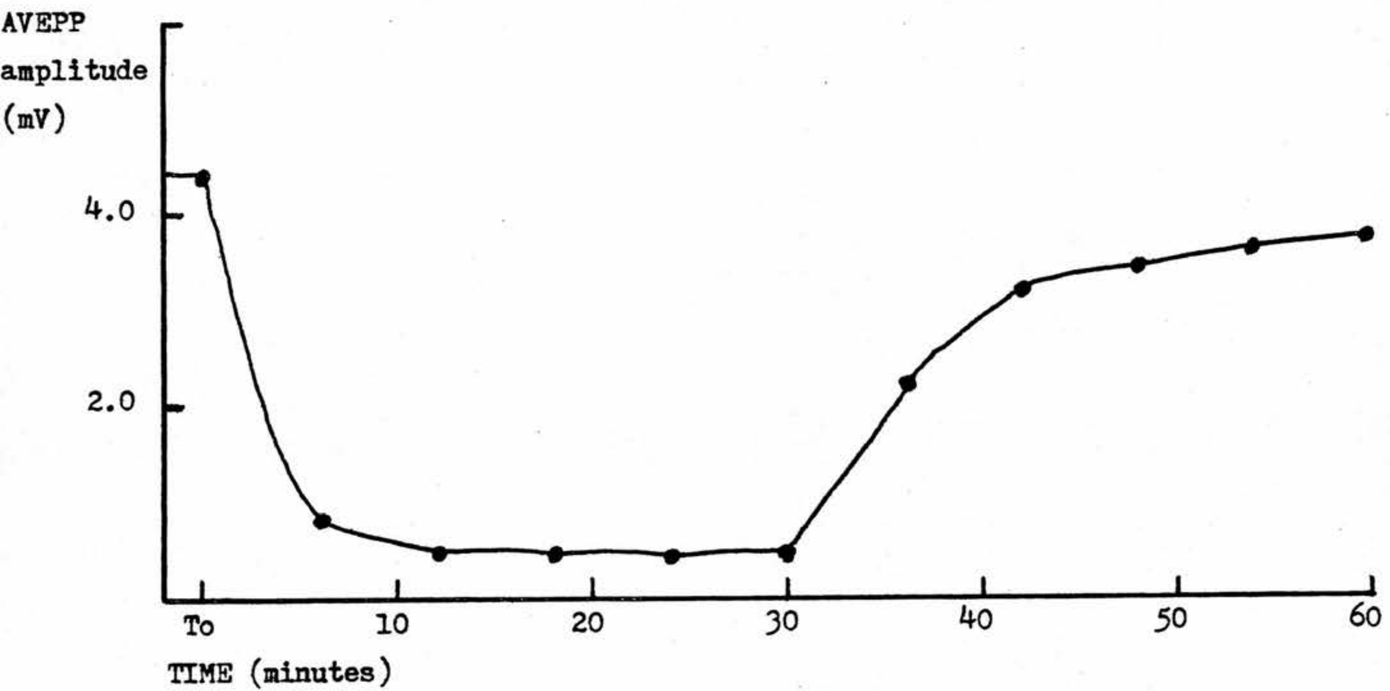
& CHLORTETRACYCLINE HCl $5.0x10^{-4}$M.

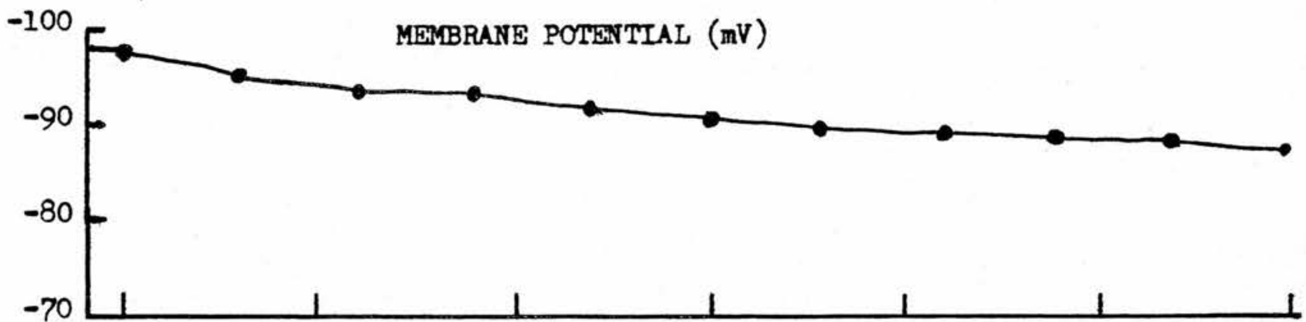
C SAME CONDITIONS AS "B" BUT WITH $[Ca^{++}]$ INCREASED TO $2.3x10^{-3}$M.

FIGURE 24

CHLORTETRACYCLINE HCl in normal and elevated calcium ion concentration.

[CHLORTETRACYCLINE HCl] = 5.0×10^{-4}M, [tubocurarine] = 2.0×10^{-6}M, pH=7.0





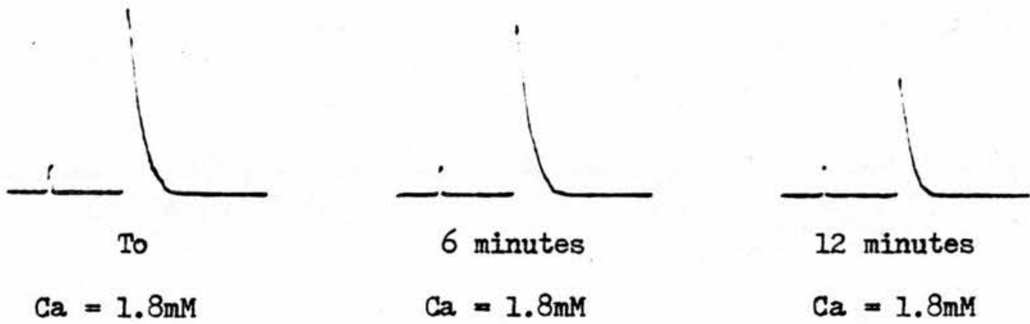
curarised Ringer [Ca^{++}] = 1.8mM

CHLORTETRACYCLINE [Ca^{++}] = 1.8mM

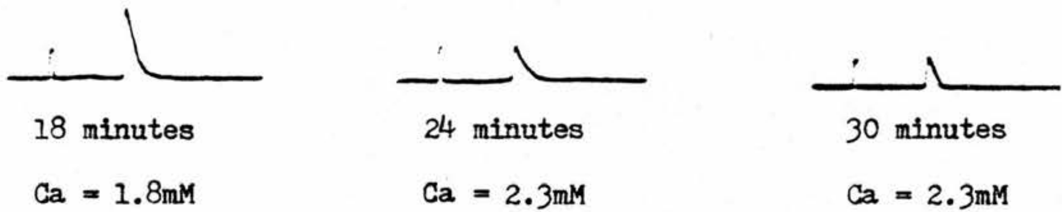
CHLORTETRACYCLINE [Ca^{++}] = 2.3×10^{-3}M

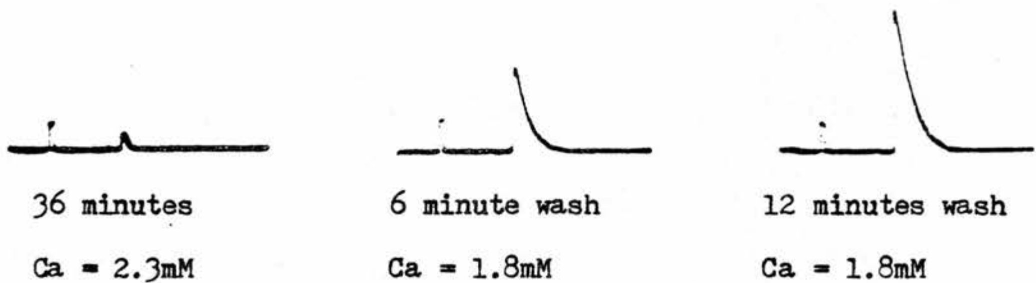
FIGURE 25

OXYTETRACYCLINE HCl (5.0×10^{-4}M), Ca^{++} INCREASED FROM 1.8 to 2.3mM.



1.0mV calibration pulse. — 5 msec.

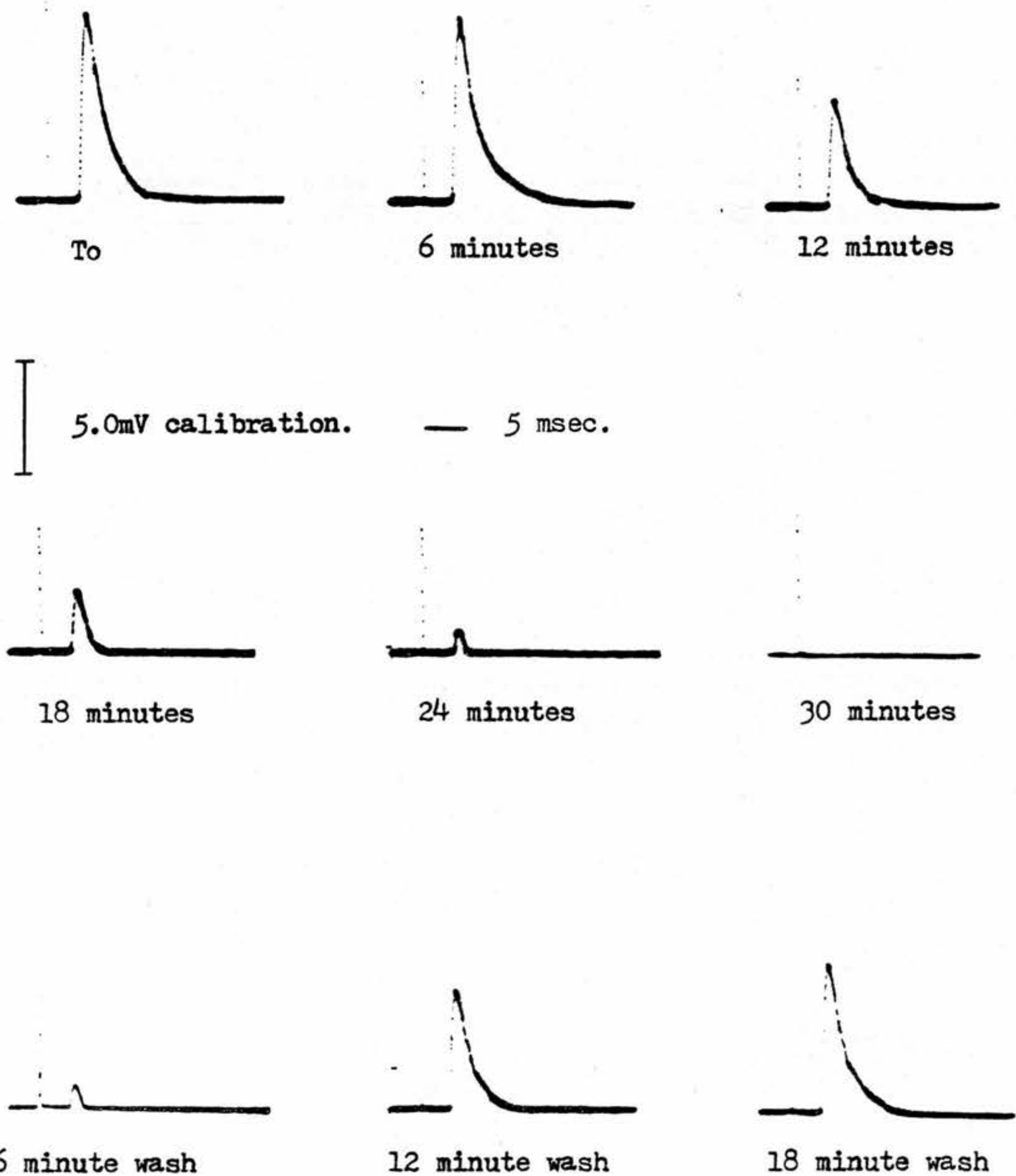




The preparation was bathed in Ringer solution buffered at pH = 7.0 and containing tubocurarine at a concentration of 2.0×10^{-6}M. Calcium concentrations were as indicated above; the increase occurred 18 minutes after To.

FIGURE 26

MINOCYCLINE ($5.0x10^{-4}M$) AVERAGED EPP AMPLITUDE.



Prior to To the preparation was bathed in HEPES buffered Ringer solution containing calcium at a concentration of 1.8mM and tubocurarine at a concentration of $2.0x10^{-6}M$. For the 30 minutes following To the Ringer solution also contained minocycline at a concentration of $5.0x10^{-4}M$ in addition to the tubocurarine.

DISCUSSION :- Both from data presented in graphical form and from sample traces of averaged EPP amplitudes it can be seen that these antibiotics exert their effect on neuromuscular transmission commencing almost immediately following their introduction into the recording chamber. EPP amplitudes progressively decrease throughout the period during which the antibiotic is present until in the case of minocycline at a concentration of 5.0×10^{-4} M there is no perceptible response to neural stimulation after 30 minutes. In all cases the EPP amplitude recovered on washing out the antibiotic, however in most cases an extended period of washing out in excess of 30 minutes was necessary in order to restore the averaged EPP amplitude to a value in excess of 80% of the preantibiotic treatment EPP amplitude level.

In some of these experiments the calcium concentration of the tubocurarine containing Ringer solution was increased from the normal level of 1.8 mM to 2.3 mM at a time after the antibiotic induced decrease in EPP amplitude had become well established. Such a 0.5 mM increase in the calcium ion concentration of the solution surrounding the exterior of the nerve terminal is sufficient to compensate for the maximum possible amount of calcium which could be rendered unavailable due to chelation by the antibiotic (assuming the formation of a 1:1 antibiotic / divalent cation complex, see discussion section of chapter 14).

The EPP amplitude continues to decrease even in the presence of this elevated calcium ion concentration, therefore these antibiotics must exert their neuromuscular blocking action through some mechanism other than by their ability to decrease the available calcium ion concentration.

CHAPTER 11

THE EFFECTS OF BLOCKING CONCENTRATIONS OF TETRACYCLINES ON QUANTAL CONTENT

THE EFFECTS OF BLOCKING CONCENTRATIONS OF TETRACYCLINES ON QUANTAL CONTENT

INTRODUCTION :- From the results of the twitch tension experiments presented in chapter 4 it can be seen that in buffered Ringer solution containing the normal calcium ion concentration of 1.8 mM and with no added magnesium tetracycline hydrochloride at a concentration of 2 x 10^{-3} M does lead to a complete loss of the development of twitch tension in response to supramaximal stimulation of the sciatic nerve. Consequently it was decided to determine what the quantal content would be under similar circumstances.

METHODS :- It will be appreciated that in the absence of a suitable blocking agent to abolish the muscle twitch, it is not possible to record EPP's from the muscle fibres, however it is possible to locate a suitable endplate without stimulating the sciatic nerve. The optical system of the microscope used was not of the Nomarski type, consequently endplates could not be visualised directly; however it was possible to follow along a fine branch of the nerve arborisation until a region likely to contain endplates was located. A microelectrode was then inserted into the fibre, if MEPP's were observed of amplitude 0.3 mV or more and with a fast rise time then an endplate had been successfully located. In order to avoid undue damage to the muscle fibre, no more than three penetrations of any particular fibre were made. If an endplate had not been successfully located then another fibre was selected. A series of MEPP's were

recorded at 30 minute intervals and the membrane potential noted. After a period of 60 minutes had elapsed since the antibiotic was introduced into the recording chamber test stimuli were applied to the sciatic nerve. On several occasions this caused a number of the muscle fibres to twitch, resulting in the microelectrode either causing damage to the muscle fibre or becoming completely dislodged; in either event recording from that particular preparation was abandoned.

RESULTS :- The results obtained from some of these experiments are presented in the accompanying tables.

TABLE 15

TETRACYCLINE HCl 2.0 x 10^{-3}M.

PREPARATION 1 Initial calcium ion concentration = 1.8 x 10^{-3}M.

HEPES buffered Ringer solution pH = 7.0

no magnesium or tubocurarine was added to the Ringer.

	before TETRACYCLINE	60 minutes in TETRACYCLINE	90 minutes in TETRACYCLINE
RP (mV)	94	87	83
MEPP freq (HZ)	2.8	2.8	2.6
AVMIN (mV)	0.71	0.53	0.44
AVEPP (mV)		2.93	1.76
$\frac{AVEPP}{AVMIN}$		5.54	4.00
$\frac{AVEPP^2}{VAREPP}$		5.86	4.28

PREPARATION 2 same conditions as for preparation 1.

	before TETRACYCLINE	60 minutes in TETRACYCLINE	90 minutes in TETRACYCLINE
RP (mV)	96	88	81
MEPP freq (HZ)	3.6	3.8	3.2
AVMIN (mV)	0.53	0.40	0.32
AVEPP (mV)		3.3	1.2
$\frac{AVEPP}{AVMIN}$		8.20	3.74
$\frac{AVEPP^2}{VAREPP}$		10.04	4.10

TABLE 16

TETRACYCLINE HCl 2.0×10^{-3}M.

PREPARATION 3 Initial calcium ion concentration = 1.8×10^{-3}M.

HEPES buffered Ringer solution pH = 7.4

no magnesium or tubocurarine was added to the Ringer.

	before TETRACYCLINE	60 minutes in TETRACYCLINE	90 minutes in TETRACYCLINE
RP (mV)	93	86	79
MEPP freq (HZ)	4.6	4.4	4.7
AVMIN (mV)	0.42	0.34	0.26
AVEPP (mV)		6.82	2.80
$\frac{AVEPP}{AVMIN}$		20.05	10.77
$\frac{AVEPP^2}{VAREPP}$		31.03	16.29

PREPARATION 4 same conditions as for preparation 3.

	before TETRACYCLINE	60 minutes in TETRACYCLINE	90 minutes in TETRACYCLINE
RP (mV)	89	82	76
MEPP freq (HZ)	1.8	1.7	2.0
AVMIN (mV)	0.47	0.38	0.32
AVEPP (mV)		3.82	2.19
$\frac{AVEPP}{AVMIN}$		10.05	6.84
$\frac{AVEPP^2}{VAREPP}$		15.24	8.16

TABLE 17

MINOCYCLINE HCl 2.0×10^{-3}M.

PREPARATION 1 Initial calcium ion concentration = 1.8×10^{-3}M.

HEPES buffered Ringer solution pH = 7.0

no magnesium or tubocurarine was added to the Ringer.

	before MINOCYCLINE	60 minutes in MINOCYCLINE	90 minutes in MINOCYCLINE
RP (mV)	96	81	76
MEPP freq (HZ)	1.5	20.3	48.2
AVMIN (mV)	0.58	0.39	0.28
AVEPP (mV)		4.85	2.72
$\frac{AVEPP}{AVMIN}$		12.43	9.71
$\frac{AVEPP^2}{VAREPP}$		18.46	13.92

PREPARATION 2 same conditions as for preparation 1.

	before MINOCYCLINE	60 minutes in MINOCYCLINE	90 minutes in MINOCYCLINE
RP (mV)	89	82	77
MEPP freq (HZ)	3.1	10.4	21.3
AVMIN (mV)	0.49	0.33	0.19
AVEPP (mV)		8.42	3.04
$\frac{AVEPP}{AVMIN}$		25.51	16.00
$\frac{AVEPP^2}{VAREPP}$		36.91	22.84

TABLE 18

MINOCYCLINE HCl 2.0×10^{-3}M.

PREPARATION 3 Initial calcium ion concentration = 1.8×10^{-3}M.

HEPES buffered Ringer solution pH = 7.0

no magnesium or tubocurarine was added to the Ringer.

	before MINOCYCLINE	60 minutes in MINOCYCLINE	90 minutes in MINOCYCLINE
RP (mV)	97	80	73
MEPP freq (HZ)	2.2	28.3	49.0
AVMIN (mV)	0.51	0.40	0.33
AVEPP (mV)		6.04	3.11
$\frac{AVEPP}{AVMIN}$		15.10	9.42
$\frac{AVEPP^2}{VAREPP}$		19.43	11.89

PREPARATION 4 same conditions as for preparation 3.

	before MINOCYCLINE	60 minutes in MINOCYCLINE	90 minutes in MINOCYCLINE
RP (mV)	91	83	76
MEPP freq (HZ)	3.4	26.4	38.9
AVMIN (mV)	0.38	0.24	0.15
AVEPP (mV)		2.83	0.98
$\frac{AVEPP}{AVMIN}$		11.79	6.53
$\frac{AVEPP^2}{VAREPP}$		12.63	7.08

DISCUSSION :- These experiments demonstrate that in the absence of any neuromuscular blocking agents such as either magnesium or tubocurarine, tetracycline HCl at a concentration of 2.0×10^{-3} M, produces a decrease in MEPP amplitude of 22.02% $\pm$ 1.70% (standard error of the mean, N = 4) over a period of 60 minutes. However there is no alteration in the rate of spontaneous release of acetylcholine throughout the 90 minute period of the experiment. In 4 of these experiments, after a period of 60 minutes the neuromuscular block had developed to the extent that no muscle fibres twitched when stimuli were applied to the sciatic nerve thus allowing a series of EPP amplitudes to be recorded and the quantal content calculated. Over the following 30 minute period the quantal content, as derived from EPP & MEPP amplitudes, decreases by a further 40.14% $\pm$ 6.21%, which is in good agreement with values derived from EPP amplitude variance:- 45.38% $\pm$ 6.93%.

Similar results were derived from measurements made on preparations blocked with minocycline; MEPP amplitude decreased by 30.96% $\pm$ 3.28% after a period of 60 minutes and decreased further to 52.19% $\pm$ 6.03% after 90 minutes in minocycline. During this interval the quantal content decreased by 35.35% $\pm$ 4.80% and 36.37% $\pm$ 4.13% as calculated from EPP & MEPP amplitudes and from the variance of EPP amplitudes respectively.

The postsynaptic component of the antibiotic induced neuromuscular blockade is demonstrated by the decrease in MEPP amplitude; the presynaptic action is demonstrated by the decrease in quantal content. Unlike the other members of the tetracycline group of antibiotics minocycline exerts another presynaptic action namely the large increase in MEPP frequency.

CHAPTER 12

THE EFFECTS OF TETRACYCLINES ON MEMBRANE CONSTANTS

THE EFFECTS OF TETRACYCLINES ON MEMBRANE CONSTANTS

INTRODUCTION :- The effects of tetracyclines on the membrane constants of frog sartorius muscle fibre were investigated. A square wave analysis technique was used in which hyperpolarising pulses were applied to the interior of the muscle fibre via one microelectrode and the resultant potential change sampled at various distances along the interior of the fibre via a second microelectrode.

When applying a current from a point source the resultant potential change varies with both time and distance from the current source. The differential equations which define this relationship were solved by HODGKIN & RUSHTON (1946) who showed that the potential change (V) produced by a steady current (I) across a membrane is given by

$$V = \tfrac{1}{2}I \sqrt{(rm\ ri)}\ \exp\left[- x/\sqrt{(rm/ri)}\right] \qquad (1)$$

where
- x is the distance separating the recording and current passing electrodes (cm).
- rm is the transverse membrane resistance of a unit length of membrane(Ω cm).
- ri is the longitudinal internal resistance of a unit length of the fibre interior ($\Omega\,cm^{-1}$).

When log V/I is plotted against the electrode separation a straight line is obtained which, when extrapolated to zero electrode separation, gives the value of the input resistance Ro from the relation

$$Ro = \tfrac{1}{2}\sqrt{(rm\ ri)} \text{ from eqn 1 when } x = 0. \qquad (2)$$

The space constant λ, which is defined as $\lambda = \sqrt{\left(\frac{rm}{re + ri}\right)}$

where ri is the resistance per unit length of the myoplasm (Ωcm^{-1}), is obtained from the slope of the straight line plot which results from the plot of log V/I versus the distance between the electrodes.

$$\log \frac{V}{I} = \frac{\log e}{\lambda} x + \log \tfrac{1}{2} (rm.ri)$$

The specific resistance of the fibre Ri was taken to be 169.5Ω cm (HODGKIN and NAKAJIMA, 1972), rather than the value 250Ωcm established by KATZ in 1948.

In order to calculate the total membrane resistance Rm ($\Omega .cm^2$) the fibre diameter must be known. Fibre diameter is difficult to determine from visual measurement via the calibrated graticule of the microscope since the fibre may be partially obscured by adjacent fibres furthermore the fibre may not necessarily be circular in cross section. However fibre diameter may be calculated from the relationship

$$d = \sqrt{\frac{4}{\pi} \times \frac{Ri}{ri}}$$

Rm the total membrane resistance (Ωcm^2) was calculated from the equation

$$Rm = \pi \, d.rm \text{ where } d = \text{fibre diameter in cm.}$$

METHODS :- Figure 27 shows in schematic form the equipment used to provide hyperpolarising current pulses of 100 milliseconds duration and the techniques used to measure both the magnitude of the hyper-

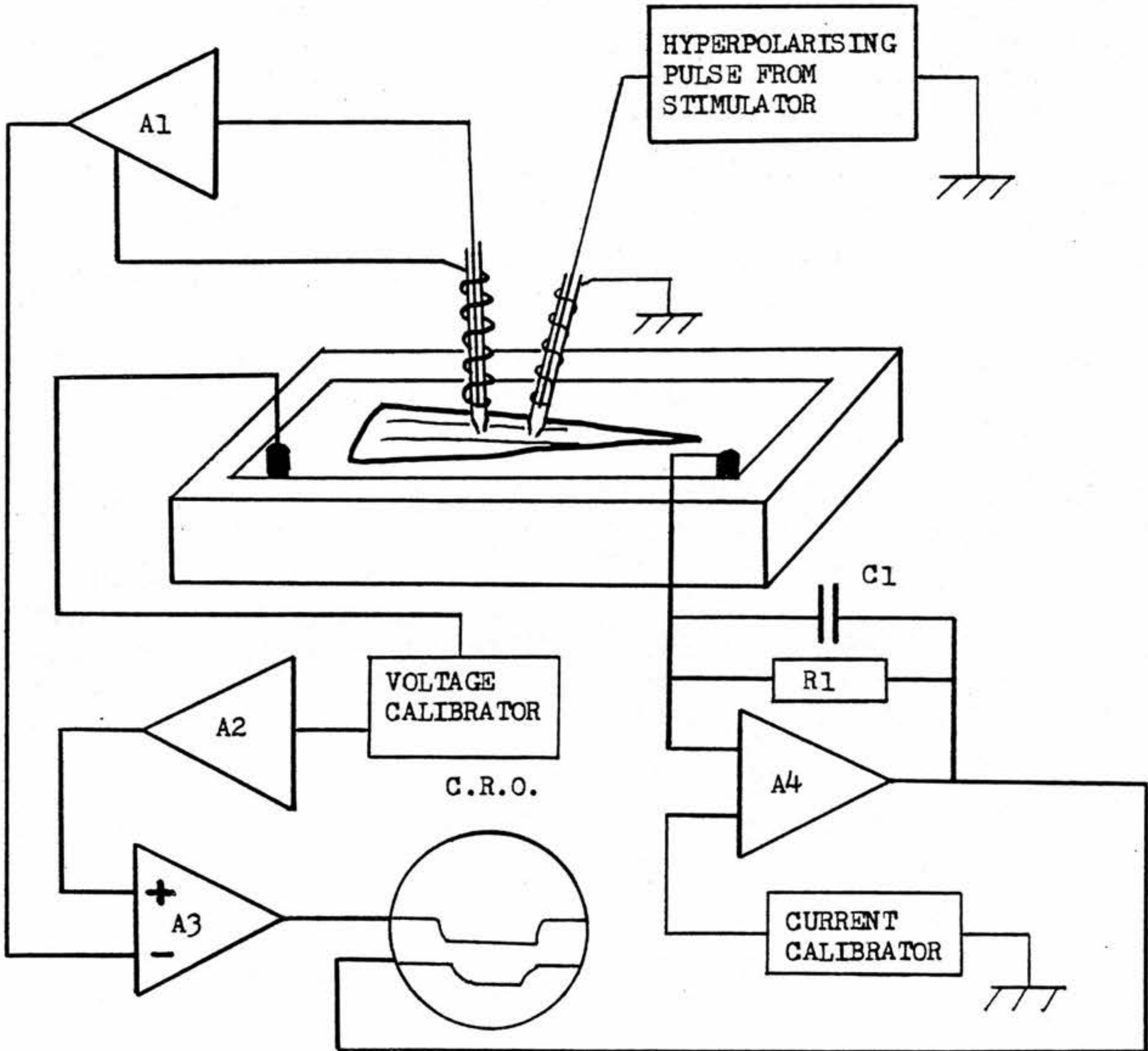
polarising current and the resultant change in membrane potential. Frog sartorius preparations (Rana pipiens) without the sciatic nerve were set up in the perspex muscle bath as previously used for electrophysiological recordings. Similarly, solutions could be changed rapidly, without disturbing the microelectrodes, by means of the perfusion system as described in a previous section.

A suitable fibre was located and the voltage measuring electrode inserted. A suitable fibre was deemed to one which was readily visualised over a distance of a centimetre or more at a site well removed from an endplate region. The current passing electrode was then inserted into the same fibre at a distance of approximately 5 millimetres. The detection of hyperpolarising pulses by the voltage measuring microelectrode confirmed that the current passing microelectrode was correctly located within the selected fibre. Electrode separation was measured by means of a calibrated scale incorporated within one of the eye-pieces of the binocular microscope. Due care was exercised in order to minimise possible errors in the measurement of microelectrode separation arising from parallax occurring between the ocular scale and the point of the electrode penetration of the surface of the fibre.

Once a series of current and voltage traces, consisting of 3 or 4 separate responses superimposed on a single frame of the 35mm film as shown in figure 28, were recorded at a particular electrode separation the current passing electrode was then removed from the muscle fibre and moved to a site closer to the voltage measuring electrode. Such a series of traces, recorded at 3 different electrode separations, were made for a chosen fibre firstly in HEPES buffered Ringer solution

FIGURE 27

MEMBRANE CONSTANTS MEASUREMENT TECHNIQUE USING TWO MICROELECTRODES



A1 = FET OPERATIONAL AMPLIFIER CONNECTED AS A SOURCE FOLLOWER PROVIDING A LOW IMPEDANCE GUARD DRIVE SIGNAL.

A2 = FET OPERATIONAL AMPLIFIER CONNECTED AS A SOURCE FOLLOWER.

A3 = DIFFERENTIAL AMPLIFIER (DC COUPLED).

A4 = FET INPUT OPERATIONAL AMPLIFIER CONNECTED IN A CURRENT TO VOLTAGE CONVERTOR CONFIGURATION.

R1 = 1M RESISTOR (AN INPUT CURRENT OF 0.1 μA RESULTS IN AN OUTPUT VOLTAGE OF 100 mV).

C1 = HIGH FREQUENCY COMPENSATION CAPACITOR.

of normal calcium ion concentration (1.8 mM); then in HEPES buffered Ringer solution containing tetracycline. The tetracycline was present for a period of 30 minutes before a second series of recordings at 3 different electrode separations were made.

In the first series of experiments, recordings were made from a single fibre before, then following a period of exposure to tetracycline; a fresh preparation being used for each experiment. In the second group of experiments a group of fibres were sampled before exposure to tetracyclines then a separate group of fibres, none of which had previously been recorded from, were sampled after exposure to tetracyclines.

ELECTRODES :- The voltage measuring microelectrode was filled with 3M KCl. The current passing microelectrode was filled with 2M potassium citrate since it was found difficult to maintain a stable level of depolarisation over the 100 millisecond duration of the current pulse when using KCl filled microelectrodes (FATT and GINSBORG, 1958). Microelectrodes with resistances in the range 10 to 12 megohms were used for both electrodes.

SOLUTIONS :- Buffered Ringer solution (3.0 mM) pH 7.0, 112.4 mM Na^+, 2.5 mM K^+, 117.0 mM Cl^-, 1.8 mM Ca^{2+}. Tetracyclines in the form of the hydrochloride were freshly made up in Ringer solution of the above composition and the pH adjusted to 7.0 with deci-normal NaOH.

As in other experiments pH 7.0 was chosen rather than the more physiological pH value 7.4 because a much smaller quantity of NaOH is required to adjust the pH to 7.0. The greater volume of NaOH required to adjust the pH to 7.4 would have added substantially to the overall Na^+ concentration of the Ringer solution.

The capacitance of the muscle fibre membrane was calculated from a knowledge of Rm and measurement of the time constant of the voltage change across the membrane in response to a step change in membrane current from the relationship

$$Cm = \frac{\tau m}{Rm}$$

τm was determined by measuring the difference in time taken for the voltage trace to reach 50% of the maximum steady state hyperpolarisation when measured at two different electrode separations.

Where

$$\tau m = 2 \frac{\Delta t}{\Delta x} \lambda$$

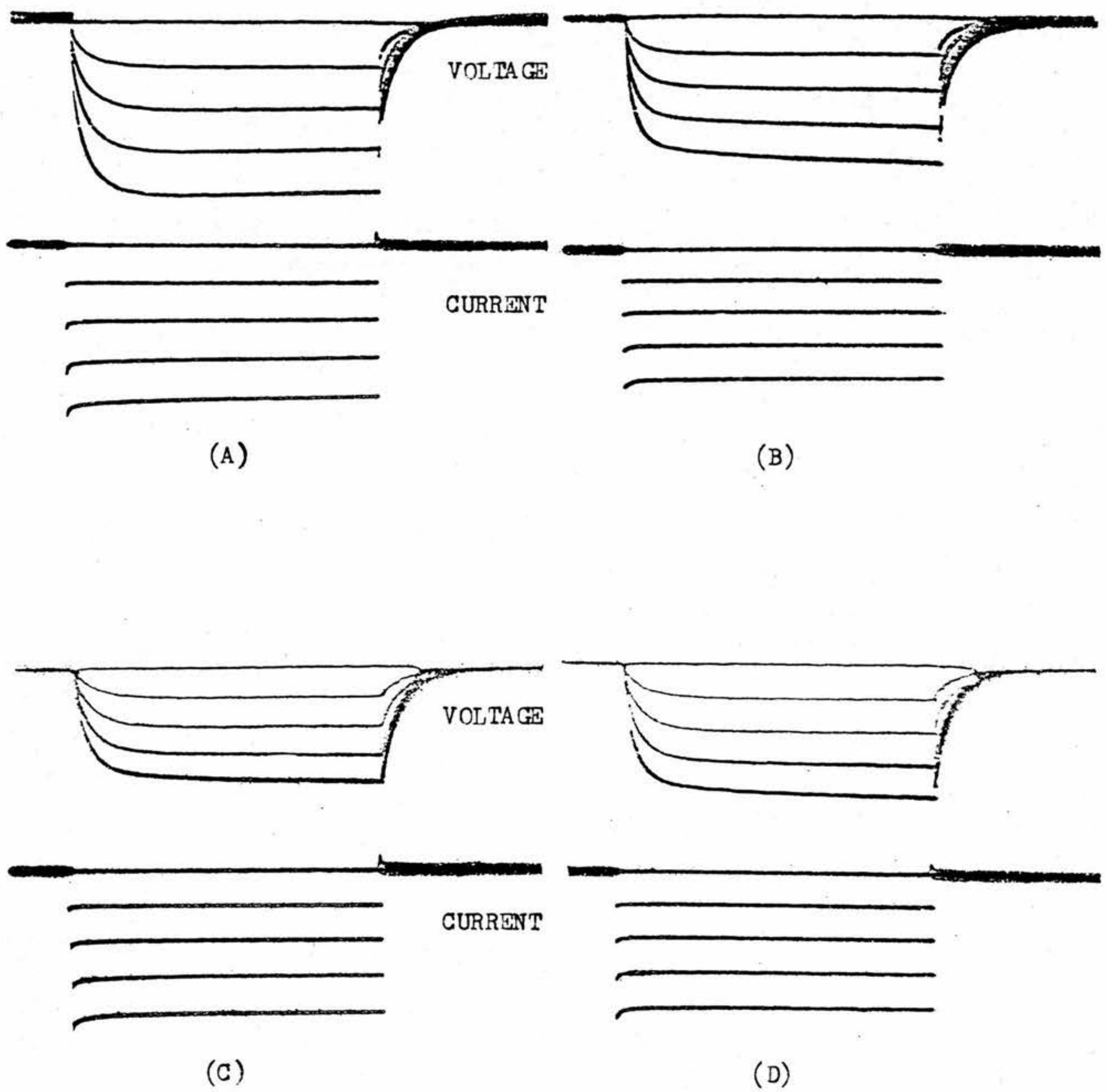
and Δt (milliseconds) is the difference in time taken to reach 50% of the steady state hyperpolarisation

and Δx (mm) is the separation between the two sites at which the membrane voltage traces were recorded.

STATISTICS :- All values are expressed as the mean ± S.E.M. Student's t test was used to analyse data. P values < 0.05 were considered statistically significant.

RESULTS :- A typical set of membrane current / voltage recordings, each taken at different electrode separations are shown in figure 28. For the purposes of measuring the rise time of the membrane voltage a much higher time base velocity than that shown in the figure was used. Table A of figure 29 lists a series of measurements taken from oscilloscope traces such as those shown in figure 28 and shows how the various membrane constants were derived from these measurements.

FIGURE 28



CALIBRATION:- Pulse duration 100 milliseconds.

VOLTAGE = 10 millivolts.

CURRENT = 0.1 microampere.

TABLE A

I(mm)	V(mm)	I(mm)	V(mm)	I(mm)	V(mm)
18	34	20	30	25	30
37	63	39	54	45	55
57	94	52	70	60	70
Σ I.V = 8301		I.V = 6346		I.V = 7425	
ΣI^2 = 4942		I^2 = 4625		I^2 = 6250	
$\Sigma \frac{I.V}{I^2}$ = 1.680		= 1.372		= 1.188	
Ω 391974		320112		277181	
log Ω 5.593		5.505		5.443	

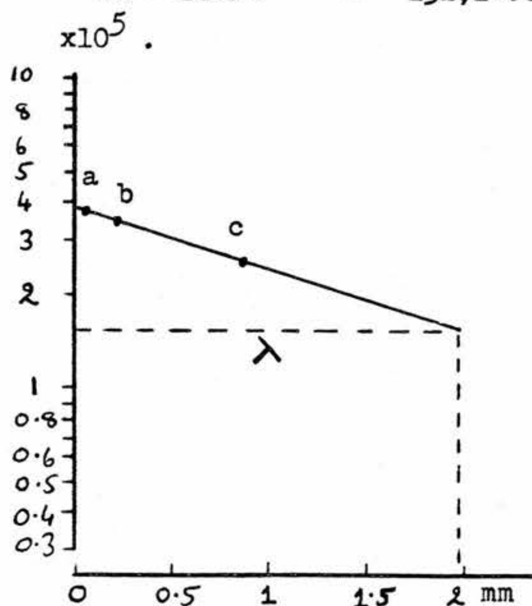
x(mm)	y log Ω
0.083	5.593
0.277	5.505
0.708	5.443

slope = -0.224 λ = 1.94 mm

intercept = 5.593 Ro = 392,114 Ω

$ri = 2 \times Ro \times 1/\lambda = 4,042,412\ \Omega cm^{-1}$ $d = \sqrt{\left(\frac{4}{\pi} \times \frac{Ri}{ri}\right)} = 73.2\ \mu$

$rm = ri \times \lambda^2 = 152,140\ \Omega cm$ $Rm = \pi \times d \times rm = 3497\ \Omega cm^2$



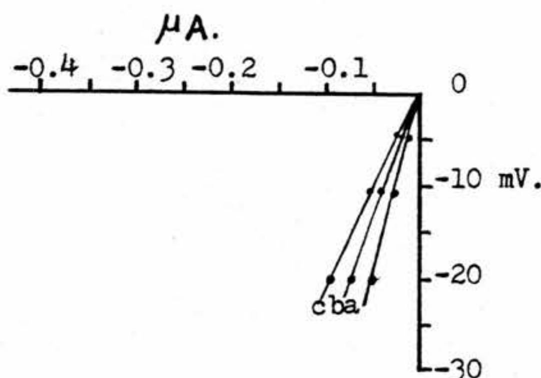


FIGURE 29

TABLE 18

THE EFFECTS OF TETRACYCLINES ON MEMBRANE CAPACITANCE Cm.

CONDITIONS	RM Ω cm^2	M (msec)	Cm μF	N
TETRACYCLINE HCl (1×10^{-3} M)	3899±396	16.52±0.71	4.24±0.38	10
MINOCYCLINE HCl (1×10^{-3} M)	4298±709	16.73±1.09	3.90±0.80	10
CONTROLS	3891±246	16.46±0.69	4.23±0.36	17

TABLE 19

MEMBRANE CONSTANTS OF MUSCLE FIBRES AFTER 60 MINUTES IN RINGER SOLUTION CONTAINING ANTIBIOTICS (TEMPERATURE 20°C Ri = 169.5Ω)

CONDITIONS	λ (mm)	RM (Ω CM^2)	DIAMETER (μ)	N
CONTROLS pH 7.0 (Ca 1.8)	1.95±0.10	3891±246	71.4±5.1	17
TETRACYCLINE 1×10^{-3} M	1.89±0.12	3795±303	65.7±5.9	19
TETRACYCLINE 2×10^{-3} M	1.73±0.10	3899±396	63.4±3.9	10
MINOCYCLINE 1×10^{-3} M	1.83±0.09	3288±308	69.5±4.0	19
MINOCYCLINE 2×10^{-3} M	1.83±0.14	4299±709	55.7±17.6	10

The values presented in tables 18 and 19 are the mean ± S.E.M. and were obtained from a number of preparations, the number of fibres studied is given by N.

Comparisons were made statistically between the control values obtained from a number of preparations before the introduction of the antibiotic and values obtained from different fibres in those preparations after 60 minutes in Ringer solution containing antibiotic. Only one concentration of a single antibiotic was used on any individual preparation.

Student's t test was used to analyse the data. P values <0.05 were considered statistically significant.

CONCLUSION :- No statistically significant differences were found between membrane constants measured in HEPES buffered Ringer solution and those measured in the presence of concentration of tetracyclines that produce a substantial degree of neuromuscular blockade.

CHAPTER 13

THE EFFECTS OF TETRACYCLINES ON MEMBRANE POTENTIAL IN THE FROG SARTORIUS MUSCLE

THE EFFECTS OF TETRACYCLINES ON MEMBRANE POTENTIAL IN THE FROG SARTORIUS MUSCLE

INTRODUCTION :- During the course of the quantal content experiments it was observed that in many instances there was a decrease in transmembrane potential following the introduction of the antibiotic into the bathing medium. This reduction in resting potential, in the region of 10 millivolts, was in most instances restored to within 2 or 3 millivolts of the initial value following a 30 minute period of continuous washing out. In the quantal content experiments the microelectrode tip had been inserted through the muscle fibre membrane for a period of two hours, consequently a small drop in resting potential would be expected as a result of the presence of a non specific leakage conductance around the microelectrode. So in order to determine whether or not the antibiotic does have an effect on membrane potential the following series of experiments were carried out.

METHODS :- Frog sartorius preparations (Rana pipiens) were set up in the normal way, however no stimulation was applied to the sciatic nerve. It was considered essential to the experimental design to eliminate any prospect of damage to the integrity of the muscle fibre membrane by either the previous or prolonged insertion of a microelectrode; therefore the preparation was notionally divided longitudinally into two parts. To serve as controls ten separate fibres in one of these parts were recorded from; five impalements were made at the tibial region, which is known to be without innervation (KATZ

& KUFFLER 1941) and the remainder in areas where neuromuscular junctions were likely to be present. In the latter situation the value of the resting potential was noted only if the presence of MEPP's confirmed that the electrode was situated close to the neuromuscular junction. After the preparation had been in contact with a particular antibiotic concentration for one hour a second series of ten recordings were taken from the previously undisturbed half of the preparation. In this manner data were collected to enable the effect of the antibiotic on the membrane potential to be determined statistically and also to distinguish between a possible difference in effect on the extrajunctional muscle fibre membrane and that in proximity of the neuromuscular junction.

STATISTICS :- All values are expressed as the mean $\pm$ the standard error of the mean. Student's t test was used to analyse the data. P values < 0.05 were considered statistically significant.

RESULTS :- Results are listed in table 20. Within the control groups, as expected, no significant differences were found between the means of the membrane potentials whether measured directly at, or some distance from the neuromuscular junction.

Following a period of exposure to antibiotics for one hour, tetracycline, chlortetracycline and minocycline all showed a statistically significant decrease in resting potential. Whereas oxytetracycline at a concentration of one millimolar was without apparent effect.

No significant differences between the means of the membrane po-

tentials, whether measured directly at, or some distance from the neuromuscular junction, were found except in the case of minocycline (1.0×10^{-3} M). In which case there is a 4.3 mV difference between the means, this difference is statistically significant at $P < 0.001$.

REFERRING TO TABLE 20.

S These values are significantly different ($P < 0.05$) from their respective control values.

S** This value is significantly different ($P < 0.05$) from the value obtained from resting potentials sampled at the neuromuscular junction.

DISCUSSION :- With the exception of oxytetracycline, the three other antibiotics tested all have a statistically significant effect on the membrane potential of the frog sartorius muscle fibres. However the magnitude of this reduction in membrane potential is numerically small, being approximately 7.0 mV for tetracycline HCl at a concentration of 2.0×10^{-3} M which, as demonstrated in chapter 4 leads to complete blockade of the neuromuscular junction.

Of the 290 fibres tested which had been exposed to either tetracycline HCl, chlortetracycline HCl, oxytetracycline HCl or minocycline only 10 fibres had a resting potential of -60 mV or less; a value below which a muscle fibre is unlikely to produce a propagated muscle action potential in response to neural stimulation and will therefore not contribute to the development of twitch tension. Since less than 4.0% of the fibres tested had resting membrane potentials of less than

TABLE 20

THE REDUCTION IN RESTING POTENTIAL OF FROG SARTORIUS MUSCLE FIBRES AFTER 60 MINUTES IN RINGER SOLUTION CONTAINING ANTIBIOTICS

CONDITIONS	EXTRAJUNCTIONAL RP (mV)	N	JUNCTIONAL RP (mV)	N
CONTROL $[Ca^{++}_o]$ 1.8 pH 7.0	93.7±0.34	66	94.8±0.41	33
TETRACYCLINE 1×10^{-3}	89.4±0.95	73	91.5±0.94	33
MINOCYCLINE 1×10^{-3}	S** 82.0±1.25	62	S* 86.3±1.50	42
CONTROL $[Ca^{++}_o]$ 5.0 pH 7.0	92.3±0.39	54	92.5±0.60	30
TETRACYCLINE 2×10^{-3}	85.7±3.20	14	S* 87.9±1.44	12
MINOCYCLINE 2×10^{-3}	75.0±3.60	18	S* 76.3±2.38	12
CONTROL $[Ca^{++}_o]$ 1.8 pH 7.0	91.3±0.41	58	92.7±0.63	40
OXYTETRACYCLINE 1×10^{-3}	90.7±0.94	25	91.0±0.71	15
CHLORTETRACYCLINE 5×10^{-4}	70.5±5.31	15	S* 73.2±4.20	9

The values of the resting potential presented are the mean ± the standard error of the mean and were obtained from a number of preparations.

N = the number of fibres studied.

Comparisons were made statistically between the appropriate control values obtained from a number of preparations before the introduction of the antibiotic and values obtained from different fibres of those same preparations after one hour in the Ringer solution containing the antibiotic. Only one concentration of a single antibiotic was used on any individual preparation.

-60 mV it is unlikely that the effect of the antibiotic on the muscle fibre membrane potential is a significant component of the neuromuscular blocking action of the tetracycline group of antibiotics.

CONCLUSIONS :- Tetracycline, chlortetracycline and minocycline all produce a decrease in the membrane potential. This decrease in membrane potential becomes numerically greater with increasing concentration of these antibiotics.

There is no statistically significant difference in the depolarisation caused at the junctional membrane as compared to that caused extrajunctionally, with the possible exception of minocycline which at concentrations in the region of one millimolar may cause a slightly greater depolarisation of the extrajunctional membrane, however, this difference is not significant when the concentration of the minocycline is doubled.

Depolarisation of the muscle membrane by the antibiotics tested does not contribute significantly to the reduction of twitch tension which occurs at these concentrations of antibiotics.

CHAPTER 14

METAL COMPLEXATION OF TETRACYCLINE HYDROCHLORIDES

METAL COMPLEXATION OF TETRACYCLINE HYDROCHLORIDES

INTRODUCTION :- It has been shown that tetracyclines interfere with neuromuscular transmission (BEZZI & GESSA 1959, 1960 & 1961), also tetracyclines are known to have a high avidity for divalent metals (ALBERT 1953, 1956). Therefore it is useful to ask to what extent can tetracyclines bind the biologically important metal ion calcium and to consider if tetracycline induced neuromuscular blockade can be explained on the basis of the tetracycline binding a sufficiently high proportion of the extracellular calcium thereby reducing the release of neurotransmitter from the motor nerve terminals to a subthreshold level.

In order to pursue such a line of investigation it is necessary to have a method whereby the avidity of tetracycline for divalent metals can be determined quantitatively. For technical reasons measurement of the free calcium ion concentration by means of a calcium selective electrode proved unreliable when used in the presence of tetracyclines. Therefore an alternative approach, utilising a potentiometric titration technique; was adapted to the problem of ascertaining the unbound calcium ion concentration.

The structural formulae of tetracycline and some of its derivatives are shown in figure 30. They are widely used in medical practise as "broad spectrum" antibiotics, their high therepeutic index depends on their selective accumulation by bacteria. The mode of action of the tetracycline group of antibiotics is now known to be dependent upon the presence of certain metal ions, primarily magnesium (FRANKLIN 1971), however the precise binding sites involved have not

been established with certainty. Also in considering the possible role of metal binding in the neuromuscular blocking action of the tetracyclines a knowledge of the particular binding site, or sites, involved in calcium chelation, the pKa associated with that site and the nature of the particular ionic form of the tetracycline taking part in the chelation process would be invaluable. However no explicit statement concerning any of these problems can be obtained from the literature. The available evidence will be reviewed with regard to the specific questions:-

What are the appropriate pKa values for a particular tetracycline and with which sites on the tetracycline molecule are they associated?

At which of the sites does calcium chelation take place?

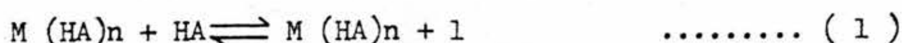
What is the stoicheometry of the calcium tetracycline complex, does it form in a 1:1 or a 2:1 metal ion to ligand ratio?

The nature of chelation is such that any substances which can bind a proton can bind a metallic cation instead. At high proton concentrations, the proton will be greatly preferred, at low proton concentrations the metallic cation may be preferred if it is present in great excess, or if a new ring can be formed by two groups in the molecule which simultaneously bind the metal. This simultaneous binding is termed chelation, and it confers stability on the complex. The substance which binds the metal is termed a ligand.

Stability constants are determined in order to assess the

affinity between various ligands and various metals. Of the several methods available for the determination of stability constants potentiometric titration was chosen. The method was introduced by BJERRUM (1941) to describe metal amine formation in aqueous solution. BJERRUM was the first to recognise that the equilibrium between a complex forming agent and an ion could be correctly represented by mass action equations since complex formation is usually thermodynamically reversible and occurs instantly, without appreciable energy of activation and usually follows a step-wise course:-

For the reaction



$$M\ (HA)n + HA \rightleftharpoons M\ (HA)n + 1 \qquad \ldots\ldots\ldots (1)$$

the general equation for the step-wise stability constants, Ki, is

$$\log Ki = p\ (HA) \quad \text{at } \bar{n} = \tfrac{1}{2} \qquad \ldots\ldots\ldots (2)$$

where (HA) = molar concentration of the complexing species

i = integer indicating the particular step-wise reaction of interest

$$\bar{n} = \frac{\text{total concentration of ligand bound}}{\text{total concentration of metal}}$$

Using this method two pH titration curves are required. The first is the potentiometric titration of the tetracycline, in the form of the hydrochloride, in the absence of the metals calcium or magnesium. The second is the titration of the same concentration of the ligand in the presence of a particular concentration of the metal ion under investigation. Because the metal cation displaces the hydrogen cation, this second titration curve lies at lower pH values than the first one for the addition of the same volume of titrant. The

stability constants and an estimate of what fraction of a particular metal ion concentration is chelated by the tetracycline can be made from the difference between these pH curves.

From the titration data obtained in the absence of the chelating metal species, pKa values are calculated.

Referring to figure 30 STEPHENS et al (1956) assigns pKa values to the groups in the oxytetracycline molecule as follows:-

C 3.27 B 7.32 A 9.11

however later workers (LEESON et al 1963, RIGLER et al 1965) reassign the groups

C 3.27 A 7.32 B 9.11

The paper of STEPHENS et al (1956) remains the most often quoted publication concerning pKa group assignment despite the fact that in the first footnote these authors state that their own more recent studies had placed their assignments for pKa 2 and pKa 3 "in serious jeopardy". However no subsequent report of any re-assignment of pKa groups by these authors appeared in the literature. Table 21 lists published values of the acidity constants for tetracycline HCl, oxytetracycline HCl and chlortetracycline HCl.

Regarding the actual sites involved in metal binding by the tetracyclines CONVER (1956), using a spectrophotometric technique, concluded that the binding group is the enolized B-diketone group at C 11 and C 12 (Group A of figure 30). On the basis of potentiometric titration studies however DOLUSIO & MARTIN (1963) favoured the dimethyl-

amino group at C 4 (Group B of figure 30) and the hydroxyl at either C 3 or C 12a. Further evidence regarding the location of the complexation sites was obtained by NEWMAN & FRANK (1976) who examined the formation of tetracycline complexes with magnesium and calcium ions. The participation of particular sites in complexation was determined by using circular dichroism to follow complex formation in a series of modified tetracyclines in which potential complexation sites were blocked. They concluded that calcium formed a 2:1 metal-ion to ligand complex, while the magnesium complex formed at a 1:1 ratio in 90% methanol buffered at pH 7.4. Formation of the calcium complex was a step-wise process involving firstly the addition of one metal ion to the C 10, C 11 site then the subsequent addition of a second metal ion at the C 12, C 1 site. The magnesium chelate occurred at the C 11, C 12 B-diketone site. When the experiments were repeated using an aqueous solvent buffered at pH 7.4 it was found that while the Mg^{++} complex of tetracycline is the same in both aqueous and methanolic solutions the formation of the calcium chelate was not as favourable as in 90% methanol. They concluded that the second Ca^{++} is not complexed significantly in aqueous solution therefore the 1:1 metal-ion to ligand will be the predominant species in an aqueous medium. The C 11, C 12 site for the formation of the magnesium chelate is in agreement with the conclusions of the earlier study by CONVER (1956).

Using a spectrophotometric method IBSEN & URIST (1962) studied the reactions of oxytetracycline with calcium and magnesium in order to elucidate the mechanism of deposition of oxytetracycline in bone.

They reported that calcium has a lower affinity for oxytetracycline than either magnesium, manganese, cobalt or any other divalent cation present in the blood. They observed that calcium and magnesium binding by oxytetracycline occurs in a step-wise fashion with the formation firstly of a 1:1 metal to ligand complex and then a 2:1 metal to ligand complex. It should be noted however that in these experiments the metal ion was present in great excess. The ratio of calcium to oxytetracycline present being greater than 150:1 for the formation of the 2:1 calcium to oxytetracycline complex. They assigned values of log K for these various complexes as:-

1:1 complex log K Ca OTC 3.0 and log K Mg OTC 3.6

2:1 complex log K Ca_2OTC 2.0 and log K Mg_2OTC 2.4

However the method used by IBSEN & URIST to evaluate the affinity constants log K1 and K2 was somewhat simplistic in approach. The formulae used were:-

$$K1 = \frac{[\mathrm{Ca\ OTC}]}{[\mathrm{Ca^{++}}]\ [\mathrm{OTC}]} \quad ; \quad K2 = \frac{[\mathrm{Ca_2\ OTC}]}{[\mathrm{Ca^{++}}]\ [\mathrm{Ca\ OTC}]} \quad \ldots (3)$$

where $[\mathrm{OTC}]$, $[\mathrm{Ca\ OTC}]$ and $[\mathrm{Ca_2\ OTC}]$ were determined spectrophotometrically and

$[\mathrm{Ca^{++}}]$ = Total calcium - $[\mathrm{Ca\ OTC}]$ - 2 $[\mathrm{Ca_2\ OTC}]$.

It will later be shown that such formulae are not an adequate description of the complex equilibrium that exists between a divalent cation and tetracycline.

The thermodynamic acid dissociation constant Ka, has a truly constant value at a given temperature, which is calculable from the activities of the components of the dissociation reaction at equilibrium. Apparent values of Ka determined from the concentrations of the components at equilibrium will be affected by the ionic strength of the solution since the activity coefficients of ions in solution are affected by the total ionic strength of the solution. As the ionic strength of the solution is decreased, the ratio of the activity coefficients approaches unity the value of the apparent Ka approaches the value of the thermodynamic (true) Ka.

pKa values were calculated from the titration data obtained in the regions of half equivalents using the equation (due to KIRK & SCHMIDT 1929):-

$$pKa = PaH - \log \frac{[Na^+] - [OH^-]}{[acid] - [Na^+] \quad [OH^-]} \qquad \text{..... (4)}$$

where $[Na^+]$ and $[OH^-]$ and the molar concentrations of the respective ions and $[acid]$ is the initial molar concentration of the antibiotic in the form of the hydrochloride.

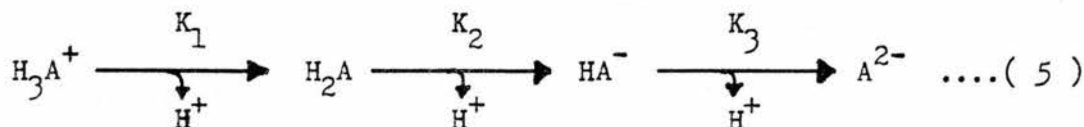
As there is some variation in the reported values of acidity constants (table 21) and since the method for calculating the amount of calcium chelated by the various tetracyclines depends on a knowledge of the appropriate acidity constants it was decided to determine values for these in Ringer solution. The values of acidity constants reported in the literature were all determined in aqueous solution, however it was considered desirable to determine values in a medium as similar as

possible to that used in the biological experiments. A secondary advantage was that small variations in ionic strength were effectively swamped by the presence of much larger concentrations of Na^+ Cl^- and K^+ ions present in the Ringer solution.

Using a potentiometric titration technique ALBERT (1953, 1956) has already calculated the stability constants of tetracycline HCl, chlortetracycline HCl and oxytetracycline HCl with various metal ions. However this particular work does not include the calcium ion at all and only one stability constant for the magnesium-oxytetracycline HCl complex was reported. In this work ALBERT points out that on the basis of trial calculations, combination of tetracycline with metals requires that the groups near pKa 7 should be ionised but that those near 3 and 9 should not be ionised. DOLUSIO & MARTIN (1963) interpretated this assertion by ALBERT as indicating that the complexing species of the tribasic tetracycline hydrochlorides, H_3A^+, Cl^-, is HA^-. DOLUSIO & MARTIN studied the metal complexation of tetracycline hydrochlorides with copper, nickel and zinc but not with either calcium or magnesium. However their methodology and equations were adapted to the requirements of the present study.

To determine the proportion of calcium bound by tetracycline, supplied as H_3A^+, Cl^-, in physiological solutions.

Suppose that the dissociation of tetracycline is governed by the relationships:-



$$H_3A^+ \xrightarrow[\downarrow H^+]{K_1} H_2A \xrightarrow[\downarrow H^+]{K_2} HA^- \xrightarrow[\downarrow H^+]{K_3} A^{2-} \quad ....(5)$$

The total concentration of tetracycline, all species, To is given by

$$To = [CaHA^+] + [H_3A^+] + [H_2A] + [HA^-] + [A^{2-}] \quad \ldots (6)$$

$$= [CaHA^+] + [HA^-]\left\{1 + \frac{H^{+\,2}}{K1\ K2} + \frac{H^+}{K2} + \frac{K3}{H^+}\right\} \quad \ldots (7)$$

$$= [\overline{Ca}] + x\,q$$

where $[\overline{Ca}]$ is the concentration of calcium bound, assuming that a 1:1 calcium tetracycline complex is formed.

x is $[HA^-]$ assuming that HA^- is the complexing species at the pH of interest (7.0 to 7.4).

q is the expression contained within the brackets $\{\ \}$ in equation 7.

The concentration of free and combined hydrogen ions $[XH]$ is given by

$$[XH] = 3To - [NaOH] \quad \ldots\ldots (8)$$

and, according to DOLUSIO & MARTIN, by:-

$$[XH] = [H^+] + [HA^-] + 2\,[H_2A] + 3\,[H_3A^+] - [OH^-] \quad (9)$$

With the exception of a very small amount of H^+ from the aqueous medium all of the hydrogen ions come from the tetracycline, therefore the total concentration of potentially replaceable hydrogen from the tetracycline will be 3 To .

By combining equations 8 and 9 and substituting the appropriate acidity constants it may be shown that

$$HA^- = \frac{3To - [NaOH] - [H^+] + [OH^-]}{\beta} \quad .... (10)$$

where $$\beta = \frac{3[H^+]^2}{K1\ K2} + \frac{2[H^+]}{K2} + 1 \quad .... (11)$$

and $\bar{n}$, the proportion of the total calcium concentration $[Ca_o]$ which has been bound by the tetracycline; is given by

$$\bar{n} = \frac{To - [HA^-]\ Q}{[Ca_o]} \quad .... (12)$$

The free calcium ion concentration available in Ringer solution containing tetracycline hydrochloride was calculated from equation 12 and presented in table 23.

TABLE 21

PUBLISHED pKa VALUES

TETRACYCLINE HCl

3.35	7.82	9.57	ALBERT (1956)
3.30	7.68	9.69	STEPHENS et al (1956)
3.69	7.63	9.24	DOLUSIO et al (1963)

OXYTETRACYCLINE HCl

3.49	7.55	9.24	REGNA et al (1951)
3.50	7.60	9.20	STEPHENS et al (1952)
3.10	7.26	9.11	ALBERT (1952)
3.27	7.32	9.11	STEPHENS et al (1956)
3.60	7.42	9.05	DOLUSIO et al (1963)

CHLORTETRACYCLINE HCl

3.4	7.4	9.2	STEPHENS et al (1952)
3.30	7.44	9.27	ALBERT (1953)
3.30	7.44	9.27	STEPHENS et al (1956)

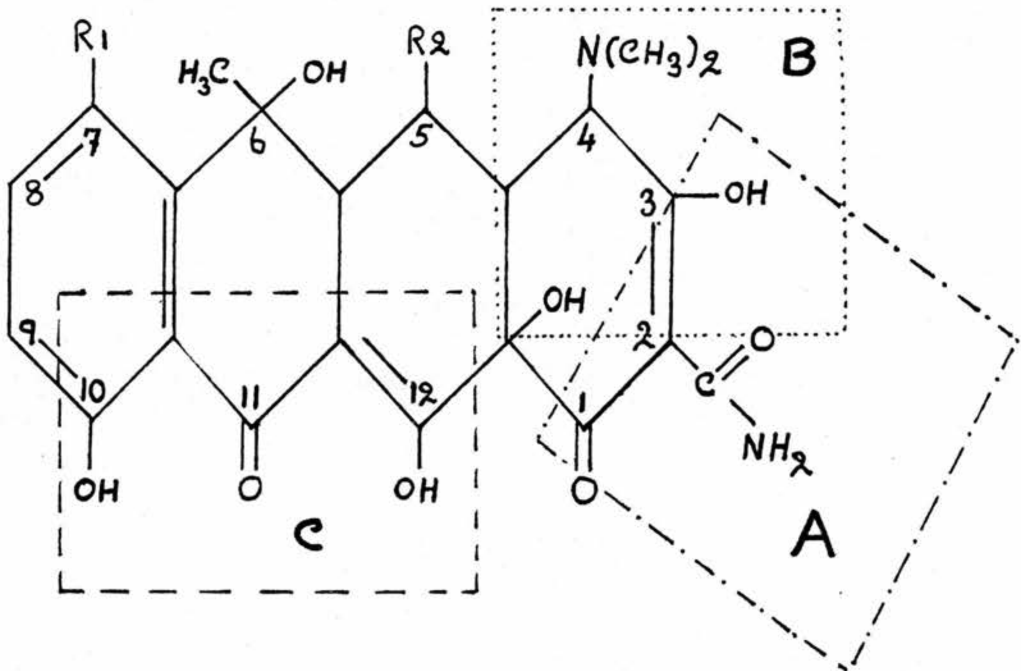
EXPERIMENTALLY DETERMINED pKa VALUES

3.64	7.73	9.46	TETRACYCLINE HCl
3.60	7.52	9.60	OXYTETRACYCLINE HCl
3.56	7.43	9.34	CHLORTETRACYCLINE HCl

FIGURE 30

STRUCTURAL FORMULAE FOR TETRACYCLINES

Tetracycline	R1 = H	R2 = H
Chlortetracycline	R1 = Cl	R2 = H
Oxytetracycline	R1 = H	R2 = OH



The tetracyclines are all very closely related structurally. The antibiotic properties of these drugs are essentially the same. When resistance arises to one of the compounds there is cross resistance with the others. The basic polycyclic structure of the tetracycline group of antibiotics is illustrated in the diagram. The functional groups labelled A , B & C are referred to in the text.

EXPERIMENTAL TECHNIQUE :- A number of samples of the antibiotic under investigation were accurately weighed out, each containing a quantity corresponding to a concentration of either 1.0 or 2.0 millimolar, as appropriate, in 20.0 millilitres of calcium and magnesium free Ringer solution which had been made with distilled water from which dissolved CO_2 had been eliminated by boiling. The first of these samples was titrated with deci-normal sodium hydroxide. During the titration rapid mixing was ensured using a magnetic stirrer. From the data obtained the values of the stability constants were determined. The pKa values for tetracycline, oxytetracycline, chlortetracycline, all at a concentration of 2.0 millimolar, are reported in table 21. Further titrations were performed on three similar samples of the antibiotic in 20 ml of calcium and magnesium free Ringer, to which small volumes of calcium chloride were added corresponding to calcium concentrations of 0.5, 1.0 and 1.8 millimolar respectively. Plots of pH versus the volume of titrant added were drawn (figures 31 to 41).

APPARATUS :- The titration vessel (Metrohm Herisau EA 87 G 20) was maintained at 22° C by means of water from a thermostatically controlled bath being continuously circulated through the outer jacket which is an integral part of this vessel. The contents of the titration vessel were maintained under an atmosphere of oxygen-free nitrogen which, before being introduced into the titration vessel, had been bubbled successively through a series of three flasks. The first of these flasks contained normal hydrochloric acid which removed any traces of amines, any volatile HCl vapour being removed

during passage through the second flask which contained normal sodium hydroxide. Finally presaturation of the nitrogen was achieved as it bubbled through a quantity of Ringer solution in the third flask which was kept at 22° C in the water bath.

Titrant was introduced in 10 microlitre drops from a motor driven pipette of 1 ml volume (Metrohm Herisau Dosimat). pH readings were noted from a digital pH meter (PYE PW (9410/10)) connected to a combined glass and reference electrode (PYE INGOLD type 401 E07). Buffer solutions of pH 4.0, 7.0 & 9.0 (E.I.L. Ltd.) were used to check and adjust the calibration of the pH meter pH electrode system over the range pH 4.0 to 9.0. Temperature compensation was achieved manually.

SOLUTIONS :- Care was taken to eliminate a potential source of error which could give rise to incorrect pH readings as a consequence of the presence of carbonic acid, a weak acid formed from dissolved carbon dioxide in solution. All solutions were made up from glass distilled, de-ionised, water from which carbon dioxide was eliminated by boiling. Before use the distilled water was allowed to cool to room temperature in a flask containing a carbon dioxide absorbent in the air inlet tube. Solutions were then made up and stored in a nitrogen atmosphere.

Samples of tetracycline HCl, doxycycline HCl and oxytetracycline HCl were supplied by Pfizer Ltd.
Samples of chlortetracycline HCl and minocycline were supplied by Lederle Laboratories.

RESULTS :-

FIGURE 31

MAGNESIUM CHELATION BY TETRACYCLINE HCl ($1.0x10^{-3}$M)

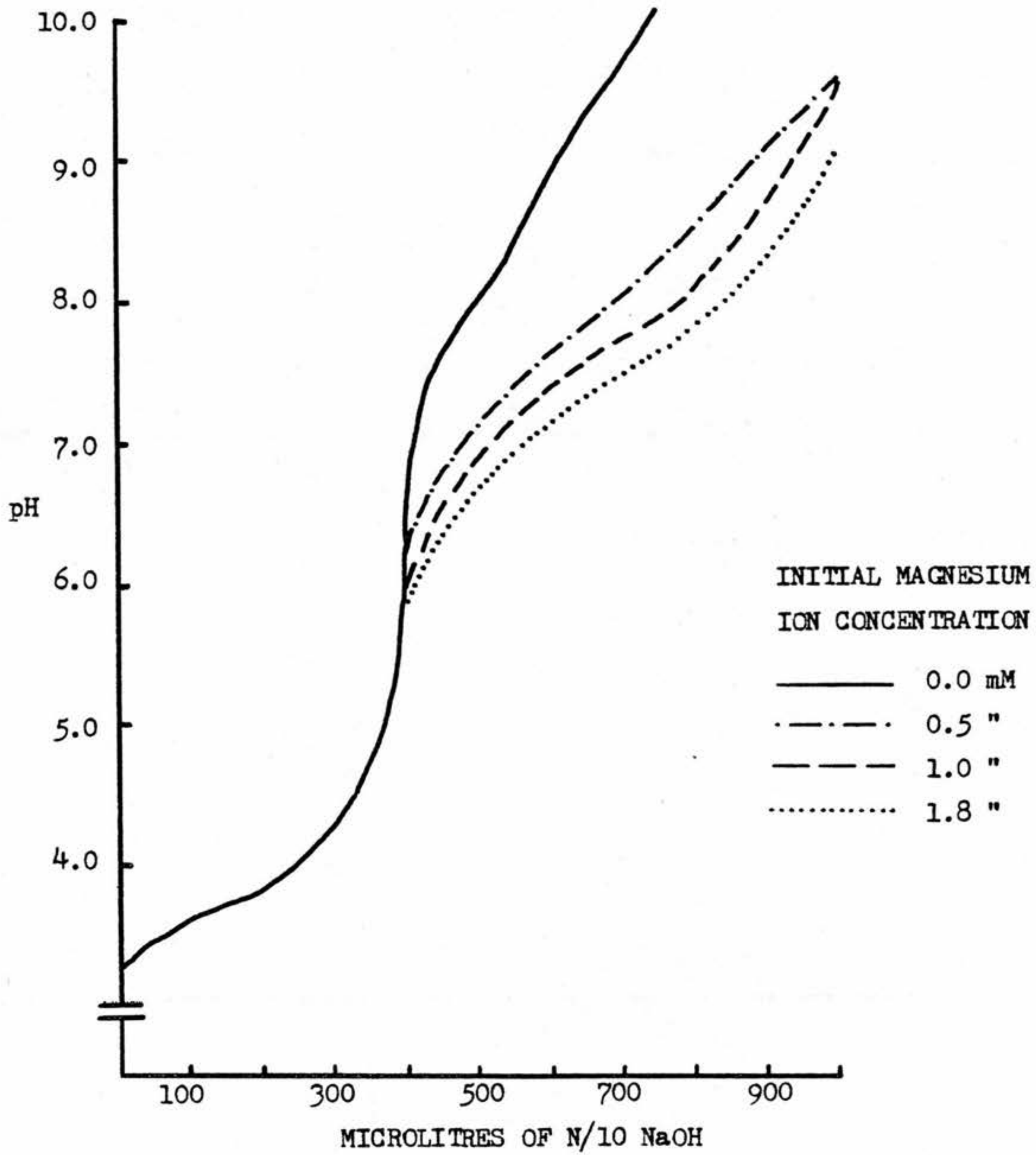


FIGURE 32

CALCIUM CHELATION BY TETRACYCLINE HCl ($1.0x10^{-3}$M)

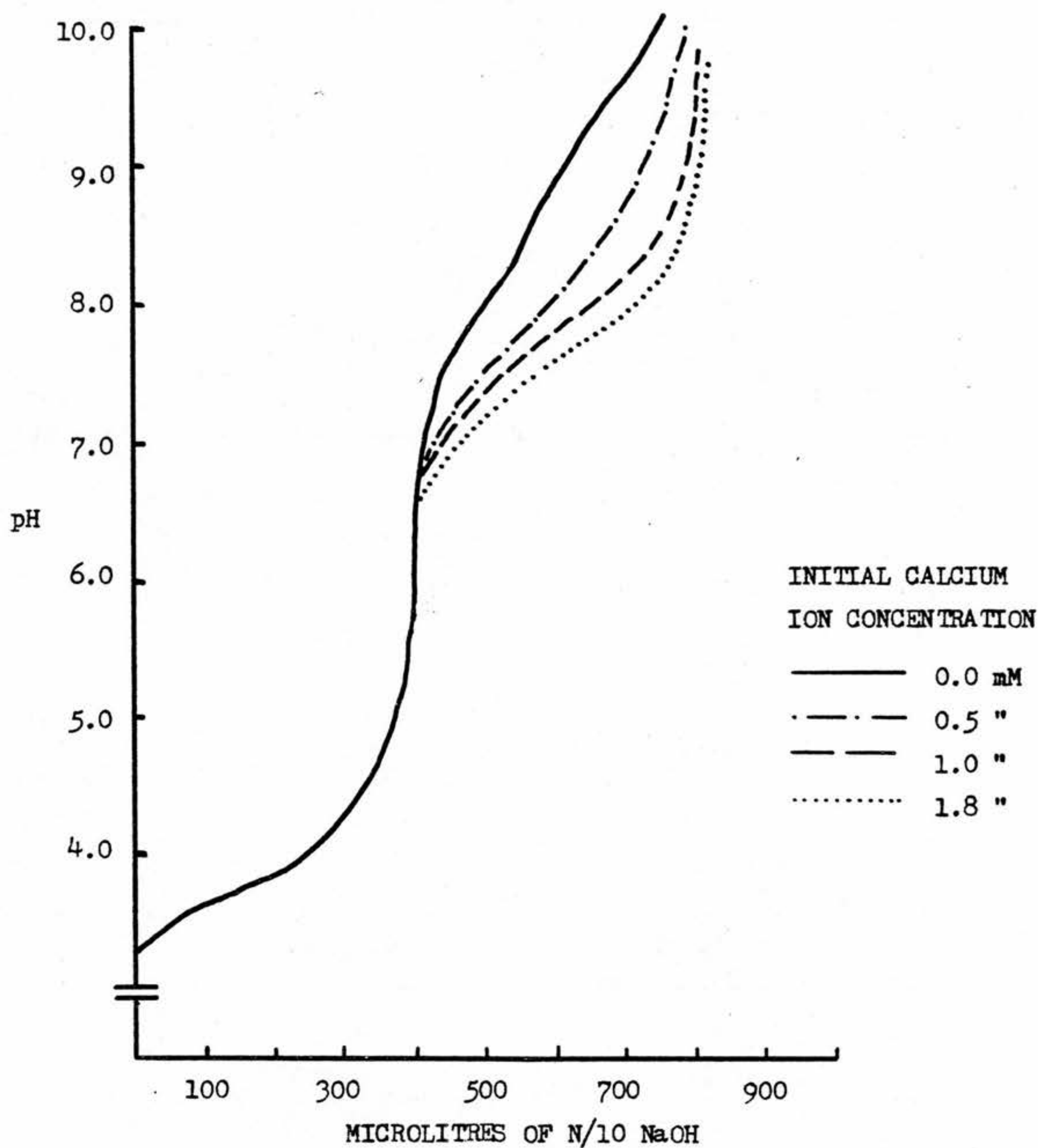


FIGURE 33

CALCIUM CHELATION BY TETRACYCLINE HCl ($2.0x10^{-3}M$)

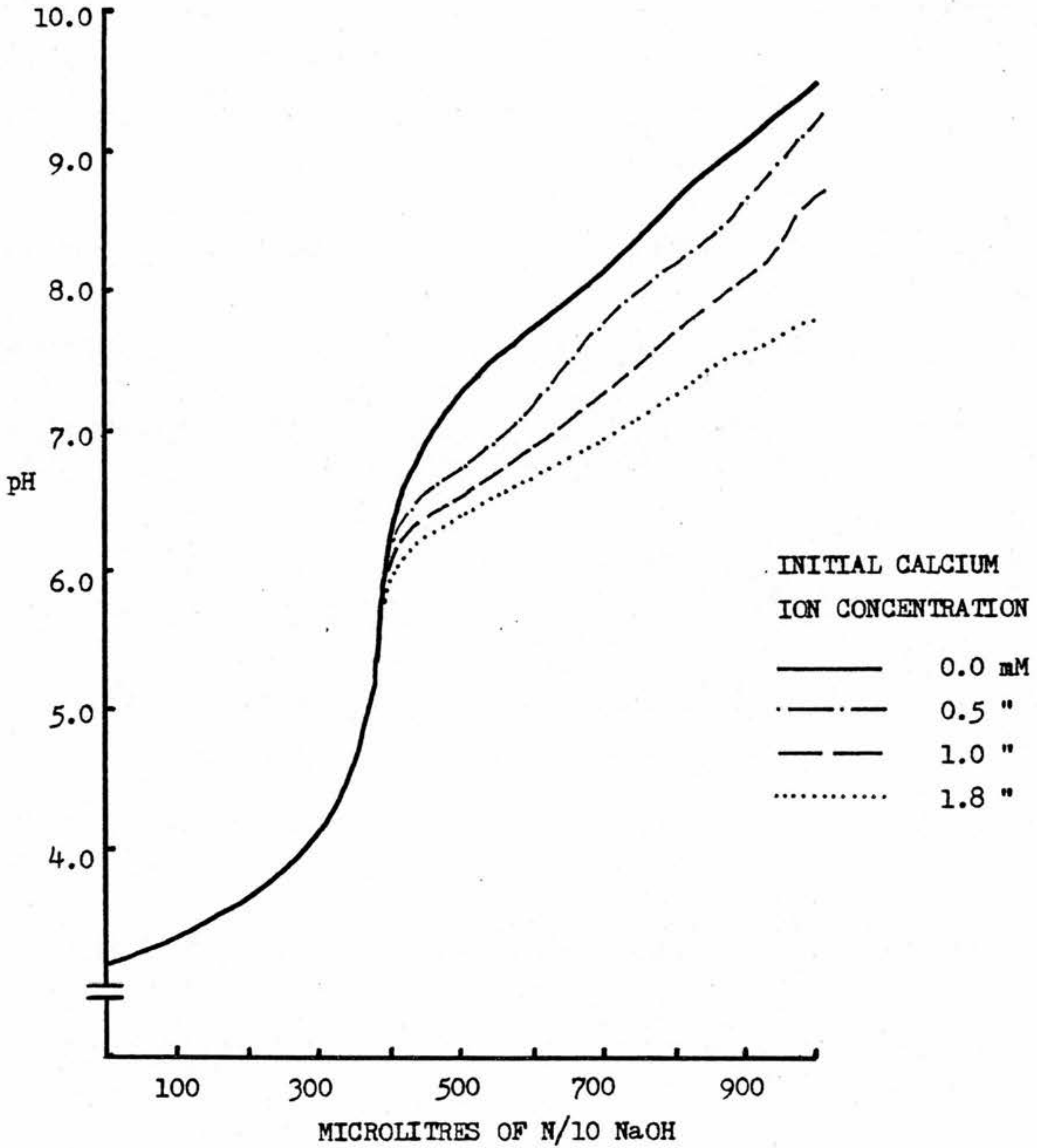


FIGURE 34

CALCIUM CHELATION BY OXYTETRACYCLINE HCl ($1.0x10^{-3}$M)

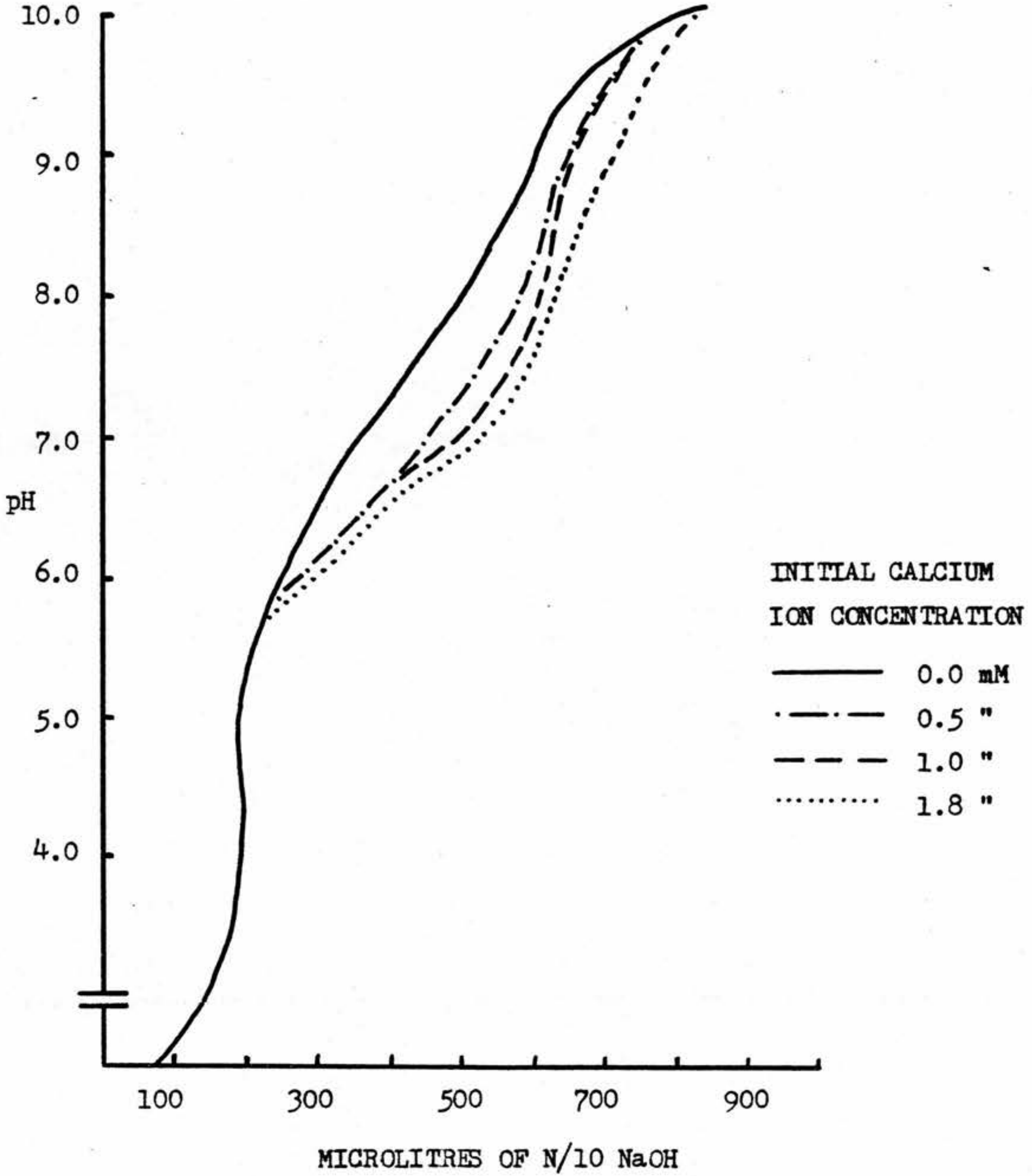


FIGURE 35

CALCIUM CHELATION BY OXYTETRACYCLINE HCl (2.0×10^{-3}M)

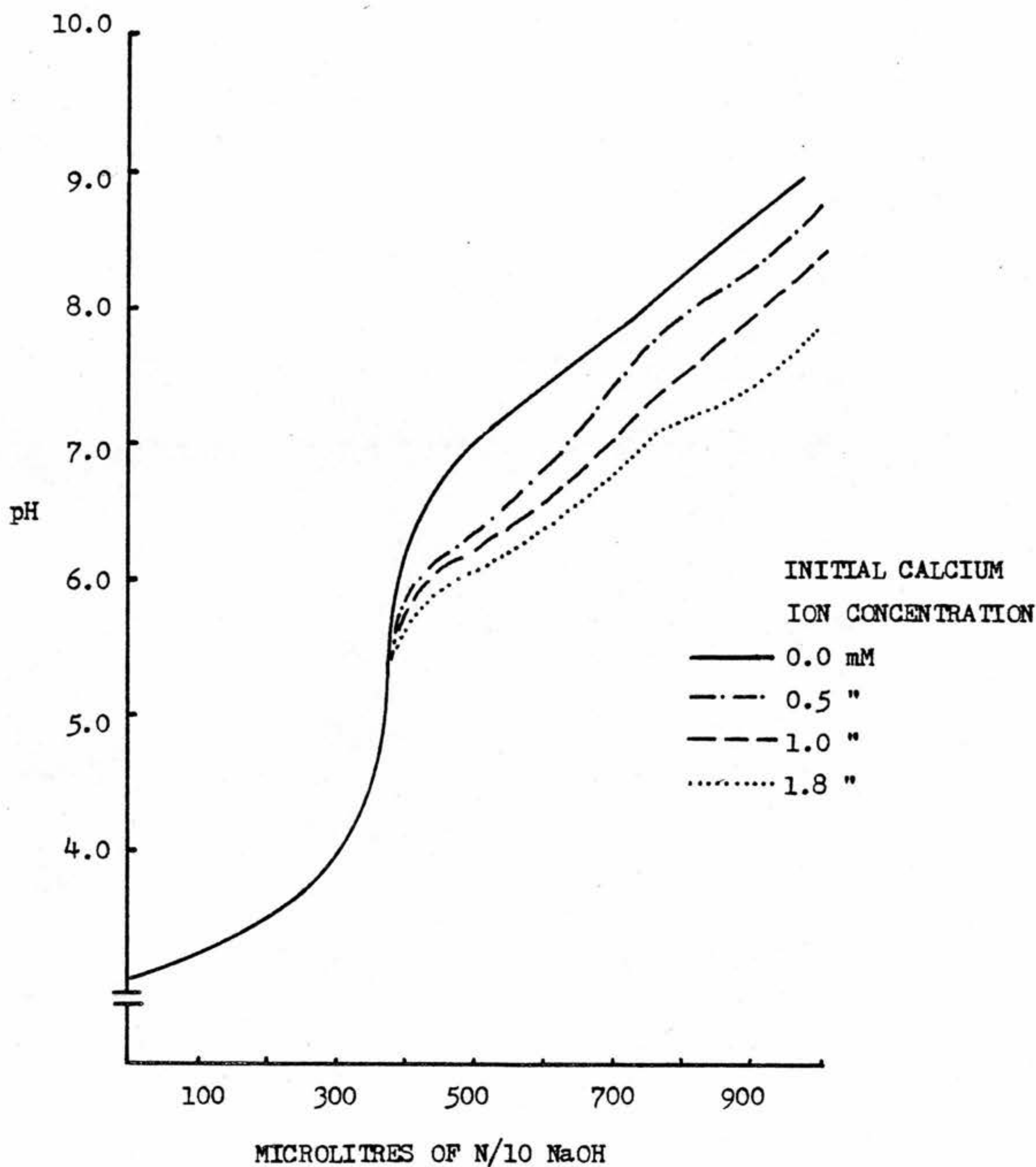


FIGURE 36

MAGNESIUM CHELATION BY OXYTETRACYCLINE HCl ($2.0x10^{-3}M$)

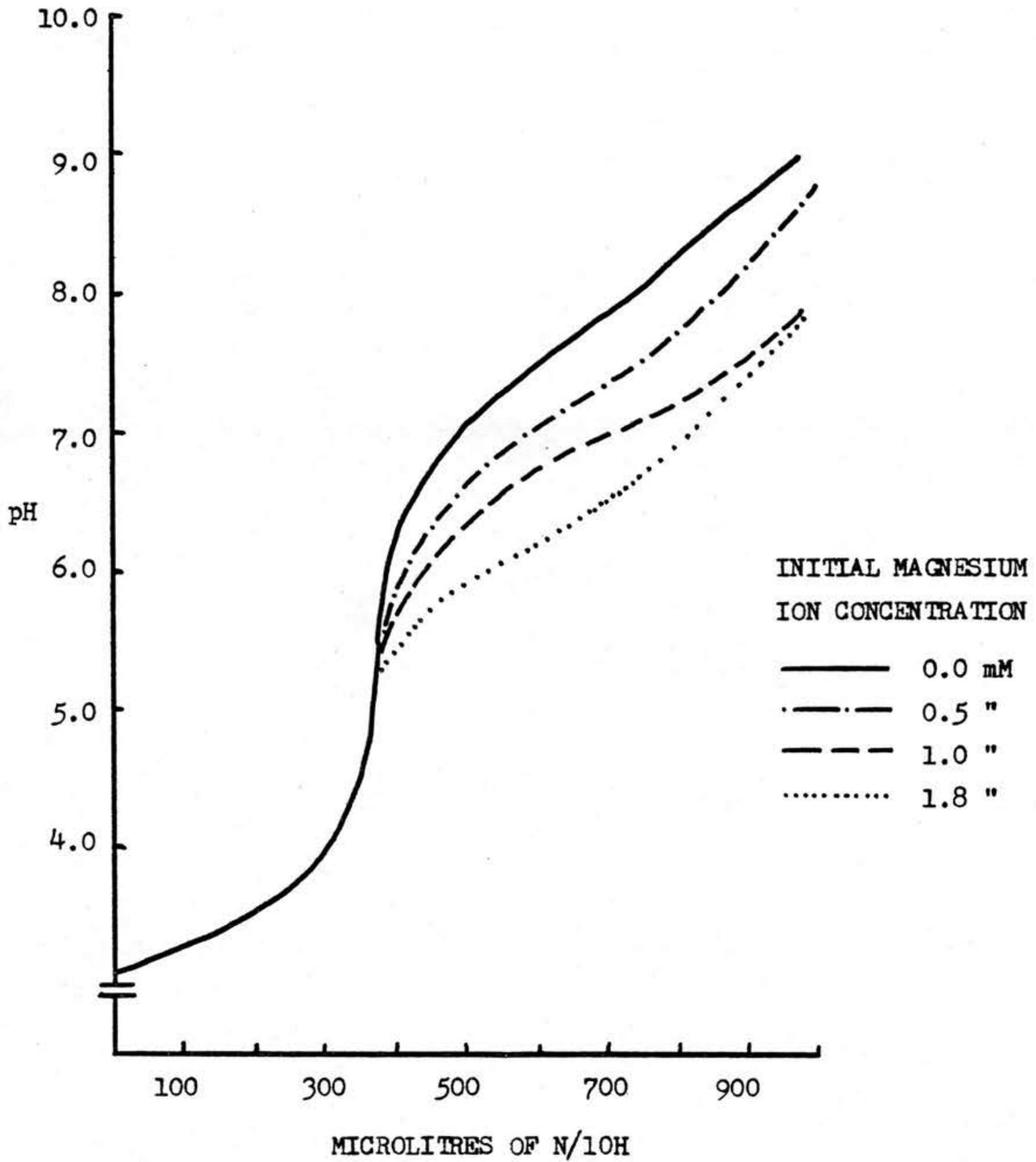


FIGURE 37

CALCIUM CHELATION BY MINOCYCLINE (1.0×10^{-3}M)

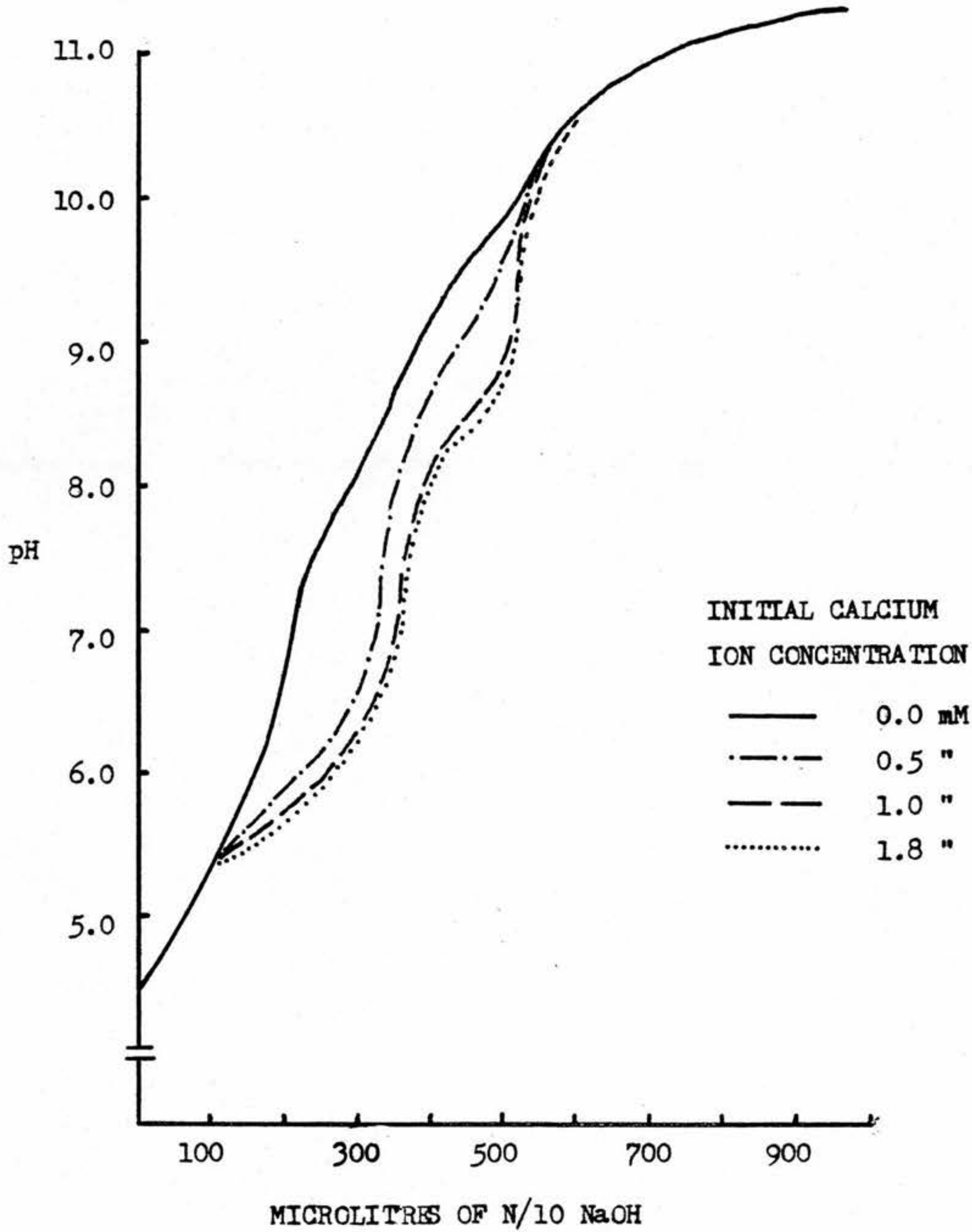


FIGURE 38

CALCIUM CHELATION BY MINOCYCLINE ($2.0x10^{-3}M$)

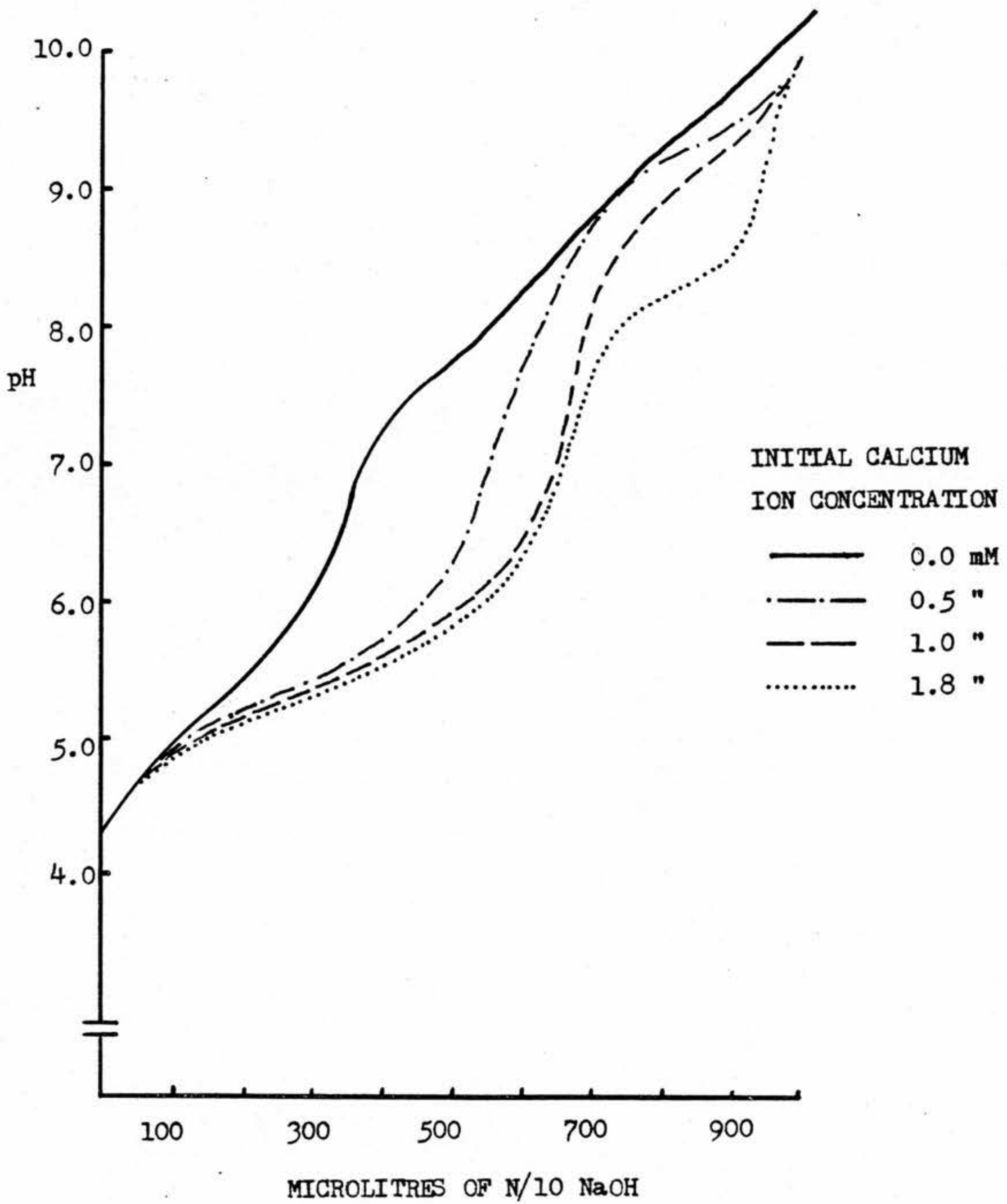


FIGURE 39

CALCIUM CHELATION BY CHLORTETRACYCLINE HCl ($2.0x10^{-3}M$)

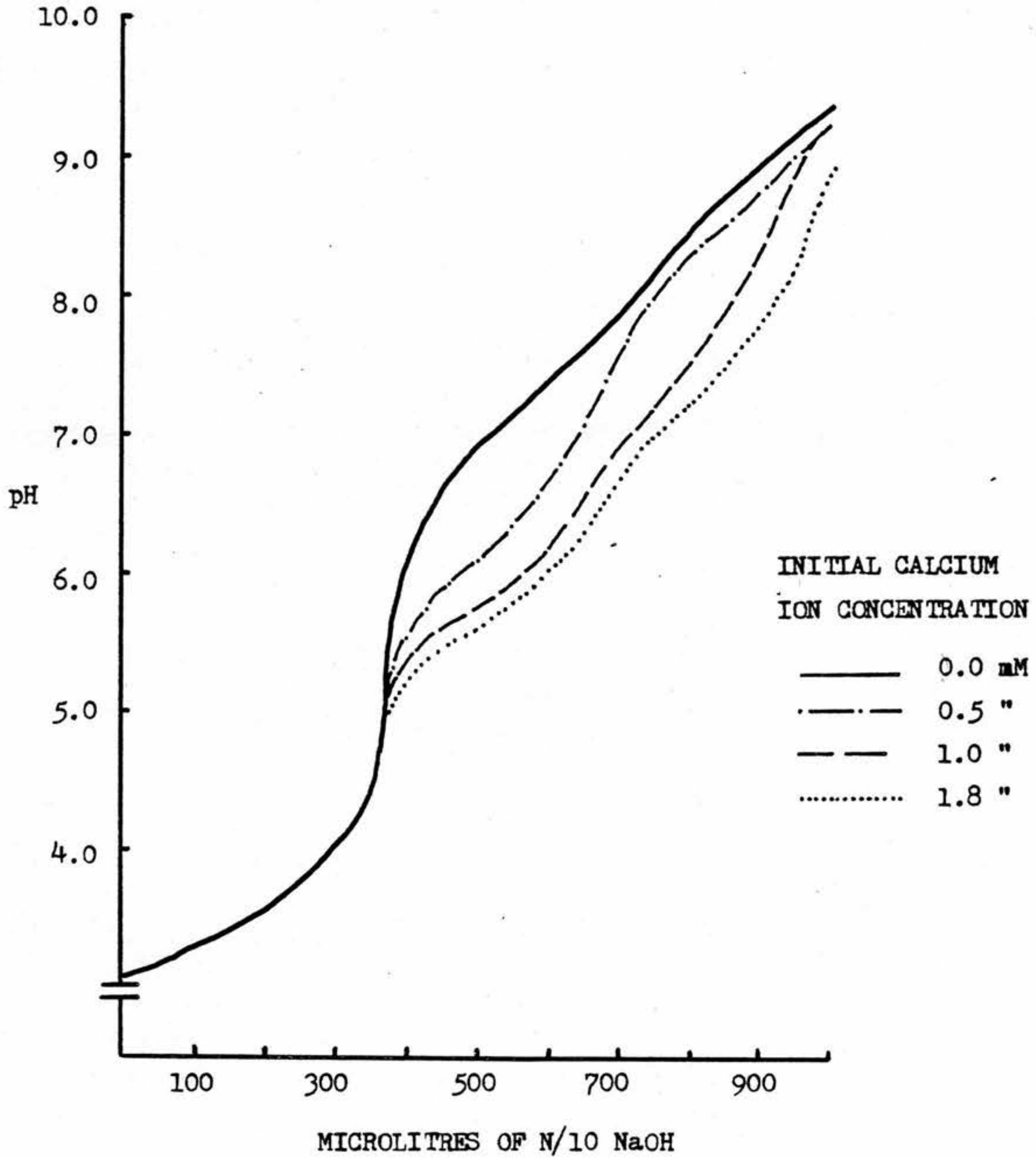


FIGURE 40

CALCIUM CHELATION BY DEMETHYLCHLORTETRACYCLINE HCl ($2.0x10^{-3}M$)

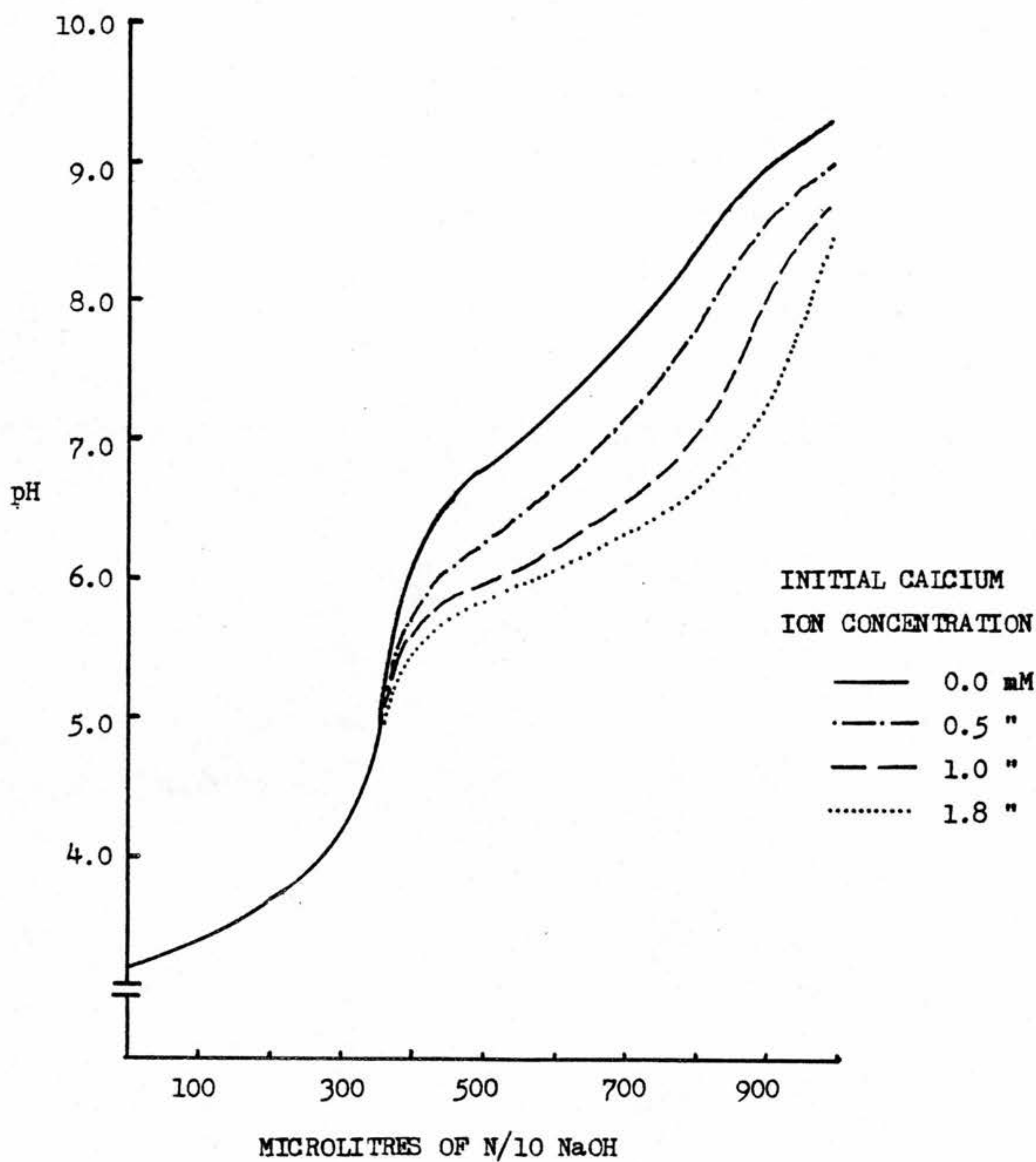


FIGURE 41

CALCIUM CHELATION BY DOXYCYCLINE HCl ($2.0x10^{-3}M$)

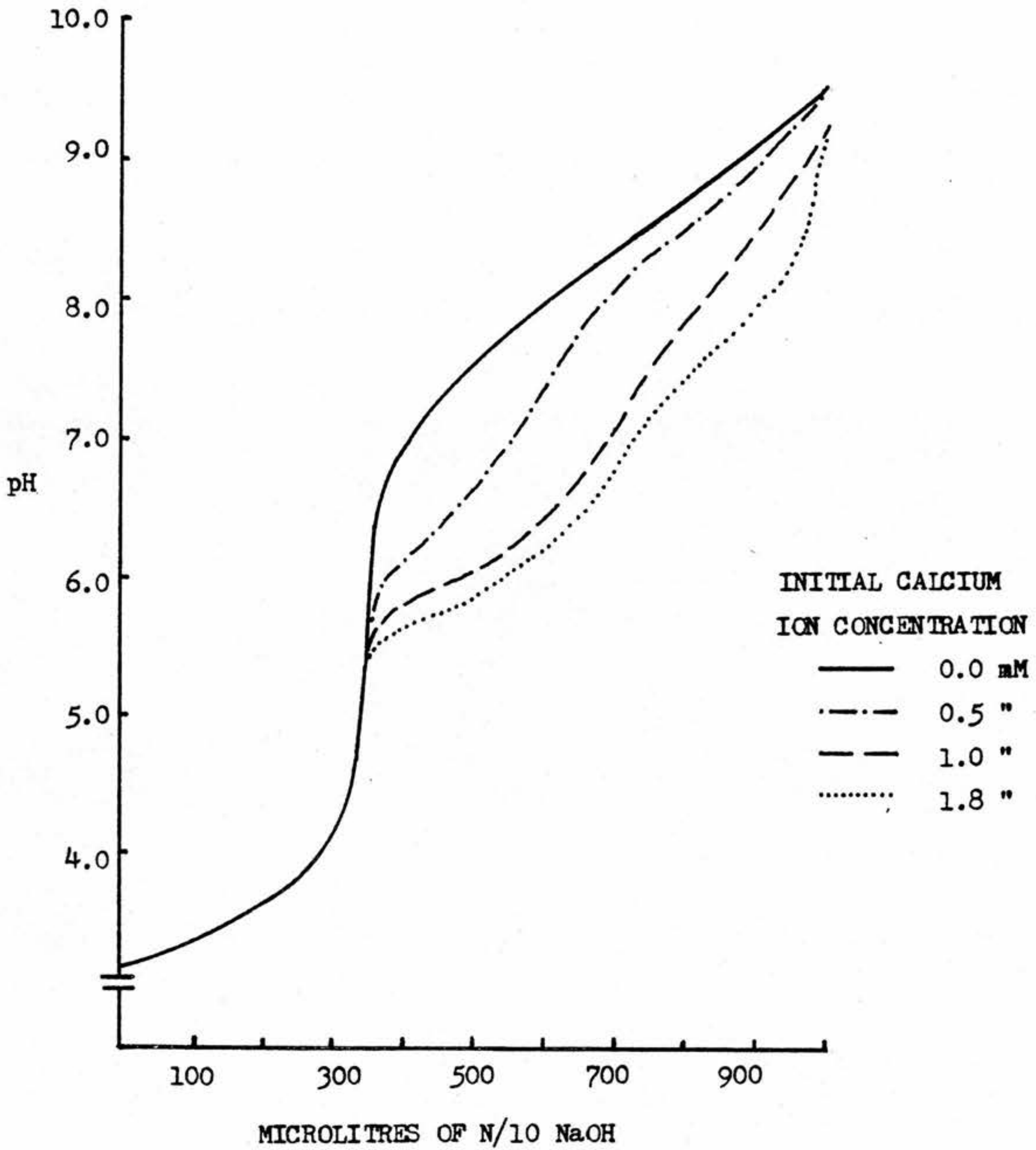


TABLE 22

TETRACYCLINE HCl (2.0 mM) TITRATION DATA

pKa values determined in Ringer solution

pKa_1	pKa_2	pKa_3
3.64	7.73	9.46
K_1	K_2	K_3
2.163×10^{-4}	2.630×10^{-8}	3.524×10^{-10}

pH 7.0

nominal calcium concentration (mM)	0.5	1.0	1.8
total volume of solution (ml)	20.77	21.04	21.50
volume corrected calcium concentration (mM)	0.482	0.950	1.674
volume corrected tetracycline concentration (mM)	1.92	1.90	1.86
volume corrected NaOH concentration (mM)	2.74	3.06	3.63

pH 7.4

total volume of solution (ml)	20.84	21.13	21.65
volume corrected calcium concentration (mM)	0.480	0.946	1.662
volume corrected tetracycline concentration (mM)	1.92	1.89	1.84
volume corrected NaOH concentration (mM)	3.06	3.47	4.31

TABLE 23

TETRACYCLINE HCl (2.0 mM)

% OF CALCIUM BOUND EVALUATED FROM DOLUISIO & MARTIN EQUATIONS

nominal calcium concentration (mM)	0.5	1.0	1.8
% calcium bound at pH 7.0	34.4	49.2	47.9
% calcium bound at pH 7.4	64.5	61.1	67.3

% OF CALCIUM BOUND EVALUATED FROM GINSBORG & WALKER EQUATIONS

nominal calcium concentration (mM)	0.5	1.0	1.8
% calcium bound at pH 7.0	109.7	101.5	104.3
% calcium bound at pH 7.4	127.7	144.9	174.2

The calculated % of the initial calcium ion concentration which is bound by the tetracycline is given by

$$\frac{\text{calculated concentration of bound calcium}}{\text{initial calcium ion concentration}} \times 100$$

TABLE 24

TETRACYCLINE HCl (1.0 mM) TITRATION DATA

pH 7.0

nominal calcium concentration (mM)	0.5	1.0	1.8
total volume of solution (ml)	20.51	20.74	21.11
volume corrected calcium concentration (mM)	0.488	0.964	1.706
volume corrected tetracycline concentration (mM)	1.01	1.00	0.983
volume corrected NaOH concentration (mM)	1.50	2.07	1.824

pH 7.4

total volume of solution (ml)	20.57	20.83	21.21
volume corrected calcium concentration (mM)	0.486	0.960	1.697
volume corrected tetracycline concentration (mM)	1.01	0.998	0.980
volume corrected NaOH concentration (mM)	1.822	2.064	2.310

TABLE 25

TETRACYCLINE HCl (1.0 mM)

% OF CALCIUM BOUND EVALUATED FROM DOLUISIO & MARTIN EQUATIONS

nominal calcium concentration (mM)	0.5	1.0	1.8
% calcium bound at pH 7.0	36.8	51.4	21.8
% calcium bound at pH 7.4	59.5	31.1	35.6

% OF CALCIUM BOUND EVALUATED FROM GINSBORG & WALKER EQUATIONS

nominal calcium concentration (mM)	0.5	1.0	1.8
% calcium bound at pH 7.0	73.0	113.0	47.1
% calcium bound at pH 7.4	139.5	115.6	91.92

DISCUSSION :- The equations used by DOLUSIO & MARTIN (1963) in their study of metal complexation by tetracycline hydrochlorides are essentially those used by ALBERT (1952) in a quantitative study of the avidity for trace metals shown by certain amino acids having three ionising groups. The constants governing the equibibria between such amino acids and the ions of heavy metals were evaluated using equations, the derivation of which was reported by J.N. PHILIPS in an appendix to ALBERT'S 1952 paper.

There is, however, a defect in these equations as they have been applied to the calculation of the proportion of divalent cation bound by tetracycline. DOLUISIO & MARTIN correctly state that:-

$$3\,To - NaOH = \text{total } [H^+] \text{ concentration}$$
$$= \text{bound hydrogen concentration} + [H^+] - [OH^-] \quad \ldots\ldots (13)$$

However, they maintain that the bound hydrogen concentration is given by:-

$$3\,[H_3A^+] + 2\,[H_2A] + [HA^-] \quad \ldots\ldots (14)$$

hence leading to equation 10. It can be shown that:-

$$3\,To - [NaOH] = \beta[HA^-] + [CaHA^+] = \beta[HA^-] + [\bar{Ca}] \quad \ldots\ldots (15)$$

where β is the expression given in equation 11. Thus the DOLUISIO & MARTIN equations may lead to an underestimate of the amount of calcium bound by tetracycline (see tables 23 and 25).

It is generally supposed that the calcium chelating species of tetracycline corresponds to HA^- giving rise to the calcium complex corresponding to CaHA, this is equivalent to supposing that the binding

of one calcium ion displaces two hydrogen ions. If this were so, it may be shown (B.L. GINSBORG AND J. WALKER, personal communication) that the concentration of free ionised calcium in the solution is given by:-

$$[Ca^{++}] = [Ca_o] - \frac{([NaOH] + [H^+] - [OH^-]) - (3 - \beta_1/\alpha)\ To}{\beta_1/\alpha - 1} \quad .... (16)$$

$$\text{where } \beta_1 = \frac{[H^+]}{K_3} + \frac{[H^+]^2}{K_2\ K_3} + \frac{3\ [H^+]^3}{K_1\ K_2\ K_3} \quad .... (17)$$

$$\text{and } \alpha = 1 + \left\{ \frac{[H^+]}{K_3} + \frac{[H^+]^2}{K_2\ K_3} + \frac{[H^+]^3}{K_1\ K_2\ K_3} \right\} \quad .... (18)$$

Substitution of experimental values from tables 22 and 24 leads to an improbably high value for the amount of calcium chelated by the tetracycline, (tables 23 and 25) which is inconsistent with the results obtained from those twitch tension experiments (chapter 4) and the averaged EPP amplitude experiments (chapter 10) in which the initial calcium ion concentration greatly exceeded that of the antibiotic (by a ratio of 4.6 : 1 in figure 23).

The most plausible defect in the theory outlined above would appear to be the assumption, first made by ALBERT (1953) and accepted by DOLUISIO & MARTIN (1963), that chelation is confined to the tetracycline species corresponding to HA^-. If the anion A^{--} also contributed and the complex CaA were also formed then the binding of one calcium ion would, on average, "displace" more than two hydrogen ions. This would explain why the forgoing method of calculation (equations 16, 17 & 18) would exaggerate the amount of bound calcium and thus underestimate the concentration of free calcium.

CONCLUSIONS :- As a method of determining the free calcium ion concentration in the presence of tetracycline the potentiometric titration technique has proved to be ineffective. This is primarily due to the probability that chelation may not, as has previously been supposed, be limited to the tetracycline species corresponding to HA^-. The complex CaA may be formed if the anion A^{--} is also involved in the chelation process. Furthermore, the evidence available from the literature on whether the calcium tetracycline complex forms in a ratio of 1 : 1, 2 : 1 or a mixture of them both is somewhat equivocal. It was therefore considered that the persuance of these questions is outwith the scope of this thesis.

CHAPTER 15

SUMMARY OF RESULTS AND CONCLUSIONS

SUMMARY OF RESULTS AND CONCLUSIONS

In chapter 1 the general scope of blockade of the neuromuscular junction by antibiotics is outlined and the literature relevant to the tetracycline group of antibiotics is reviewed. In chapter 2 the data from the in vitro experiments on the isolated hemidiaphragm preparation of the rat confirm the initial observations reported by BEZZI & GESSA (1959, 1960) that tetracyclines potentiate the paralysing action of tubocurarine. The work has been extended to include minocycline and also to demonstrate that at a sufficiently high concentration these antibiotics can bring about a decrease in twitch tension even in the absence of tubocurarine.

It was considered that numerous practical advantages were to be gained by using the isolated sciatic nerve sartorius muscle preparation of the frog rather than continuing to use the phrenic nerve hemidiaphragm preparation of the rat. A series of experiments were conducted in order to establish that the tetracycline group of antibiotics also exert a neuromuscular blocking action in the frog sartorius preparation; the experimental evidence is presented in chapter 3.

Tetracycline and its derivatives are only slightly soluble in water. A 1% solution of these antibiotics in water has a pH of 1.8 to 2.8 and since Ringer solution has no inherent buffering capability it is essential to utilise a buffering agent to maintain the pH of the solution at a more physiologically suitable value. Solutions were buffered at pH 7.0 rather than 7.4 since tetracyclines are slowly destroyed in solutions of pH 7.0 or higher; in general the destruction being more rapid the more alkaline the pH. The buffering agent HEPES was selected on the grounds

that it exerted no detectable effect on the quantal content of evoked release from the frog sartorius preparation and also that it has no reported effect on calcium ion concentration.

That the reduction in twitch tension developed in the frog sartorius preparation in response to stimulation of the sciatic nerve is due to some action of tetracycline exerted at the neuromuscular junction rather than by some action upon either the excitation contraction coupling mechanism or directly upon the contractile apparatus of the muscle fibres themselves is demonstrated in those experiments in which the muscle fibres remain responsive to direct stimulation but do not respond to stimulation via the nerve.

In what has been designated by PITTINGER (1972) as "the chelation hypotheses" CORRADO (1963) postulated that the neuromuscular blockade by kanamycin, neomycin and streptomycin was due to the ability of these antibiotics to reduce the level of ionised calcium in solution. CORRADO'S concept was consistent with the known potentiality of kanamycin, neomycin and streptomycin to form complexes with calcium.

Since tetracycline and its derivatives also possess chelating properties (ALBERT 1953, 1956) does CORRADO'S assertion that streptomycin, neomycin and kanamycin bring about neuromuscular blockade by virtue of a reduction in the level of ionised calcium also apply to the neuromuscular blocking action exerted by the tetracycline group of antibiotics? In order to answer this question it was necessary to determine:-

(A) just how low the calcium concentration of the extracellular solution must be before there is any perceptible effect on twitch tension,

(B) the avidity with which the tetracycline antibiotics form complexes with calcium, thereby leading to a decrease in the calcium ion concentration available in the extracellular fluid.

In chapter 5 the effect of calcium concentration on the development of twitch tension in the indirectly stimulated sciatic nerve sartorius muscle of the frog was investigated. It was found that at the low stimulation rate of one stimulus every 30 seconds used in the twitch tension experiments no perceptible reduction in the twitch tension occurred when the calcium concentration was decreased from the normal value of 1.8 mM to 0.5 mM. However when the calcium concentration was reduced to 0.25 mM the twitch tension decreased by 90%, indicating that if tetracyclines effect their neuromuscular blocking action in the manner proposed by CORRADO for streptomycin and other antibiotics, tetracyclines must be capable of reducing the calcium ion concentration of Ringer solution from the normal value of 1.8 mM to some level rather less than 0.5 mM.

In their 1976a paper WRIGHT & COLLIER state that the neuromuscular blocking potency of most of the tetracyclines could not be tested because of their poor solubility in aqueous media. They limited their investigation of the neuromuscular blocking effects of these antibiotics on twitch tension in the rat phrenic nerve hemidiaphragm preparation to two soluble tetracyclines, rolitetracycline and oxytetracycline. Rolitetracycline, they found, was the more potent blocking agent, producing 50% inhibition of the response to nerve stimulation, with concentrations in the range 5 to 8 x 10^{-4} M. No figures were quoted, however, concerning the concentration of oxytetracycline required to produce a 50% decrease in the response. From the results of the twitch tension experiments on

the rat diaphragm preparation as detailed in chapter 2 rolitetracycline is a more potent neuromuscular blocking agent than either minocycline or tetracycline hydrochloride. When tested in the absence of d-tubocurarine a concentration of minocycline of about 1.3×10^{-3} M was required in order to bring about a 50% decrease in the twitch tension developed in response to stimulation via the phrenic nerve. A similar concentration of tetracycline hydrochloride brought about only a 38% decrease in the response.

One feature of the neuromuscular blocking action of the tetracyclines reported by WRIGHT & COLLIER (1976a), namely that both rolitetracycline and oxytetracycline produced an initial augmentation of the twitch tension developed in response to stimulation via the nerve, was not observed in the experiments described in chapter 2. SINGH et al (1978b) also observed an initial augmentation of the twitch response of the mouse phrenic nerve hemidiaphragm preparation in response to stimulation via the nerve when either tetracycline or oxytetracycline was present. They also reported that wash-out of the tetracyclines was often accompanied by a strong contracture. No such contracture was observed in the rat diaphragm experiments of chapter 2, however the concentrations of the antibiotics used in these experiments were 1.0 mM or less compared to the 10 to 20 mM used by SINGH et al (1978a,b).

In keeping with the comment made by WRIGHT & COLLIER (1976a) concerning the poor solubility of some of the tetracycline antibiotics in aqueous media some difficulty was experienced in obtaining solutions of these antibiotics in excess of 4 mM in Ringer solution. SINGH et al apparently encountered no such difficulty since they were able to use

Krebs-Henseleit solution containing tetracycline hydrochloride at a concentration of 20.5 mM. However their solutions were maintained at 32° C as opposed to the 20° C of the Ringer solution.

The concentrations of antibiotics used by SINGH et al to produce between an 80 and 90% block were, oxytetracycline 10.4 ± 0.2 mM and tetracycline 20.5 ± 0.3 mM. Concentrations of antibiotic which greatly exceeds the calcium concentration of 2.6 mM present in the Krebs solution. It is therefore probable that virtually all of the calcium in the extracellular fluid has formed chelation complexes with the tetracycline. In skeletal muscle there is a considerable reserve of calcium ions stored in the sarcoplasmic reticulum thus rendering skeletal muscle less dependent on extracellular calcium ions to support contraction. The decrease in twitch amplitude in response to direct stimulation of the muscle as reported by SINGH et al (1978b) is probably due, in part, to the depletion of the calcium stores in the sarcoplasmic reticulum.

As WRIGHT & COLLIER (1976a) observed in the rat diaphragm preparation, figures 7 and 9 of chapter 4 show that also in the frog sciatic nerve sartorius preparation the twitch amplitude elicited in response to direct stimulation of the muscle is slightly greater than that elicited by stimulation via the nerve. These records also demonstrate that although the antibiotics may have some direct effect on the muscle as indicated by the slow decrease in the twitch amplitude of the directly stimulated response, the rate of decrease in the indirectly stimulated twitch amplitude is very much greater. However in the frog sartorius preparation there was no initial period of twitch augmentation or contracture of the muscle during wash-out

following exposure to tetracyclines as reported by SINGH et al (1978b) during their experiments with the mouse hemidiaphragm preparation.

For various technical reasons measurement of the free calcium ion concentration by means of a calcium ion selective electrode proved to be unreliable when used in the presence of tetracyclines. An alternative approach (as detailed in chapter 14) was adopted which involved the determination of stability constants for the tetracycline calcium chelation complex. It has been generally supposed that chelation is confined to the tetracycline species corresponding to HA. Applying the DOLUISIO & MARTIN equations to titration data indicates that tetracycline HCl at a concentration of 2.0 mM would reduce the free calcium ion concentration by about 50% at pH 7.0. However, a particular shortcoming in the derivation of the DOLUISIO & MARTIN equations has been demonstrated. The alternative approach suggested by GINSBORG & WALKER, when applied to the same data, indicates that all of the calcium in the Ringer would be bound by the tetracycline (table 23). This, however, is inconsistent with results from experiments such as those illustrated in figure 8 and in chapter 10 where the initial calcium ion concentration exceeds that of the antibiotic by factor of 2 or 3 : 1. As a method of determining the free calcium ion concentration in the presence of tetracycline the potentiometric titration technique was not satisfactory. It is probable that more than one species of tetracycline is involved in the chelation process.

Evidence that the tetracyclines do not bring about neuromuscular blockade merely by means of a reduction in calcium ion concentration is demonstrated in those experiments in which in order to compensate for the reduction in the calcium ion concentration resulting from chelation

by 2.0 x 10^{-3} M tetracycline HCl the calcium concentration of the Ringer solution was raised from 1.8 to 5.0 mM, but complete loss of twitch tension still occurred. Clearly CORRADO'S hypotheses of antibiotic blockade of the neuromuscular junction solely by means of the formation of an antibiotic calcium complex does not apply to the neuromuscular blockade effected by tetracycline and its derivatives.

The "competitive hypotheses" postulated in relation to the neuromuscular blocking action of neomycin and gentamicin by VITAL BRAZIL & PRADO-FRANCESCHI (1969) likens the antibiotic effects to those of magnesium ions. This hypotheses recognises the decreased sensitivity of the motor endplate to the depolarising action of acetylcholine but stresses the inhibition of its prejunctional release. It is to this latter effect that the "competitive" aspect of the hypotheses relates.

Electrophysiological recording techniques were employed to further investigate the neuromuscular blocking action of tetracyclines. The estimation of the number of quanta of acetylcholine released per impulse is discussed in chapter 6 and the electrophysiological recording techniques which were adopted are described in chapter 7. From the results tabulated in chapter 9 it can be seen that tetracycline HCl, chlortetracycline HCl, oxytetracycline HCl and minocycline all exert both a presynaptic and a postsynaptic effect. The postsynaptic effect is demonstrated by the reduction in MEPP amplitude; the presynaptic effect is demonstrated by the reduction in the quantal content of the evoked release of acetylcholine and also, in the case of minocycline, the large increase in MEPP frequency. The time-course of the reduction in both EPP and MEPP amplitudes was followed in the experiments detailed in chapter 10 where it was found that these antibiotics exert their

effect on neuromuscular transmission commencing almost immediately following their introduction into the recording chamber. Further evidence against CORRADO'S hypotheses is obtained from those experiments in which the calcium concentration of the tubocurarine containing Ringer solution was increased from the normal concentration of 1.8 to 2.3 mM at a time after the antibiotic decrease in EPP amplitude had become established. The EPP amplitude continuing to decrease even in the presence of the increased calcium ion concentration.

A complicating factor in the quantal content experiments was that in nearly all instances there was a decrease in trans-membrane potential, in the order of 10 millivolts, following the introduction of the antibiotic into the bathing medium. In most of the electrophysiological experiments the microelectrode remained in a particular muscle fibre, in close proximity to an endplate zone, for considerable periods of time. A small amount of damage to the membrane must inevitable be associated with the insertion of a microelectrode. Therefore in order to eliminate the possibility of the decrease in resting potential being an artefact due to the antibiotic gaining access into the interior of the muscle fibre via the damaged membrane surrounding the recording electrode the experiments detailed in chapter 13 were conducted.

The results of these experiments confirmed that the decrease in the resting potential of the muscle fibre membrane associated with the presence of tetracycline, chlortetracycline and minocycline is a statistically significant effect; oxytetracycline however produced no statistically significant decrease in membrane potential. Of

some 290 fibres whose resting potentials were sampled following a one hour exposure to antibiotic only 10 fibres had a resting potential of -60 millivolts or less; a value below which a muscle fibre is unlikely to produce a propagated muscle action potential in response to neural stimulation and will therefore not contribute to the development of twitch tension. Since less than 4.0% of the fibres sampled has a resting potential of less than -60 mV (B.L. GINSBORG, Personal communication) it is unlikely that the effect of the antibiotic on the muscle fibre membrane potential is a significant component of the neuromuscular blocking action of the tetracycline group of antibiotics.

This reduction in resting potential has however taken into consideration when calculating values for the quantal content of evoked transmitter release in the presence of tetracyclines. From the various sample traces of EPP and MEPP recordings it can be seen that the level of background noise is quite low, resulting in a good signal to noise ratio. Consequently it was considered unnecessary to subtract the variance of the noise level from the EPP variance when calculating values for quantal content from the variance of EPP amplitudes.

Despite the decrease in the resting potential of the muscle fibre membrane in the presence of tetracycline HCl, chlortetracycline HCl and minocycline, no statistically significant alteration in the values of the muscle fibre membrane constants ($P < 0.05$) were observed under these conditions.

The twitch tension experiments described in chapter 4 demonstrate that tetracycline HCl at a concentration of 2.0×10^{-3} M results in a complete loss of twitch tension in response to supramaximal stimulation

of the nerve. In chapter 11 a series of experiments were conducted in order to determine what the quantal content of evoked release would be under similar circumstances. When an endplate was located, utilising the technique and acceptance criteria as detailed in chapter 11, the development of the postsynaptic effect of the tetracyclines could be followed by observing the decrease in the average MEPP amplitude.

Once the neuromuscular block due to the tetracycline had become established to the extent that the recording microelectrode was not disturbed by movement of the muscle fibres when supramaximal stimuli were applied to the nerve, the presynaptic action of tetracycline in bringing about a reduction in the quantal content could be monitored. Various estimates (chapter 6) give a value in the region of at least 100 for the number of quanta normally released per nerve impulse. Following 60 minutes exposure to tetracyclines the average quantal content was reduced to 11.5 ± 2.8 (standard error of the mean, $N = 8$). Minocycline also produces a further presynaptic effect, namely the substantial increase in the rate of the spontaneous release of acetylcholine.

Evidence has been presented which demonstrates that the tetracycline group of antibiotics give rise to blockade of the neuromuscular junction by means of both a pre and postsynaptic action; also that the presynaptic action is not primarily due to a reduction in the ionised calcium concentration as a consequence of the formation of chelation complexes with the antibiotic.

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