CONTRACTION OF MUSCLE IN ALTERED IONIC ENVIRONMENT

by

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TABLE OF CONTENTS

SUMMARY

This thesis contains three experimental sections concerned with changes that occur in the twitch response of mammalian muscle to alterations in the local environment of the cell. The isolated rat diaphragm bathed in Synthetic Interstitial Fluid was used as the test preparation.

GENERAL INTRODUCTION:

The processes involved in the elicitation of a mechanical response from a muscle stimulated electrically, are reviewed. The areas of this process - Excitation Contraction Coupling - that are of particular relevance to this study are defined as the sarcolemma membrane, and the sarcotubular system.

SECTION A:

The effects of temperature on the twitch response of the preparation to direct stimulation, have been observed. Parameters of the isometric myogram ie. Tension, Contraction Time and Half Relaxation Time were assessed at temperatures from 5 to 45°C and were compared with similar literature values. The twitch demonstrates increased speed of both contraction and relaxation as temperature rises while twitch tension displays peaks near 15 and 40°C. The rat diaphragm is a muscle consisting of similar proportions of both slow and fast fibres which demonstrate differential temperature dependence. The peak in twitch tension near

15°C is associated with the fast muscle fibres, while the slow muscle component is responsible for the anomolous peak near 40°C.

SECTION B:

The effects of some potential stabilising agents were assessed on the impermeant anion, 'chloride free', treated preparation. This preparation simulates the responses of myotonic muscle. High K^+ (25 mM), while it did not produce contracture, eliminated the delayed relaxation of mechanical activity normally observed when the impermeant anion was substituted for chloride ion, provided it was added prior to Cl^- replacement. γ - amino-butyric acid (GABA) and glycine were ineffective in opposing the chloride free effect which became apparent in this preparation when Cl^- concentration in the bathing solution was less than 42 mM.

SECTION C:

2,4-Dichlorphenoxyacetate (2,4-D) and 20,25-Diazacholesterol (DAC) treated muscle was examined for evidence of in vitro myotonic mechanical activity. Interaction of these two drugs produced significant myotonia - delayed relaxation in response to single stimuli. Short trains of stimuli were effective in eliciting myotonic responses from 2,4-D treated muscle; these were opposed by addition of GABA to the bathing solution. Since one significant myotonic effect of 2,4-D (and DAC) is to cause a decrease in muscle membrane resting chloride conductance, the GABA effect may indicate a small specific action of this amino acid to increase chloride conductance in skeletal muscle, thereby acting as a weak though specific myotonic membrane, stabiliser.

DECLARATION

I declare this thesis to be a record of original work containing no material that has been accepted for the award of any other degree or diploma in any University.

To the best of my belief and knowledge, no material previously published or written by another person has been included without due reference in the text of the thesis.

R. G. D. Roberts

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My greatest debt of gratitude is to my wife, Carol.

GENERAL INTRODUCTION

Since the rediscovery of the sarcoplasmic reticulum by Bennett and Porter (1953), and the establishment of its structural and functional significance in muscle cells; (Retzius, 1881; Bennett, 1955; Porter and Palade, 1957; Huxley and Taylor, 1958; Porter, 1961; Ebashi and Lipmann, 1962; Costantin, Franzini-Armstrong and Podolsky, 1965;) investigation of the link between electrical or ionic membrane effects and mechanical contractile activation of muscle - electromechanical coupling (EMC) or excitation-contraction coupling (ECC) - has been greatly stimulated; Sandow, (1965; 1970). Formerly the experimental techniques employed usually involved investigation of the electrical properties of muscle membranes at rest, during activity and in recovery in one preparation, while an analysis of contractile mechanisms was made in another. A causal connection between the former and the latter was then inferred, but remained somewhat tenuous without knowledge of the structure and function of the linking mechanism. The situation is now clearer in that the area of ignorance has been localised to the sarcotubular system (STS), observations into both electrical and mechanical events can be carried out in vivo in the same preparation and explanations of ECC on a macro-molecular basis are being developed with considerable predictive success.

While there are several other areas of interest which have a bearing on muscle contraction, in particular neuromuscular junction activity, calcium activation of contractile mechanisms, tension development, etc., this thesis will be concerned mainly with activity in the sarcotubular

system during ECC since it is in this region that alterations to the in vitro ionic environment of the muscle employed in these experiments are expected to have their major effect.

The following general description of ECC has been developed mainly from experimentation on mammalian skeletal muscle and fast amphibian twitch muscle, and although differences from this model have been observed in some other muscle types (Hoyle, 1969), there is much accumulating evidence to suggest that the basic mechanisms are similar in all muscle fibres (Hoyle, 1962; 1969) and that many of these mechanisms are functional adaptions of basic universal cellular characteristics. See for example Weber (1966), Ebashi and Endo (1968), Ebashi, Endo and Ohtsuki (1969) and Needham (1971) for sarcoplasmic reticulum properties and contraction activitation generally; Sandow (1965; 1970) and Close (1972) for twitch skeletal muscle; Brady (1968) and Brutsaert and Meijler (1971) for heart muscle; Hoyle (1969) for a variety of invertebrate muscle; Hess (1970) for vertebrate slow muscle and Somlyo (1972) for smooth muscle.

The suggestion of Pringle (1960) as modified in Fig. 1 will be used as a convenient model for the clarification of the sequence of events occurring in the normal excitation and contraction of twitch skeletal muscle.

1. Following the initiation of electrical activity in the cell

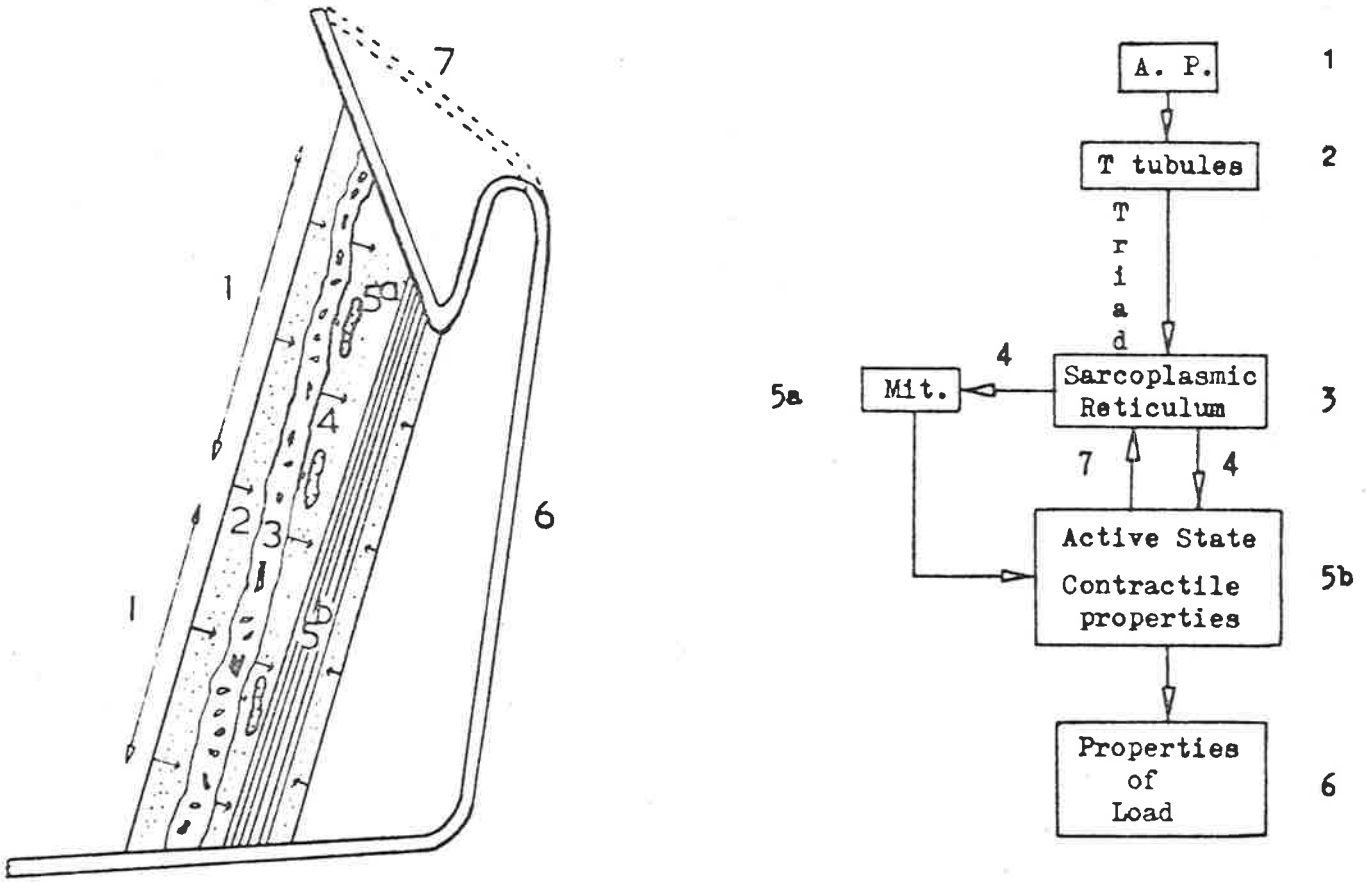


Fig. 1: Pictorial diagram (a) and block diagram (b) to illustrate excitation contraction coupling in skeletal muscle. Modified from Pringle, (1960).

membrane of the sarcolemma, via an excitatory post-synaptic stimulus (usually electrical or chemical), various ionic changes occur in accordance with the propagation of a depolarising current along this outer membrane. The wave of potential change which usually reverses momentarily the intracellular and extracellular potentials - normally determined as the value of the resting membrane potential (E_m) - has been attributed to a sudden change in the permeability of the membrane to Na^+ ions (Hodgkin and Katz, 1949a; Nastuk and Hodgkin, 1950; Hodgkin and Huxley, 1952; Adrian, Chandler and Hodgkin, 1970) permitting an influx of positive charge sufficient to cause lowering of the E_m to enable a threshold of activation to be reached at which Na^+ conductance (gNa) becomes so great that the cell depolarises to a positive potential. This momentary change in gNa is very quickly shut down and reversal of the membrane potential occurs with the onset of a sudden rise in the permeability of the membrane to K^+ ions - an increase in gK - (Hodgkin and Huxley, 1952; Hodgkin and Horowicz, 1959a; Adrian, Chandler and Hodgkin, 1970). This efflux of K^+ which is slower in onset yet longer sustained than the gNa change and which has the tendency to make the inside of the cell again more negative than the outside, (repolarisation) may be accompanied by a further repolarising current (Hodgkin and Huxley, 1952) probably involving movement of Cl^- ions into the cell (Hodgkin and Horowicz, 1959a,b; Hutter and Noble, 1961; Dudel et al, 1967; Dudel and Rüdel, 1969; Bretag, 1970) the two processes transferring sufficient

charge to ensure repolarisation of the cell to the E_m within 2-3 m.sec. of the initiation of depolarisation.

The action potential as described above is propagated along the membrane surface at a rate of 3-5 m.sec.$^{-1}$ (Podolsky, 1965) by the development of these local circuits of ionic current. (Katz, 1966).

2. Continuous with the surface but running transversely into the muscle fibre is an organisation of tubules - called 'T' tubules (Porter and Palade, 1957; Andersson-Cedergren, 1959; Porter, 1961) because of their anatomical position in relation to the sarcolemma - which invade at the level of the Z line or the A-I junction, (Porter and Palade, 1957) depending on the species. These tubules are implicated in the spread of excitation into local areas of the muscle cell (Huxley and Taylor, 1958; Huxley, 1964; Page, 1964; Peachey and Schild, 1968; Gage and Eisenberg, 1969). It has recently been proposed that the spread of excitation within these tubules is propagated,as it is along the surface membrane,(Gonzalez-Serratos, 1966; 1971; Costantin, 1970; Costantin and Taylor, 1971; Bezanilla et al, 1972) so that depolarisation of the tubules will lead to large current flow across the membrane of the tubules, deep into the fibres. It is possible that this kind of activation is essential for the further relaying of the signal into the muscle cell, since from consideration of temporal aspects of ECC a non-propagated, electrotonic, spread of excitation originating from a full-sized AP at the

surface membrane would only be just sufficient to permit activation of the contractile mechanism within the observed time. (Falk and Fatt, 1964; Falk, 1968; Adrian, Chandler and Hodgkin, 1969; Adrian, Costantin and Peachey, 1969).

3. The next stage in the initiation of contraction, probably the least well understood, concerns the spread of the stimulus into the muscle cell proper. Each bundle of contractile filaments is surrounded by an enclosed membranous structure - the sarcoplasmic reticulum (SR) which runs longitudinally the length of the sarcomere in repeating units. These units have been elegantly described by Porter and Palade (1957) for rat sartorius and rat diaphragm. (Fig. 2). The reticulum consists of loosely arranged longitudinal tubules connected by a vesicular membranous structure overlying the centre of the A band. At the I band end of the tubules the reticulum forms a cisternal arrangement of apparently homogenous structure which lies in close proximity (500Å) to a similar transverse cisternal arrangement that connects via thin vesicular elements to the reticulum of the sarcomere and hence overlies the Z line. Jammed between these transverse cisternae lies the adjoining transverse tubule which circles the bundle of filaments and connects with the plasma membrane. In longitudinal section the two terminal cisternae of the sarcoplasmic reticulum and the central 'T' tubule make up a functional triad, there being two triads per sarcomere. The nature of the struc-

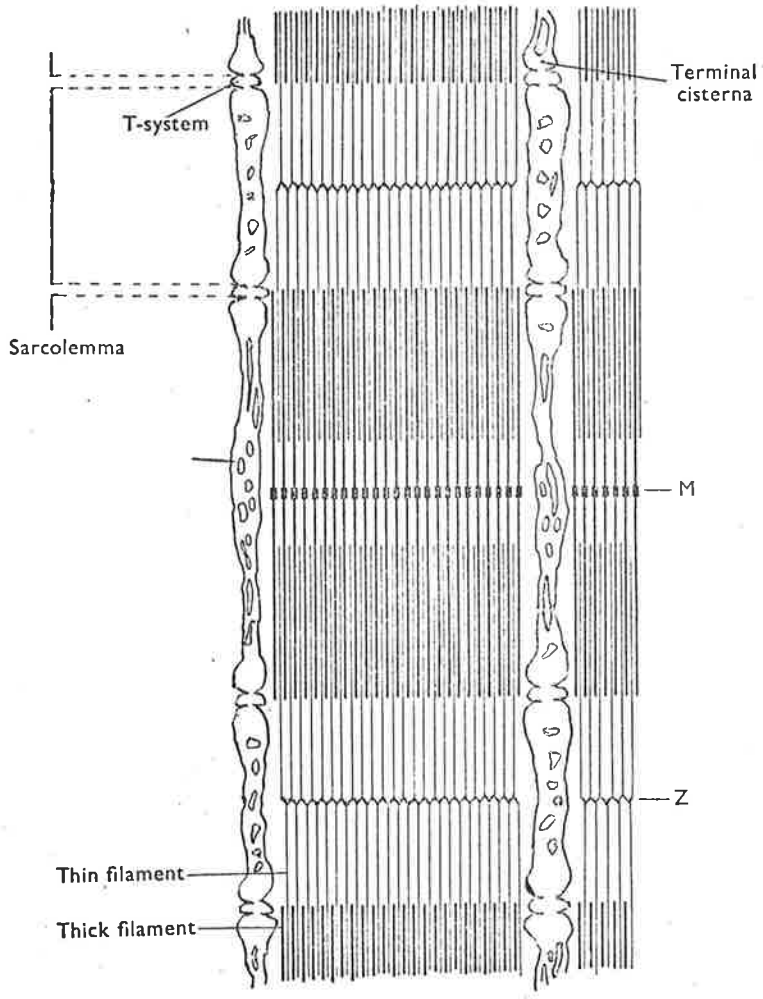


Fig. 2. Schematic diagram of proposed arrangement of sarcotubular system and myofilaments in rat skeletal muscle. Adapted from Porter and Palade (1957) and Ebashi, Endo and Ohtsuki (1969).

tural connection between the central element of the triad and its adjacent two terminal cisternae is not clear as yet, making it difficult to say with confidence what functional mechanism of connection operates here. There is accumulating evidence to suggest that the reticular and tubular membranes may be connecting and that the interior of the sarcoplasmic reticulum is, like the T tubule, actually extracellular (Natori, 1965; Birks and Davey, 1969). Even if this is not the case the two structures are so close together that ionic changes in the T tubular membrane could effect changes in activity across the cisternal membrane e.g. as described by Schneider and Chandler (1973). A functional connection could also be triggered by means of ions released from the inside of the plasma membrane and connecting tubular membrane - in cardiac muscle, smooth muscle and slow tonic muscle, Ca^{++} ions (evidently bound in such a position) have been shown to be released by potential changes across the cell membrane (Hess, 1970; Brutsaert and Meijler, 1971; Somlyo, 1972) - which could pass quickly to sites on SR membrane to initiate activity there, following spike activity in the surface membrane (Bianchi, 1969; Huxley, 1969). Since it has been shown in skinned muscle fibres that the SR membrane is probably polarised and may be capable of conducting some kind of a propagated potential, (Natori, 1965; Costantin and Podolsky, 1967; Podolsky, 1968) it is likely that if a structural connection does exist between the elements of the triad it would be expected that ionic activity would

spread throughout the membranes of the SR at a rapid rate, in a manner similar to that already described for the plasma membrane or more slowly by electrotonic spread.

4. Further events in this sequence are related to the release of an activator for contraction from the membranes of the SR. Once the connection across the structures of the triad has been made there follows rapid and synchronous release of Ca^{++} from special sites in the membrane of the SR - probably in the terminal cisternal region. (Jöbsis and O'Connor, 1966; Ashley and Ridgeway, 1968). This Ca^{++} may have been free in the solution within the vesicles of the SR, or it may be released from membrane binding sites where it has accumulated during cellular quiescence or relaxation. As stated above the nature of the mechanism of this release remains in the realm of speculation, (Weber, 1966; Bianchi, 1969; Endo, Tanaka and Ogawa, 1970; Sandow, 1970) and is a central problem in this field. Several theories have been proposed and these will be discussed when reviewing the results of the present study.

5. Release of Ca^{++} into the myoplasm becomes then the signal for and the agent of initiation of the active state of the muscle fibre. The rise in intracellular Ca^{++} concentration above its resting level of approximately 3×10^{-7}M to the threshold level at which contractile activity will occur - 2×10^{-6}M (Ebashi and Endo, 1968) - is very rapid and easily accounts for the observed rate of mechanical activation - onset

of the active state. It has been clearly shown that Ca is responsible for the two processes essential for contractile activity:

a. Energy Production.

Ca^{++} at levels observed in the myoplasm during contraction - 2×10^{-6}M - is capable of activating phosphorylase b kinase thereby eventually stimulating production of the active phosphorylase a which permits glycogenolysis to proceed hence providing continuing sufficient source of ATP for the developing mechanical activity (Ozawa et al, 1967; Ebashi and Endo, 1968) and

b. Contractile apparatus activation.

The following model is based on the work and reviews of Weber (1966), Ebashi and Endo (1968), Huxley (1969) and Ebashi, Endo and Ohtsuki (1969) propounded within the theory of sliding filament contractile activity. The sites of contractile activity in striated muscle appear to be centred in regions of the myofilaments that make up the regular pattern of sarcomere striations. In their resting state these filaments lie in longitudinal array in a strict lattice formation so that interdigitation of fibres may occur. These contractile filaments composed of proteins of high molecular weight and having highly ordered structure, have been shown to consist of two basic structural elements i.e. thin and thick filaments.

The thin filaments lying in the A band of the sarcomere, consist

of aggregations of separate myosin molecules, each having a tail region joined to a head section on which Ca^{++} and Mg^{++} activated ATPase activated is localised. The molecules aggregate with a consistent orientation, tail regions aligned together and pointing towards the centre of the A band and head regions toward the I band to give a bar-bell shape to the thick filament. Across the border of the A and I bands the thick filaments interdigitate uniformly with thin filaments which lie in the I band but extend into the A band to a depth dependent on sarcomere length, thereby overlapping with thick filaments for a variable portion of their length.

These thin filaments consist of a protein, actin, which is in close association with 'native tropomyosin' - a combination of sub-elements which connects the Ca^{++} receptive small globular protein troponin to the F - actin form of the molecule at intervals of 400 Å along the filament, by means of the binding protein tropomyosin.

In this form, when the level of Ca^{++} in the vicinity of troponin is low - probably less than 10^{-7}M, there is steric and possibly electrochemical inhibition between the myosin heads and the active sites - possibly the troponin - on the actin filaments, hence development of connecting linkages between filaments is inhibited. However, following the increase in myoplasmic Ca^{++} ion concentration to approximately 2×10^{-6}M, troponin is affected in such a way that it readily combines with and exchanges Ca^{++} thereby releasing the inhibition between actin and myosin,

permitting their interaction in the form of cross linkage formation between myosin heads and actin receptive sites. Cross linkages are made and broken at a rapid rate, depending on the supply of Ca^{++} and ATP and either tension development and/or movement is the result.

6. The development of tension within the muscle - termed the active state (Hill, 1938; 1949; Taylor, 1969) precedes the useful work of the muscle since the series elastic elements - tendons, contractile filaments, viscous forces - (Jewell and Wilkie, 1958) must be stretched and overcome before external movement or tension development can be initiated. (See Section A).

7. Following this rise in tension a decrease or relaxation is observed which is preceded by several significant events within the muscle fibre. The basic event in relaxation would seem to be re-accumulation of Ca^{++} by elements of the SR but the trigger for this uptake has not been identified as yet. It is possible that the repolarisation of the plasma membrane - and connecting T tubules - acts as a signal for the longitudinal elements of the SR to alter their affinity or permeability to Ca^{++} in the myoplasm thereby initiating the relaxing process.

There is no established mechanism for the initiation of relaxation in muscle. While it is known that repolarisation of the surface membrane

promotes excitation, it is apparent that the SR can sequester Ca^{++} from the myoplasm even though depolarisation is maintained, e.g. during a potassium contracture (Hodgkin and Horowicz, 1960b; Curtis, 1964) i.e. the SR seems to have functions that need not necessarily be coupled to external potential changes - in particular with K^{+} flow during delayed rectification. (Heistracher and Hunt, 1969, a,b; cf. Adrian, Costantin and Peachey, 1969).

The mechanisms for release and re-accumulation of Ca^{++} by the SR may be independent to some extent as Ca^{++} taken up is not available for re-release until repolarisation of the surface membrane - and perhaps STS - has occurred. (Ebashi and Endo, 1968). Further possible mechanisms of initiation of relaxation may involve energy requirements and electro-chemical interactions at the sites of cross bridge formation (Huxley, 1969; Huxley and Simmons, 1971; Abbot, 1972).

Most of the Ca^{++} diffuses from its combination with troponin in the overlap region - probably in exchange for Mg^{++} - and is actively taken up probably at special sites on the SR outer membrane (Martonosi, 1968) - some Ca^{++} may be attracted to sites on the inner surface of the sarco-lemma or to mitochondria in the vicinity. The Ca^{++} uptake process has been shown to consist of two stages i.e. the first, combination with SR membrane sites involving the utilisation of ATP - the process may be one of combination with a membrane Ca^{++} activated ATPase or involve ATP hydrolysis - and the second possibly not requiring ATP specifically but

involving movement of Ca^{++} to 'storage' sites within the membranes or remaining 'labile', free in the lumen of the SR. It is probable that the first process takes place in the longitudinal sections of the SR - close to the overlap regions - while the second process is slower and takes place during recovery in the terminal cisternae. (Carvalho and Leo, 1967; Carvalho, 1968; Winegrad, 1968, 1970; Connolly, Gough and Winegrad, 1971).

These events outlined above reduce myoplasmic Ca^{++} concentration at a rapid rate (Jöbsis and O'Connor, 1966; Ashley and Ridgeway, 1968) much faster than the observed rate of tension decrease, and probably faster than observed rates of fall of the active state (Sandow, 1970). While the damping effects of the series elastic element can help explain these observations, a more satisfactory explanation will involve a re-evaluation of the 'classical' active state concept. (See Pringle, 1960; Walker, 1960; Desmedt and Hainaut, 1968; Edman, 1970; Close, 1972 and Section A of this Study for discussion on the intensity of the active state in a twitch.)

The cycle of twitch development and relaxation is thus completed, to be repeated when a further threshold stimulus excites the sarcolemma. Physiologically, this sequence is of course more complicated since the majority of muscular responses involve repetitive stimulation.

PRESENT INVESTIGATIONS.

In the above outline there are several obvious gaps in the state of knowledge and at all stages further depth in the level of understanding is required. While it is realised that attempts must be made to seek solutions to these problems at the molecular and even submolecular level, the following work has been undertaken with a view to further characterising various aspects of excitation - contraction coupling.

This thesis is divided into three sections in which a mammalian mixed muscle - the rat diaphragm - has been used in vitro in an examination of some environmental influences on the mechanogram of isometric twitch.

The following lines of approach have been examined :

(1) Observations on the effects of temperature on muscle function have often been attempted. However, much of the information now available from these studies is both contradictory and confusing. The effects of changing temperature over a wide range, on the isometric twitch of a mammalian muscle consisting of different fibre types however, have not been documented systematically, and so an attempt has been made to gather the necessary information and to explain it in relation to the E-C model.

(2) The Myotonic model (Bretag and Potter, 1969; Bretag, 1970, 1971) has been used to examine further the effects of low chloride sol-

utions on membrane and contractile functions. The chloride depleted 'myotonic' preparation has been antagonised by agents reported to increase membrane chloride conductance and other agents which have been shown to affect other low chloride preparations during observations on isometric twitch development.

(3) The plant growth stimulant (and insecticide) 2,4-Dichlorophenoxy-acetic acid and the cholesterol analogue 20, 25-Diazacholesterol have been used in further attempts to develop tissue preparations with myotonic characteristics. The effects of various potential myotonic agonists and antagonists have been evaluated against the above drugs by observations on isometric twitch development.

TECHNIQUES AND MATERIALS.

EXPERIMENTAL ANIMALS.

Hooded Norwegian rats, Rattus norvegicus, and albinos of Wistar strain were used in the present study. These animals had been bred at the Waite Institute Central Animal House, and supplied with tap water and Charlick's F85 Mouse Cubes ad libitum from weaning.

Both male and female rats were used, their weights being between 200 and 400 gm., unless otherwise stated.

SOLUTIONS AND DRUGS.

The physiological bathing solution used was the Synthetic Interstitial Fluid (S.I.F.) of Bretag (Bretag, 1969, 1970). As well, diacetamido-triiodobenzoate substituted solution was used (S.I.F.H.), the sodium salt being substituted for sodium chloride in S.I.F. on a molar basis (Bretag, 1970; Table 1).

Alteration to the potassium content of the bathing solution was effected by adding or deleting the required multiple of the normal concentration of potassium chloride in the preparation of bathing solution.

Variations in the proportion of S.I.F. to S.I.F.H. in the bathing solution, were made by pre-mixing the required proportions of the two solutions and adding the mixture to the bath when required.

Sodium 3,5-diacetamido-2,4,6-triiodobenzoate (Hypaque sodium) was

obtained from Winthrop Laboratories, Sydney.

The drugs used were tetrodotoxin (Sankyo), xylocaine (Astra), dimethyl tubocurarine iodide (Lilly) and dimethyl tubocurarine chloride (Burroughs-Wellcome), 2,4-dichlorphenoxyacetate (sodium salt - Laboratory Supplies, Adelaide; acid-Ajax, 20,25-diazacholesterol (Searle-Chicago), tetra-ethylammonium chloride (Laboratory Supplies, Adelaide), γ-aminobutyric acid (Nutritional Biochemicals Corporation, Cleveland) and glycine (B.D.H., Poole, England.).

pH MEASUREMENTS.

An Orion Research pH meter was used to estimate the effects of temperature on the hydrogen ion concentration of S.I.F. while being bubbled with carbogen (95% O_2, 5% CO_2) gas mixture. The contribution to the acidity of the bathing solution of glycine, GABA and 2,4-D was also determined in this way.

TISSUE PREPARATION.

The rat was killed by a sharp blow to the back of the neck and bled at the throat. The diaphragmatic strip was then dissected out from the animal and placed in S.I.F. bubbled with carbogen at 25°C. The rat diaphragm preparation consisted of a strip approximately 1 cm. wide of

the medial costal region of the right or left hemidiaphragm. The strip was cut parallel to the muscle fibres from the rib to central tendon. Usually the strip was mounted immediately in the organ bath, however, on a few occasions, when both left and right strips were dissected out, satisfactory responses could be obtained from the other strip even after two to three hours.

On a few occasions the extensor digitorum longus muscle of the rat was used, the dissection being somewhat more difficult and slower than extraction of the diaphragm strip, however these preparations responded quite satisfactorily also.

TWITCH TENSION RECORDS.

Diaphragm strip preparations were stimulated directly with supra-maximal square pulses via platinum electrodes immersed in the bathing solution. The pulses were of short duration (0.5-1 m.sec.) and were delivered singly (frequency 0.1/sec.), except in section C where trains of impulses consisting of 3 (1 m.sec. duration) stimuli each separated by 2 m.sec. were delivered at the required intervals. The stimulation unit was either a Grass Model S4G or an Eilco stimulator (some of Section C).

Approximately isometric tension was recorded using a Grass strain gauge Model FT 03C and Beckman or Grass ink-writing recorder. In some of the experiments in Section C tension was measured via a Both isometric

force transducer and recorded on a Both ink-writing recorder.

Muscle resting tension was adjusted to 2 gm. wt. and temperature was maintained by a constant temperature water jacket. The temperature of the water jacket could be altered easily by adjusting the temperature of the reservoir of circulating water either by adding ice or by increasing the thermostat adjustment on the Braun (Melsungen) heater to the required temperature. Changes in the temperature of the bathing solution were measured by thermometer or more usually by a thermocouple secured in the bath and connected to a channel of the recorder.

DIAZACHOLESTEROL TREATMENT.

A small group of young rats (3 weeks old, 70-80 gm.) was treated with daily injections of 20,25-diazacholesterol (DAC) - 10mg./kg. - by subcutaneous injection of a 2 mg./ml., solution of the drug. (Burns, Dale and Langley, 1965; Winer et al, 1966).

STATISTICAL PROCEDURES AND COMPUTATIONS.

Standard statistical methods were employed in the determination of means, standard deviations and standard errors. Computations were performed on an Olivetti Programma 101 computer.

TABLE 1.

COMPOSITION OF BATHING SOLUTIONS

Chemical	S.I.F. (mm/L.)	S.I.F. (per L.)	S.I.F.H. (mm/L.)	S.I.F.H. (per L.)
NaCl	107.7	6.3 gm.	-	-
KCl	3,48	0.26 gm.	4.0	.30 gm.
$CaCl_2$ 10% solution	1.53	1.7 ml.	1.89	2.1 ml.
$MgSO_4 \cdot 7H_2O$	0.69	0.17 gm.	-	-
$NaH_2PO_4 \cdot 2H_2O$	1.67	0.26 gm.	1.67	.26 gm.
$NaHCO_3$	26.2	2.2 gm.	26.2	2.2 gm.
Na gluconate	9.64	2.1 gm.	9.64	2.1 gm.
glucose	5.55	1.0 gm.	5.55	1.0 gm.
Sucrose	7.6	2.6 gm.	-	-
Hypaque sodium 50% soln.			115	146 ml.
Na_2SO_4			0.7	0.1 gm.

SECTION A.

TEMPERATURE EXPERIMENTS.

INTRODUCTION

It is a common observation that although movement can be facilitated by heating, it is invariably hampered considerably by severe cold. Such empiricisms have been cited as the bases for the controversial inclusion of 'warm up' prior to athletic events (Garfoot, 1969; Neuberger, 1969) and a cause of muscle 'cramp' occurring after prolonged cold exposure (Pugh and Edholm, 1955; Pugh, 1967). Apart from these apparent human limitations there are obvious demonstrations of the ability of living organisms to exist in extremes of temperature, being well adapted to carrying out physiological processes similar to those in man with adequate efficiency. (See Dill, 1964 for examples). Contractile activity in skeletal muscle has been demonstrated in vitro in the temperature range $-3^{\circ}C$ to $+53^{\circ}C$ (Varga, 1946; Hajdu, 1951). However, in vivo the limitations at least in vertebrates appear to be somewhat more restricted, particularly in the upper range (Hoffman, 1964; Costill, 1972). Like all other biological processes, we might expect that the efficiency of muscular contraction would vary over this range of temperature both between species and also between different types of muscle in the same species.

Since the development of the electrical theory of excitable tissues, initiated by the work of Matteucci, du Bois-Reymond and others (Brazier, 1959), there have been unnumerable studies made on the effects of tem-

perature on the excitability of nerve and muscle. The general stimulus to study the temperature dependence of any physiological process occurs as Heilbrunn explains (Heilbrunn, 1943).

"...partly because the experimental procedure involved is usually simple, and even more because of the hope that a study of the temperature coefficient of a process might furnish a clue as to the real nature of the process."

Unfortunately his further observation that

"Such a hope, often vigorously expressed, has been realised only to a limited extent"

would still seem to be true in the area of muscular activity.

Early workers seemed to be concerned mainly with the effects of cooling and cold on nerve tissue and the consequent changes in conduction velocity. Pflüger had noticed that cold, of unspecified extent, increased considerably the excitability of frog nerve (Pflüger, 1859) and later it was shown that prolonged electro-motive changes could be recorded from frog sciatic nerve in response to single mechanical stimuli in an animal which had been cooled for some hours (Steinach, 1894). The definitive work of Gotch and Macdonald (Gotch and Macdonald, 1896) established that a curarised frog sartorius preparation showed increased mechanical isotonic responses to various electrical stimuli at 5°C compared with its reactions at 26°C. These workers also stressed that conflicting results which had previously been published were almost certainly caused by failure to use sufficiently high resistance stimulating electrodes - the current and

hence the response being reduced by low temperature when electrode resistance is low. Furthermore their experiments highlight the importance of stimulus duration, since opposite results were obtained under conditions where the stimulus duration was shorter than 2.5 m.sec. Although no attempt was made to explain these changes in excitability the problem was tackled by Bernstein (Bernstein, 1902, 1908) especially for changes in muscle response following an increase in temperature.

Bernstein identified the processes in muscle contraction as being either physical or chemical and he proposed that the chemical processes have a positive temperature coefficient while the physical processes have a negative temperature coefficient. This analysis enabled him to give some order to the conflicting evidence that both he and other workers had accumulated on the effects of temperature on the rate and strength of tension development in frog skeletal muscle. With this clarification of the situation, it might appear that a further advance would be the identification of the steps underlying muscle contraction in order to produce a reliable method of predicting the temperature dependence of the overall process. Such analyses have now identified the basic outline of E-C coupling (see introduction) in which there appears to be several particular processes showing variations in temperature dependence in accord with Bernstein's predictions.

The temperature dependence of a biological process can be expressed

in terms of the Q_{10} or temperature coefficient. This is the ratio of the velocity constant of a process or reaction at a given temperature to the velocity constant at a temperature 10°C lower. The calculation of Q_{10} may be made from the formula

$$Q_{10} = \frac{[k_1]}{[k_2]}^{\left(\frac{10}{t_1 - t_2}\right)}$$

where k_1 and k_2 are the values of the parameter in question at the two temperatures t_1 and t_2 with t_1 being higher than t_2.

According to van't Hoff's rule the Q_{10} of a chemical reaction lies between 2 and 3 whereas physical changes usually show either higher or lower - less than 1.4 - values (Howell, 1913; Heilbrunn, 1943). Although these divisions may be somewhat artificial there may be some significance in the observations that biological phenomena often have Q_{10}'s between 2 and 3.

TEMPERATURE DEPENDENCE OF IONIC PHENOMENA.

Following the work of Bernstein (1902) on the proposed Donnan equilibrium that existed at rest across the border of excitable cells, and the expression of the resultant potential difference between the inside and the outside of the cell by the Nernst equation, it became obvious that this membrane potential should have a theoretical Q_{10} in the vicinity of 1.03.

Since

$$E_m = \frac{RT}{F} \ln \frac{[K^+_i]}{[K^+_o]}$$

where E_m = the resting membrane potential

R = the gas content

F = the Faraday constant

T = absolute temperature

$[K^+_i]$ = the intracellular concentration (activity) of K^+ ions

and $[K^+_o]$ = the extracellular concentration (activity) of K^+ ions

E_m is thus proportional to T. The resting membrane should therefore hyperpolarise slowly with an increase in temperature.

The situation here is however grossly simplified as in accord with the potassium theory of Bernstein's it has been assumed that permeability to the cell at rest by ions other than K^+ is so low as to have no effect on the potassium potential. It is now well known (Boyle and Conway, 1941) that both K^+ and Cl^- ions contribute to the resting membrane potential in muscle cells to a considerable though varying extent, in different muscles (Hutter and Noble, 1960; Caldwell, 1968; Rüdel and Senges, 1972b). It is probable therefore that interactions between these ions - and other membrane effects (see below) - will be induced by either changes in temperature or exposure to different temperatures outside the usual operational range of the tissue.

del Castillo and Machne (1953) for example have recorded the effects of temperature on some passive membrane properties of single sartorius muscles of the frog and although they found very little change in membrane capacitance (Cm), with a temperature rise from 3° to 20°C the transverse membrane resistance (Rm) decreased with a Q_{10} of -3.5 over this range. Such a change in Rm may induce increased excitability of the muscle at lower temperatures with a reduced active ionic current flow sufficient to overcome the threshold potential of excitability. It should be noted however that while Boyd and Martin (1959) obtained similar results to those of del Castillo and Machne in frog muscle, their observations on a mammalian preparation - cat tenuissimus - gave opposing results since Rm increased between 22°C and 37°C with a Q_{10} of 1.6.

The basis for Rm increase with decrease in temperature in frog muscle, probably lies in the decrease in permeability of the muscle cell membrane to Cl^- and K^+ ions as temperature drops (Harris, 1965; Sperelakis, 1969). Also in accord with these findings, since Hutter and Warner (1967) have demonstrated the dependence of gCl on pH in frog muscle, it would be expected that the reduction in pH which occurs in a bicarbonate/CO_2 buffer solution with a decrease in temperature (Creese, Scholes and Taylor, 1958) would also contribute to a decrease in gCl. While it is agreed that frog sartorius displays a differential permeability ratio to Cl^- and K^+ at different temperatures, there is

apparent disagreement as to the relative contribution of each ion species to the total conductance as temperature changes. The data from the electrical measurements of Sperelakis (1969) indicating a higher Q_{10} for gK than gCl appear to be more satisfactory however, and can account for the results of tracer measurements by Harris (1965).

An additional factor involved in the maintenance of the Em is the metabolic requirement associated with the redistribution of ions following activity - i.e. efficient activity of the 'sodium' pump - and any energy needed to retain the semipermeable properties of the membrane against the intra-cellular and extracellular ionic gradients. Since it has been shown in several tissues that the temperature dependence of the Em is altered by blocking the Na^+ pump (Gorman and Marmor, 1970; Marchiafava, 1970; Fischbarg, 1972), alteration in its rate of operation with temperature should affect the Em.

It is apparent that a Q_{10} determination for the Em near the predicted Nernst equation value of 1.03 would be fortuitous rather than expected, since it must be a combined value of several perhaps independent or inter-dependent processes. Ling and Woodbury (1949) have managed to arrive at such a low Q_{10} by bathing frog muscle in high K^+-PO_4^{3-} Ringer, but most other determinations of the temperature coefficient of Em in excitable tissues give slightly higher values in the middle ranges with variable results at either very high or very low temperatures (Apter and Koketsu, 1960; Nakanishi and Norris, 1970).

Apter and Koketsu (1960) have suggested that the conditions for application of the Nernst equation or Goldman's constant field equation - accounting for differential permeability of the cell membrane to different ionic species (Goldman, 1943) - can not apply in the light of a number of observations which they cite. They propose instead that temperature variations cause changes in Em by changing the concentration of Ca^{++} associated with membrane sites; a high membrane bound Ca^{++} concentration being associated with an increase in Em. Regardless of the mechanisms involved in the temperature dependence of resting membrane potential it is not surprising to find that different species appear to have adapted in such a way that amphibian and invertebrate tissue display less temperature dependence at lower temperatures (Hodgkin and Katz, 1949b; Ling and Woodbury, 1949; Macfarlane and Meares, 1958) than does mammalian muscle, while the latter is more resistant to the effects of temperature above 35°C than are the other tissues. (Creese, Scholes and Taylor, 1958; Nakanishi and Norris, 1970).

Action currents in excitable tissues are also affected by temperature. An assessment of this temperature dependence was included in Hodgkin and Huxley's analysis of the action potential in the squid giant axon, (Hodgkin and Huxley, 1952; Huxley, 1959) and in later similar analyses on frog skeletal muscle (Adrian, Chandler and Hodgkin, 1970) and rat diaphragm muscle (Bretag, 1970). On the basis of exper-

imental observations (Hodgkin and Katz, 1949b; Hodgkin, Huxley and Katz, 1952) these workers assigned a Q_{10} of 3 to the ionic current activity coefficients - thereby producing computed solutions which agreed reasonably with experimental action potentials at least in terms of rise time and spike height. A number of workers have made observations on the temperature dependence of the action potential in a variety of excitable tissues (Hodgkin and Katz, 1949b on squid axon; Coraboeuf and Weidman, 1954 on mammalian cardiac muscle; Macfarlane and Meares, 1958 on frog skeletal muscle; Tasaki and Spropulous, 1957 on frog nerve; Schoffeniels, 1958 on the isolated electro plax of the electric eel; Dalton and Hendrix, 1962 on lobster giant axon and Bretag, 1970 on rat diaphragm to mention but a representative few). Pleasingly, there is considerable agreement among these workers as to the effects of temperature on action currents in these preparations, a multiplicity of explanations of these effects, however, present themselves.

The rate of rise of the spike, the spike height and the rate of decay are all temperature dependent. The rate of repolarisation however displays a temperature dependence far greater than the other two indicators of spike activity, so that the duration of the action potential e.g. at half spike height, has a high temperature coefficient. These observations indicate that the ionic currents responsible for depolarisation are less affected by temperature than are those currents

responsible for repolarisation. With a drop in temperature most tissues exhibit a small decrease in the rate of rise of the action potential, (Schoffeneils, 1958; Falk, 1961; Bretag, 1970) with very little if any alteration in the spike height (Coraboeuf and Weidman, 1954; Tasaki and Spyropoulos, 1957; Schoffeneils, 1958; cf. Hodgkin and Katz, 1949b;Bretag, 1970). The inward flowing current responsible for depolarisation - a Na^+ current in most of the tissues studied, seems to be little affected by temperature so that the excitability of the tissue may be maintained at low temperatures and remain normal at high temperatures. However, part of the apparent independence of spike height from temperature may develop from the high temperature dependence of the repolarising currents.

The recovery phase of the spike involves an outward directed K^+ current and in some tissues a contribution from influx of Cl^- ions, these processes assisting in the recovery of the Em. This phase may display a positive (hyperpolarising) or a negative (depolarising) after-potential which will delay the onset of full recovery for a time dependent on its duration, and thus contribute to the overall duration of the spike. The spike therefore usually displays an initial rapid phase of repolarisation followed by a slower negative after potential which eventually reduces until the Em is restored.

The high temperature coefficient for this overall process is probably caused by a highly temperature dependent initiation and rate

of rise of the outward K^+ current (Falk, 1961) - with a contribution from influx of Cl^- at low temperatures (Hutter and Noble, 1960) - coupled with a highly temperature dependent after-potential. Macfarlane and Meares (1958) have concluded on the basis of metabolic inhibitor studies that the after-potential is determined by active energy processes within the cell, an increase in temperature increasing the voltage of the after-potential while decreasing its duration. Whatever the mechanism the outcome of this overall temperature dependence of repolarisation is that at lower temperatures spike activity is maintained above threshold for an increased length of time. This occurs because the slow onset of repolarisation allows full expression of the action potential amplitude, since it has a much lower Q_{10} than the recovery phase, and also as a direct result of delayed repolarisation. Macfarlane and Meares suggest that in frog muscle at high temperatures - above $38^{\circ}C$ - the voltage of the negative after-potential may be sufficient to produce spontaneous secondary spike development thereby also maintaining the membrane at above threshold activity for an increased length of time. Both of these mechanisms may be responsible for affecting excitation contraction coupling (see discussion).

The variable effects of temperature on ionic currents at rest and during activity have been stressed above. Interaction between membrane sites for transport of different ionic species may be caused by direct effects of temperature on membrane structure and the ability of membranes

to bind different species both at the outer cell surface and within the cell. These aspects are reviewed by Chapman (1967) and in brief he concludes that the effects of temperature on membrane structure vary with membrane content. The membrane moiety particularly affected is the phospholipid component. Membranes with high saturated lipid content are more suited to withstand higher temperatures whereas high unsaturated lipid content occurs in membranes of animals adapted to cooler conditions.

Unless freezing occurs, ice crystal formation is unlikely to disrupt membrane function, however some membrane lipoproteins may denature at lower temperatures - below the usual functional range - thereby affecting normal function. At higher temperatures lipoprotein complexes have been shown to undergo various structural transitions which could be expected to throw membrane organisation into disarray. These changes may be reversible or irreversible depending on the severity of the temperature change and its speed of onset. The extent to which these structural alterations affect the normal membrane function of excitable cells at present remains unknown.

THE EFFECT OF TEMPERATURE ON THE ACTIVE STATE.

The mechanics of the active state have been studied in some detail by a number of workers using a variety of technical tricks of progressing sophistication. The most illuminating information about this

process has come however from the extensive use of the now classical techniques of quick stretch in contraction - and quick release in relaxation. (Gasser and Hill, 1924; Hill, 1949; Ritchie, 1954b; Ritchie and Wilkie, 1955; Huxley and Simmons, 1971). Estimations of the time course of the active state have thus been made on a number of different muscle preparations and information concerning the temperature dependence of this process has also been published. (See Close, 1972).

The significance of these data resides in the interpretation that active state changes are a direct reflection of contractile activity within the muscle cell, preceding the overt signs of tension change, e.g. in an isometric twitch, since the latter is delayed by the requirement to overcome the compliance of series elastic elements. On this basis the active state is a measure of the time course of Ca^{++} activation of the troponin-tropomyosin complex, while the time between stimulation and the onset of the active state is determined by the series of events which lead to the Ca^{++} protein interaction which effects mechanical response. - See introduction and Ebashi and Endo (1968). While there is some doubt as to the potential value of retaining the active state concept (Hill, 1965) there is sufficient evidence to show that it certainly does have a physiological counterpart and that some clarity of the contractile mechanism is achieved by its retention. (Bahler, Fales and Zierler, 1967; Brady, 1968).

Examination of the temperature dependence of the active state during an isometric muscle twitch (Hill, 1951; Ritchie, 1954b; Gabel, Carson and Vance, 1968) has confirmed the early suggestions (Hartree and Hill, 1921) that the rate of rise in the intensity of the active state is affected less by temperature changes than is its rate of fall. The conclusion can then be drawn that the Q_{10} for the chemical reactions involved in the production of the contractile response is less than that for the removal of Ca^{++} ions from active sites and their reaccumulation in the sarcoplasmic reticulum. It is interesting in this regard to note that the rate limiting step in the decline of the active state is generally considered to be Ca^{++} uptake by the SR, while release of Ca^{++} by the SR is probably the rate limiting step of the delay in onset of the active state (Endo and Ebashi, 1968). Ritchie has demonstrated that these two processes display a similar temperature dependence - assuming that the series elastic element does not display any mechanical hysteresis - (Ritchie, 1954b) indicating that these two functions of the SR may be dependent on a common property of its structural components. Analysis of the temperature effect on intracellular Ca^{++} levels during a twitch may provide further physiological confirmation of the active state concept. (See however the complications with higher temperatures on active state intensity and duration in human muscle-Desmedt and Hainaut, 1968).

TEMPERATURE DEPENDENCE OF DIFFERENT FIBRE TYPES.

It was recognised some time ago that muscle groups often displayed some variation in individual fibre type, a difference in colour and staining being obvious to light microscopists. Further functional variations in fibres from twitch skeletal muscle have been noted from time to time (Denny-Brown, 1929; Gordon and Phillips, 1953) and clarification of the terminology of these fibre differences has recently been accomplished (Close, 1972). The conclusion to be drawn is that there are basically two different speeds of contraction in twitch muscle, with small fibres usually contracting quickly and long fibres slowly, although there appear to be some fibres with intermediate properties. Inherent speed of contractile activity appears to be controlled by both innervation and pattern of activity (Buller, Eccles and Eccles, 1960; Gutmann, Hajek and Horsky, 1969), a change in either of these, especially during development, having a profound effect on the consequent speed of contraction.

It has been suggested that fast twitch and slow twitch fibres differ in their myosin ATPase activity (Guth and Samaha, 1969; Barany and Close, 1971) and also in macromolecular composition and activity of SR membranes (Mommaerts, Buller and Seraydarian, 1969; Margreth, Salviati and Curraro, 1973) and that these differences are responsible for their differing contractile properties. As it has been

indicated above that the activity of both the SR and the contractile proteins have differing temperature coefficients within the same fibre, it is to be expected that the twitch characteristics of different types of muscle fibre would have differing temperature dependences, e.g. as described by Close and Hoh (1968). Although there are a few muscles with virtually homogeneous fibre composition, most muscles are composed of various proportions of fast, slow and intermediate fibres so that they would demonstrate a response to e.g. temperature that is a mixture of at least two separate responses (Gordon and Phillips, 1953; Biscoe and Taylor, 1967).

EXPERIMENTAL DESIGN

Rat diaphragm strips, bathed in S.I.F. at a resting temperature of 25°C were employed as described in techniques.

Three series of experiments were undertaken. The first involved establishing the normal response of the preparation to heating (to 45°C) and cooling (to 0°C). This series entailed changing the temperature of the bath from 25°C up or down gradually at various rates in order to examine changes in the myogram. When the highest or lowest temperature has been reached the bath was then returned to its normal 25°C. It was found that the changes observed were independent of rate of temperature change provided that any change was not sudden, e.g. as produced by placing large quantities of ice in the circulating water.

The treatment was then reversed in a number of experiments so that muscle responses in a single preparation over the entire range of temperature (0 - 45°C) were recorded. Repetition of the treatment (25 - 45°C only) was again effected with the addition of curare (2-5 ug/ml.) to the bath in order to establish whether any of the observed mechanical changes were due to changes in nerve terminal activity.

The second series of experiments consisted of attempts to characterise the temperature dependent myogram changes by repeating the initial procedures with the addition of various drugs in the bathing solution.

The third series of experiments entailed observations on the

effects of temperature change on responses of diaphragm strips from rats treated with 20,25-diazacholesterol.

ANALYSIS OF THE MYOGRAM.

1. Maximum isometric twitch tension (Pt).

The height of the myogram was estimated with a ruler to the nearest mm. Average values at a given temperature were calculated and compared with the response of that muscle recorded at 25°C before the temperature treatment. This latter value is considered to be 100%.

2. Contraction Time (Tc)

The time from the first sign of mechanical activity to the time at which maximum twitch tension (Pt) is reached. This was calculated by enlarging the trace by epidiascope to 50 times original size and assessing the required distance to an eventual accuracy of ± 2 m.sec.

3. Half relaxation time ($T\frac{1}{2}R$)

The time for decay of tension from the peak of the isometric twitch to one half of the peak tension. This was calculated by the same method used for Tc.

pH AND TEMPERATURE.

Table A-1 describes the effect of temperature changes and prolonged exposure to different temperatures on the pH of carbogen (5% CO_2/95%O_2) bubbled S.I.F. and S.I.F.H.

RESULTS.

The twitch tension response of the preparation to cooling and heating is shown in Figs. A-1 and A-2 respectively. Although these traces show responses from two different preparations they illustrate the general pattern observed in all similar experiments. Except for the re-warming phase in Fig. A-1, the traces were compiled from groups of responses which were recorded at the temperatures indicated; for easy comparison they have been assembled in a continuous record.

In Figs. A-3 and A-4 respectively the changes in contraction time (Tc) and half relaxation time ($T\frac{1}{2}R$) in response to temperature are shown. These myograms were recorded from a single preparation, however reference to the summaries in Table A-2 and Fig. A-5 shows that they are representative as typical responses.

Q_{10} values for the three parameters of mechanical activity being investigated are shown for the various temperature ranges in Table A-3.

COOLING EFFECTS.

The effect of slow cooling on the maximum isometric twitch tension (Pt) of the preparation is shown in Fig. A-1 which samples groups of three responses at 1°C intervals from 25°C to 3°C and includes the continuous response to rapid re-warming. On reducing the temperature, twitch height first rises to a maximum between 16°C-13°C and then drops

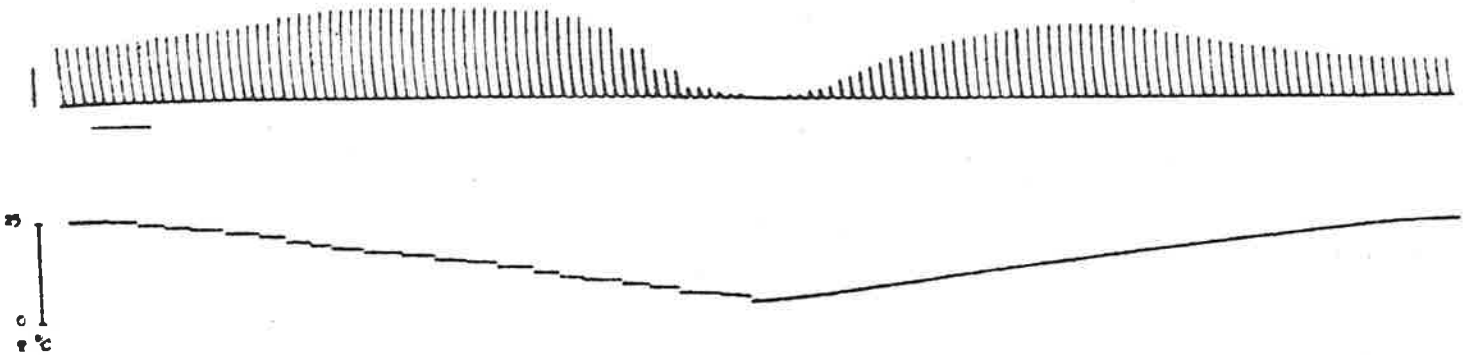


Fig. A-1: Effect of cooling and rewarming on isometric twitch tension record from isolated rat diaphragm strip bathed in S.I.F. and stimulated directly with supramax. stimuli. Upper trace, tension. Calibration: 5 gm-wt, 1 min. Lower trace, temperature. Sequence left to right, composite trace retouched. For explanation see text.

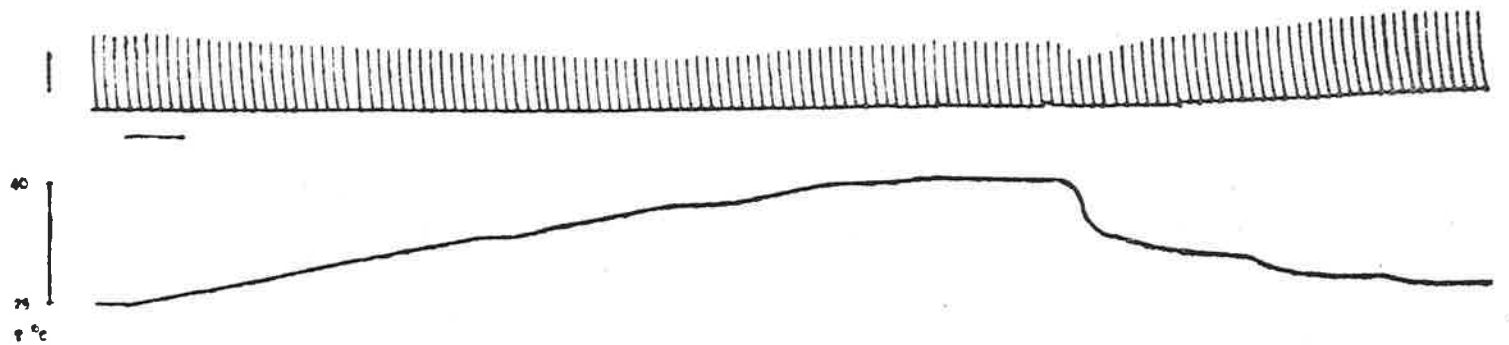


Fig. A-2: Effect of heating and recooling on isometric twitch tension of isolated rat diaphragm strip bathed in S.I.F. Upper trace, tension. Calibration: 5 gm-wt, 1 min. Lower trace, temperature. Composite trace, retouched.

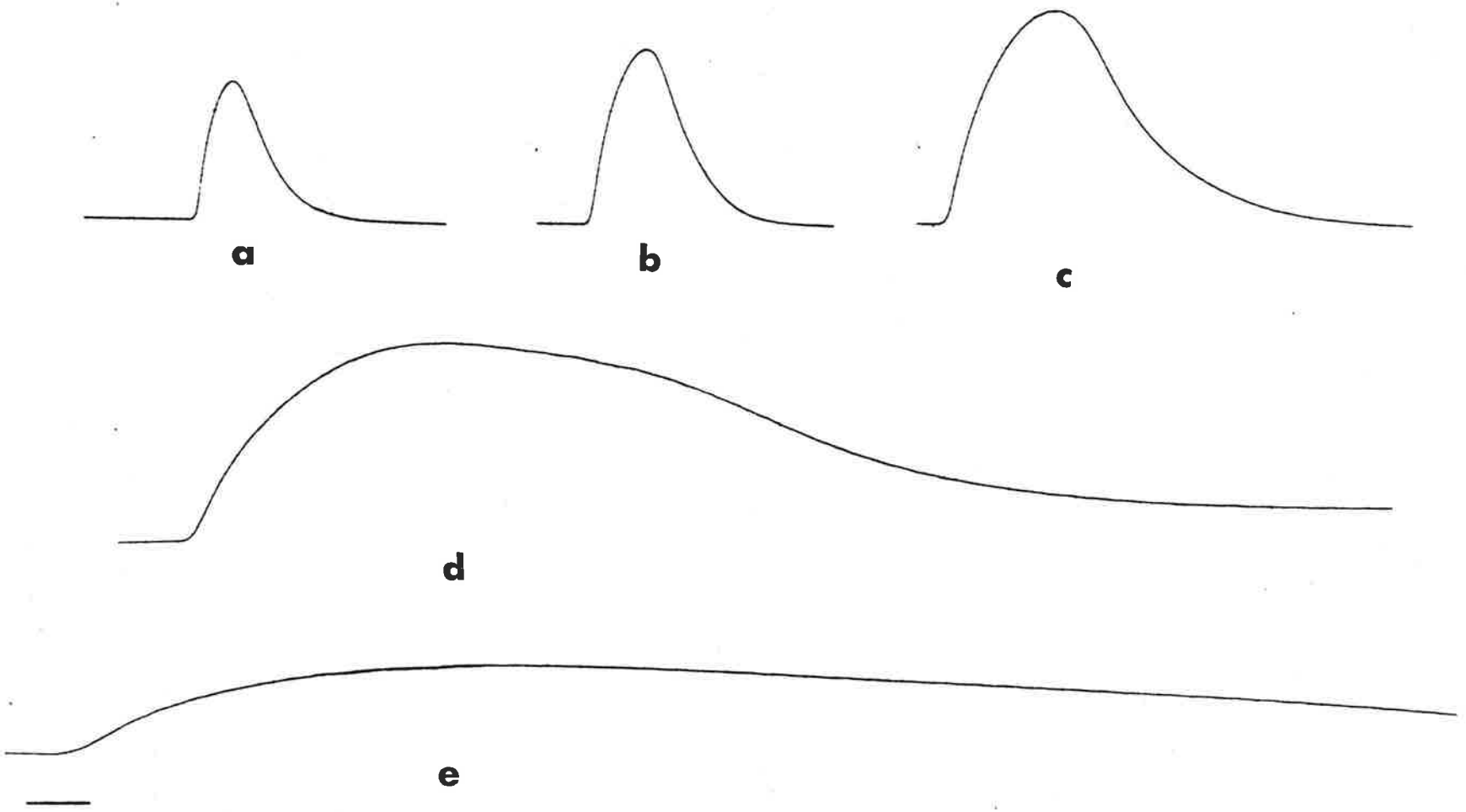


Fig. A-3: Effect of cooling on isometric twitch tension time course. a: 25°C, b: 20°C, c: 14°C, d: 9½°C, e: 5°C. Calibration: 5gm-wt, 100 m.sec.

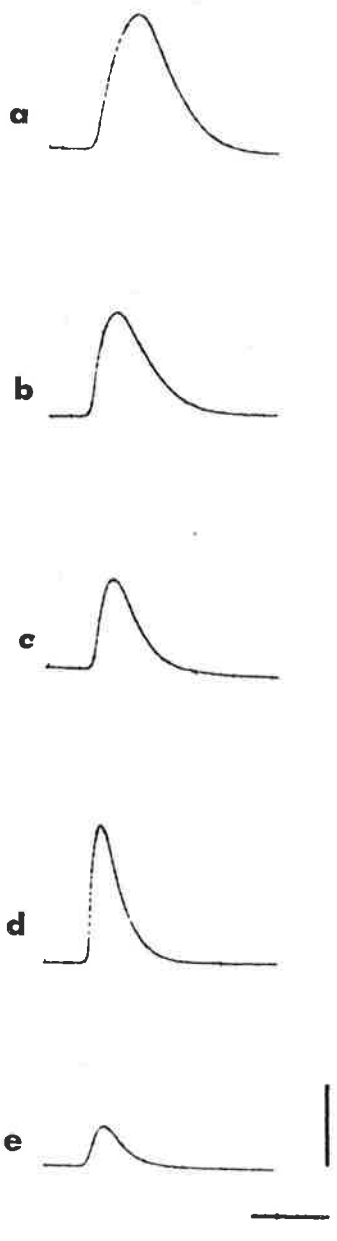


Fig. A-4. Effect of heating on isometric twitch tension time course. a: 25½°C, b: 30½°C, c: 35°C, d: 39½°C, e: 43°C. Calibration:5 gm-wt, 100 m.sec.

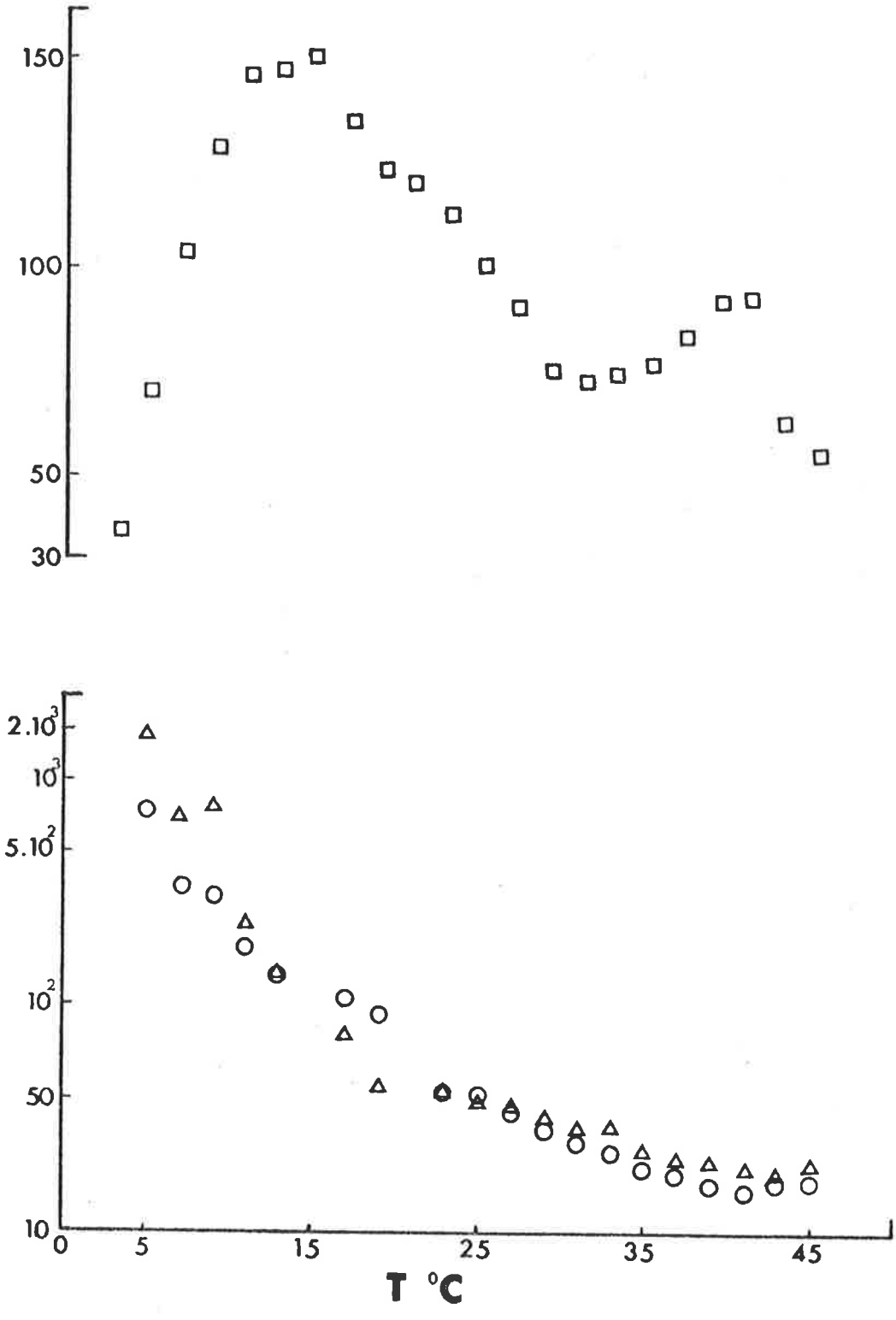


Fig. A-5: The effects of temperature on twitch tension parameters. Tension, □ ,(as % of Pt at 25°C) is plotted against temperature from 3 to 45°C. Similarly, contraction time, ○ , and half relaxation time, △ , (both in millisecs.) are shown on a semi-logarithmic scale.

rapidly until the response is eliminated near 3°C. Re-warming the preparation induces rapid recovery, again showing a peak tension near 15°C and returning to a stable level at the original temperature, although at a slightly reduced tension.

That this increasing twitch tension could be caused, at least in part, by a 'chloride free' effect (Cheah, 1961; Juttner and Philpot, 1965; Bretag, 1970) was tested by the addition of xylocaine (20 ug/ml) to the bathing solution. Fig. A-7 illustrates the response to this procedure and a comparison with Fig. A-1 indicates that xylocaine has no effect on the qualitative twitch tension changes induced by lowering temperature. (See Section B for explanation of 'chloride free' effects.)

Changes in the myogram in response to cooling are shown in Fig. A-3. An increase in Tc and $T\frac{1}{2}R$ is apparent, with the greater effect being on the latter stage of the twitch. The respective temperature co-efficients for Tc and $T\frac{1}{2}R$ over the ranges observed, indicate a difference between the processes underlying these parameters in terms of temperature dependence (Table A-3).

An interesting observation was recorded in the myograms of muscles at temperatures below about 14°C, i.e. at and below the temperature at which peak twitch tension occurred. Here the relaxation phase of the twitch develops a secondary peak which interrupts the normal smooth curve of tension decrease (Fig. A-8 a). The separation of the secondary

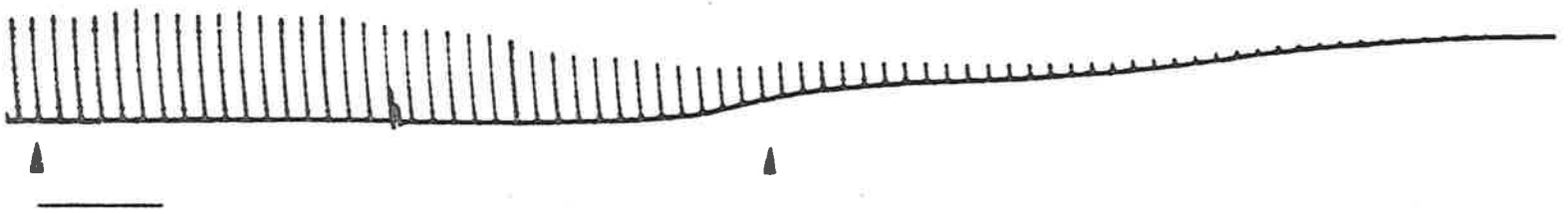


Fig. A-6: Heat contracture superimposed on diaphragm twitch response. Temperature raised from 39°C to 48°C between arrows and maintained there. Trace from left to right. Calibration: 5gm-wt, 1 min.

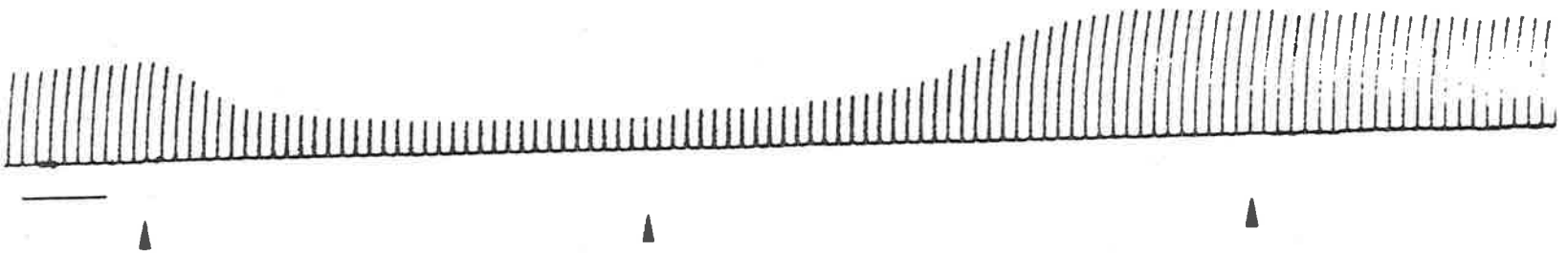


Fig. A-7: Isometric twitch response of diaphragm strip at low temperature in the presence of xylocaine (20 ug/ml). Trace from right to left. Between first two arrows, strip cooled from 15°C to 8°C, then warmed to 18°C (third arrow). Calibration: 5 gm-wt, 1 min.

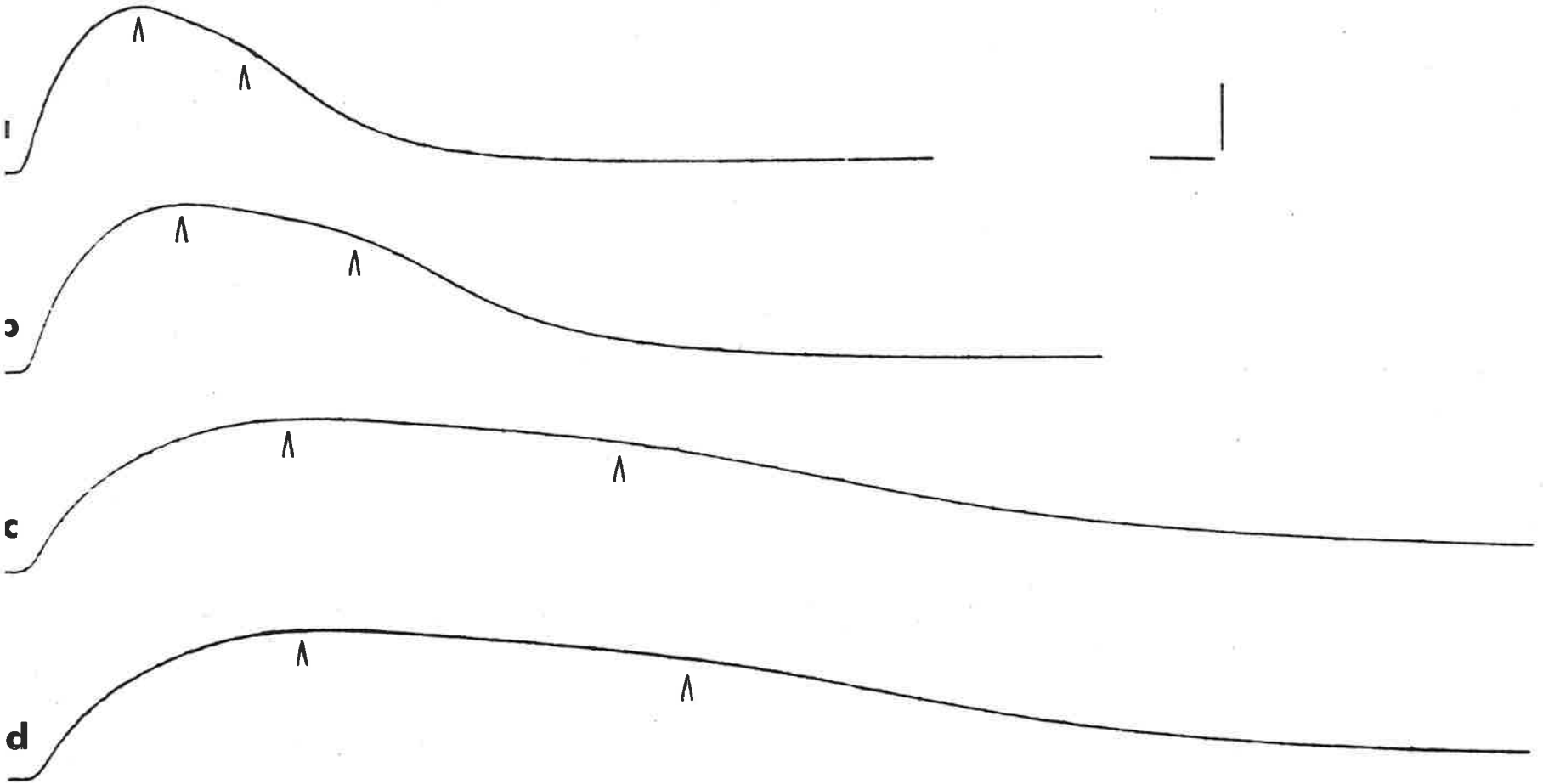


Fig. A-8: Twitch tension time course at lower temperatures. Development and separation of two components is demonstrated. ∧ a: 13 °C, b: 11½ °C, c: 9 °C, d: 8½ °C. Calibration: 5 gm-wt, 100 m.sec.

peak from the primary peak of maximum twitch tension becomes greater as the temperature falls (Fig. A-8 a-d), however, since the relaxation phase itself is increasingly prolonged, the secondary peak never appears to be very obvious. Since only a few observations were made of the myogram at temperatures below 15°C, and in these traces the accuracy of pin-pointing the time of maximum secondary peak tension is limited, estimations of the time to secondary peak were not made.

HEATING EFFECTS.

The twitch tension response of the preparation to heating is shown in Fig. A-2. Twitch tension decreases initially until the temperature reaches about 32°C whereupon it rises to a new peak near 40°C after which it is rapidly reduced again as the muscle goes into heat contracture, irreversible above 45°C (Fig. A-6). If the heating of the bathing solution is reversed before the onset of contracture and gradually reduced, the twitch tension first decreases - if the temperature change is rapid - then recovers to follow the pattern of change that occurred on heating. The response of the preparation usually settled down then to its former state of activity. From the graph in Fig. A-5 it is clear that the relationship between twitch tension and temperature appears to be inversely linear from 15 to 30°C, while a direct linear relationship apparently holds from 30°C to 40°C.

Since this latter rise in twitch tension between 30°C and 40°C was a somewhat unexpected result, (Truong, Wall and Walker, 1964; Close and Hoh, 1968) several treatments were used in order to identify the factors responsible for the change. The effects of curare (5 ug/ml), xylocaine (10 ug/ml) and tetrodotoxin (5.10^{-5}mM) were examined and the results are compared in Fig. A-9. Examination of the traces indicates that in normal S.I.F., the greatest rise in twitch tension in the range 30-40°C usually occurs between 38 and 40°C. Hence a comparison was made of the effects of the above three treatments on twitch tension over this range and is depicted in Fig. A-10.

From these two figures it is apparent that curarisation of the preparation does not affect the normal response, while both TTX and xylocaine modify it in the concentrations used. TTX treated muscle showed a decline in twitch tension from 32-41°C while xylocaine preparations produced a small peak similar to TTX around 32-34°C and a secondary smaller peak in the normal 38-40°C range.

Diaphragm preparations from rats previously treated with 20,25-diazacholesterol were also assessed for twitch parameter temperature dependence in the range 22-42°C. The results of twitch tension changes in these experiments also appear in Figs. A-9 and A-10. The DAC treatment produced a modification of the normal response in that the twitch tension usually decreased with increasing temperature until around

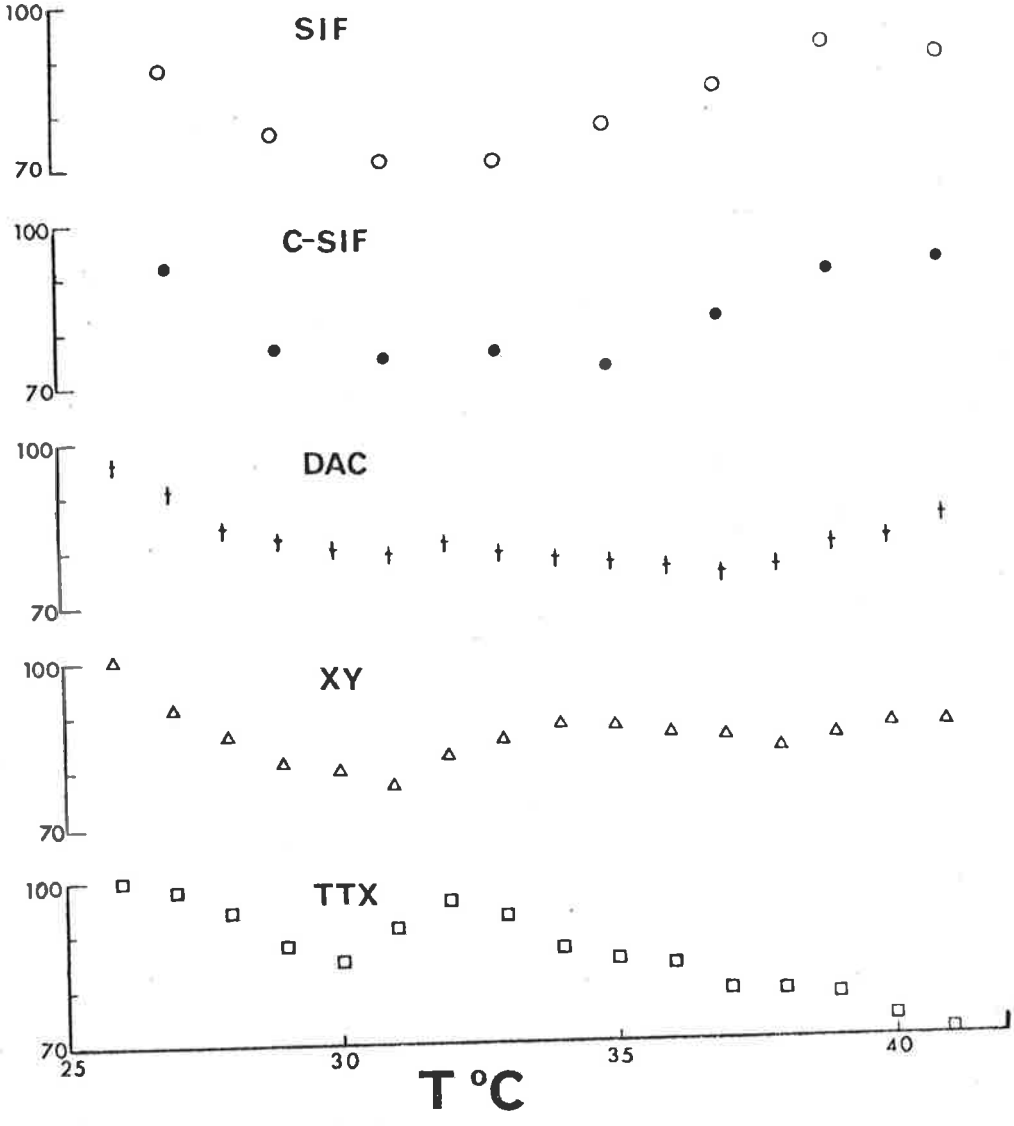


Fig. A-9: Isometric twitch tension responses to heating under various conditions, as indicated. Tension expressed as % of response at 25°C., temperature raised from 25°C to 41°C.

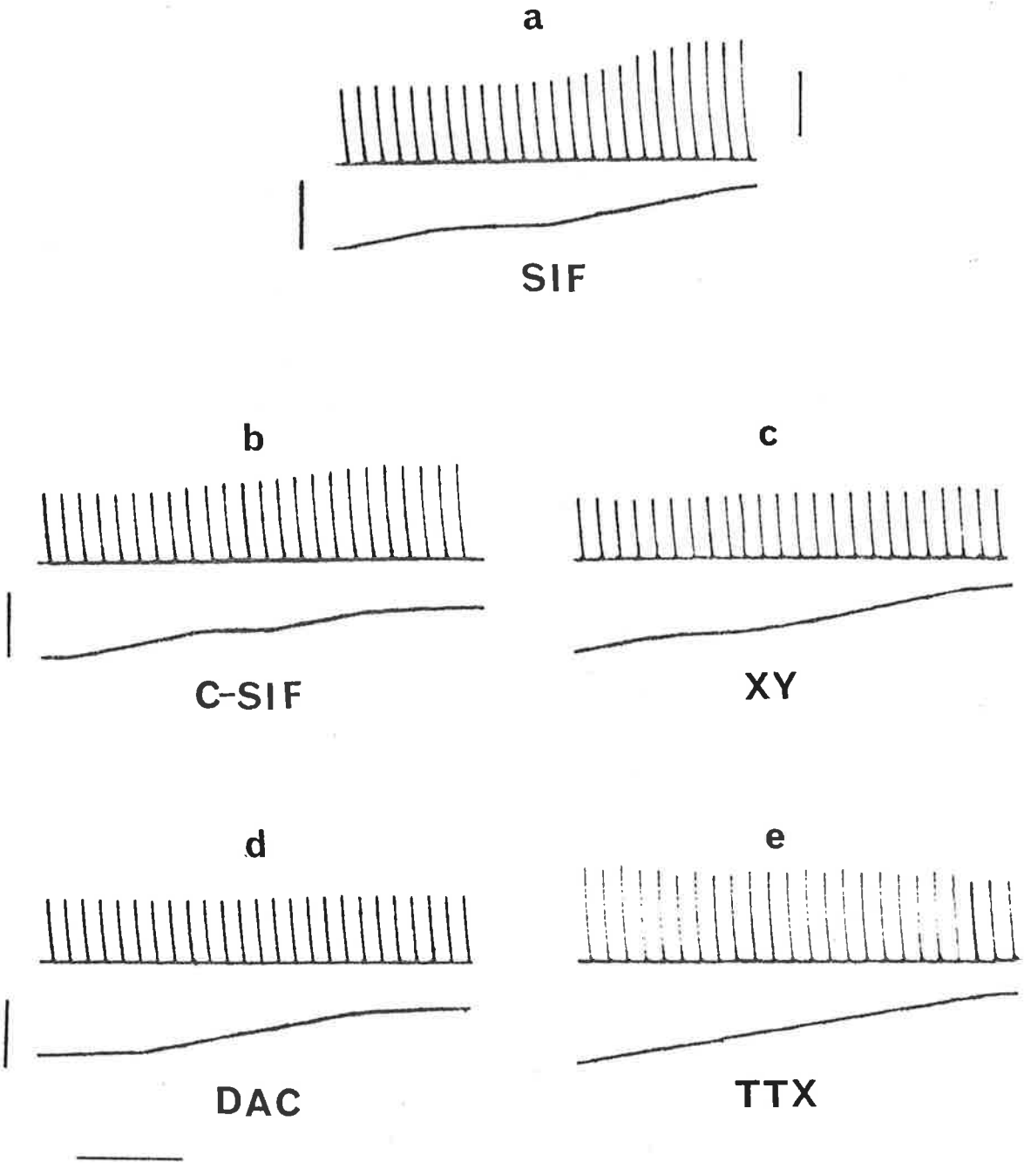


Fig. A-10: Twitch tension changes between 38-40° C. Muscle treated as indicated. In each trace the upper graph is twitch tension and the lower, temperature change. Calibration: 5gm-wt, 1 min. (a-e). Temperature 5°C. a,d,e = 35-40°C and b,c = 37.5-42.5°C reading from bottom to top.

38°C whereupon a slight increase occurred, similar to that observed in xylocaine treated preparations. Further observations were made on Tc and $T\frac{1}{2}R$ of the twitch myogram during heating. Some of these results are shown in Fig. A-4 and a summary appears in Table A-2 and Fig. A-5. From these data the Q_{10}'s shown in Table A-3 were calculated.

The temperature dependence of the processes underlying these two parameters appear to be similar between 20°C and 40°C, although $T\frac{1}{2}R$ may not be a valid indicator of the whole of the relaxation phase. Furthermore the relationship between temperature and these two processes appears to be constant over this range (Fig. A-5) despite the fact that the Q_{10} values suggest otherwise. This discrepancy, however, is inherent in the method of calculation of Q_{10} and is hence more apparent than real. The preparation displays a shortening of both Tc and $T\frac{1}{2}R$ with increasing temperature indicating that the speed of both contraction and relaxation is increased by this treatment.

Above 40°C heat contracture develops at slightly different temperatures from one preparation to the next, producing a variety of changes generally including the aforementioned decrease in Pt, and an increased Tc with little change in $T\frac{1}{2}R$. Figs. A-6, A-4, Table A-2.

The experiments with curare, TTX, xylocaine and DAC indicated that these drugs, in the concentrations used, had no significant effect on the normal changes described above in Tc and $T\frac{1}{2}R$ that occurred with heating.

TABLE A-1

EFFECT OF TEMPERATURE ON pH

Temperature °C	Solution S.I.F.	S.I.F.H.
5	7.1	7.4
25	7.3	7.6
40	7.4	7.7

TABLE A-2

TEMPERATURE AND TWITCH PARAMETER VALUES

Temp. °C	Max. Twitch Tension (Pt) (cf.100% at 25°C)	Contraction Time (Tc) m.sec.	½ Relaxation Time ($T\frac{1}{2}R$) m.sec.
0	-	-	-
5	70 ± 10	820	1880
10	137 ± 13	331 ± 116	582 ± 362
15	150	149 ± 40	134 ± 73
20	122 ± 2	96	57
25	100	53.6 ± 7.3	49.0 ± 8.9
30	73.9 ± 2.0	36.9 ± 6.3	41.9 ± 8.4
35	75.6 ± 2.2	28.0 ± 5.4	33.0 ± 5.5
40	91.3 ± 0.8	21.9 ± 4.6	28.3 ± 4.1
45	55 ± 7.1	23.6 ± 2.2	28.2 ± 4.0

TABLE A-3

TEMPERATURE COEFFICIENTS (Q_{10}) OF TWITCH PARAMETERS.

Temperature Range °C	5-10	13-31	33-40	40-45
Pt	-3.45	1.54	-1.55	3.14

Temp. Range	5-10	10-20	20-30	30-40	40-45
Tc	5.9	3.0	1.70	1.63	-1.33
$T_{\frac{1}{2}}R$	11	11.3	1.34	1.51	-

TABLE A-4

ISOMETRIC TWITCH PROPERTIES OF RAT SKELETAL MUSCLE

Reference	Muscle	Tc (m.sec.)	$T\frac{1}{2}R$ (m.sec.)	Comment
Ritchie (1954a)	D	22-23	-	In vitro, 37°C Isotonic, Fig.3
Doudoumopoulos & Chatfield (1959)	G	25	55	In vivo, 38°C Fig.1A
Walker (1960)	TS	15	30	In vivo, 36.5. Fig.10
Truong, Wall & Walker (1964)	TS	16	16	In vivo, 37°C Fig.3A
Close (1967)	EDL	11.8 ± 1.3	7.7 ± 1.2	In vivo, 35-36°C
Close (1967)	SOL	36.0 ± 2.0	51.0 ± 4.3	
Edstrom & Kugelberg (1968)	TA	13	-	A & B fibres In vitro, 37°C
Sant' Ambrogio & Saibene (1970)	D	18.3 ± 0.8	-	In vivo, 37°C
Gutmann (1970)	EDL	11.6 ± 0.5	-	
Gutmann (1970)	SOL	22.2 ± 0.5	-	
Bass, Gutmann & Hanikova (1971)	D	15.5 ± 0.5	19.1 ± 1.0	In vitro, 37°C 6 weeks old
Vyskocil and Gutmann (1972)	D	20.7 ± 0.6	22.3 ± 1.0	In vitro, 37°C
	SOL	41.6 ± 0.7	53.9 ± 1.0	
Present Study	D	25.8 ± 4.6	31.4 ± 4.4	In vitro, 36-38°C

TABLE A-5

* TEMPERATURE VS TWITCH Tc AND $T\frac{1}{2}R$ OF SOME MAMMALIAN MUSCLES

Reference	Muscle	40-35		35-30		30-25		25-20		20-15		15-10		Comment
		Tc	$T\frac{1}{2}R$	Tc	$T\frac{1}{2}R$	Tc	$T\frac{1}{2}R$	Tc	$T\frac{1}{2}R$	Tc	$T\frac{1}{2}R$	Tc	$T\frac{1}{2}R$	
Maclagan & Zaimis (1957)	Cat TA	27.5 (36)	47	33 (30)	62									Fig.1
Doudoumopoulos & Chatfield (1959)	Rat G	25 (37)	55			40 (26.5)	95							Fig.1a,b
Walker (1960)	Rat TS	15 (37)						75 (24)						
Truong, et al (1964)	Rat TS	20 (35)	18	23 (30)	20	31 (25)	31	46 (20)	55	87 (15)	94	109 (12)	130	Fig.2a
Hainaut (1968)	Human AP	90 (36.5)	150			100 (28)	210							Figs.1,2
Close & Hoh (1968)	Rat SOL EDL	28 11 (35)	36 9					122 38 (20)	170 42					
Buller et al (1968a)	Cat SOL FDL	90 27 (38)	195 40			140 45 (28)	330 70							Fig.2
Present Study	Rat D	28 (35)	33	37 (30)	42	54 (25)	49	96 (20)	57	149 (15)	134	331 (10)	582	

* Actual recording temperature in brackets

TABLE A-6

* Q_{10} VALUES OF ISOMETRIC TWITCH TENSION IN MAMMALIAN SKELETAL MUSCLE

Reference	Muscle	Temperature Ranges and Q_{10} values
Maclagan and Zaimis (1957)	Rat TA	30 ← (-1.51) 36
Doudoumopoulos et al (1959)	Rat G	28 ← (1.17) 38
Walker (1960)	Rat TS	24 ← (1.54) 37
Truong et al (1964)	Rat TS	12 ← (-1.4) 20 ← (1.34) 30 ← (1.53) 40
Hainaut (1968)	Human AP	28 ← (-1.66) 36.5
Gabel et al (1968)	Mouse RA	15 ← (-1.44) 20 ← (1.71) 30 ← (2.42) 35
Buller et al (1968a)	Cat FHL SOL	28 ← (1.47 / -1.28) 38
Buller et al (1968b)	Cat FDL SOL	28 ← (1.21 / -1.38) 38
Close & Hoh (1968)	Rat EDL SOL	20 ← (1.35 / -1.06) 30 ← (1.56 / -1.03) 35
Isaacson et al (1970)	Rat EDL SOL	4 ← (-3.27 / -2.38) 20 ← (1.37 / -1.12) 37
Present Study	Rat D	5 ← (-3.45) 10 ; 13 ← (1.54) 31 ; 33 ← (-1.55) 40

* -ve sign indicates that Pt decreases as temperature decreases

DISCUSSION.

FIBRE TYPE.

Early investigations of diaphragm muscle were centred around clarifying its function in relation to respiration (Lee, Guenther and Mellany, 1916; Briscoe, 1920). More recently detailed comparative histophysiological studies have been undertaken in an attempt to relate muscle fibre structure to function. The comparative study of Gauthier and Padykula (1966) has shown that the rate of diaphragm contraction seems to be inversely proportional to body size and that there is a variety of muscle fibre types within diaphragms from different animals. Confirmation of their work comes from the findings of Sant' Ambrogio and Saibene (1970), Gauthier (1969) and Bass, Gutmann and Hanikova (1971), these studies indicating that in animals with very high metabolic rates e.g. the mouse, small, red, highly oxidative muscle fibres compose almost the entire structure of the diaphragm, whereas in larger animals the predominant muscle type is a larger, more slowly contracting white muscle fibre. As is to be expected in an animal like the rat, the diaphragm has contractile and histological properties intermediate between the two extremes. The detailed descriptions of Gunther (1952), George and Susheela (1961) and Padykula and Gauthier (1963) have shown that although rat diaphragm muscle consists of three different fibre types histologic-

ally, there are within these three fibre types only two rates of contraction. Gunther has indicated that the slower contracting fibres (42%) are concerned with maintaining tone of the diaphragm while the rest of the fibres, being fast contracting, are the prime movers of respiratory movements.

The fast and slow fibres of the diaphragm owe their properties to certain fundamental differences. It is to be expected that as their pattern of activity differs, their innervation may differ, there may also be differences in the calcium sequestering and accumulating properties of the SR from the two different fibre types, while myosin ATPase differences have been demonstrated between fast and slow muscle fibres (Sreter et al, 1973). As described above each of these sites is a potential location for temperature to act so that it would not be surprising to find that slow and fast muscles demonstrate different responses to temperature. That this is in fact the case has been shown by Close and Hoh (1968) and Buller, Ranatanga and Smith (1968 a,b). Their investigations on the effects of temperature on contractile properties of fast and slow fibres from rat and cat respectively, are summarised in parts of Tables A-5 and A-6. Fast muscle fibres (EDL in rat and FDL and FHL in cat) demonstrate the usual decrease in tension and shortening of both Tc and $T_{\frac{1}{2}}R$ with increase in temperature in the range 20 to 35°C whereas slow muscle (Soleus in both rat and cat) shows

if anything, an increase in Pt with temperature within this range together with the usual changes in Tc and $T_{\frac{1}{2}}R$.

The results of the present study (Figs. A-5, A-9 and Tables A-2 and A-3) indicate that the rat diaphragm preparation at 37°C has isometric twitch tension parameters intermediate between those of fast and slowly contractile rat muscle fibres (Table A-4). Tc and $T_{\frac{1}{2}}R$ values appear to be a little greater than those from other studies on rat diaphragm, (cf. Levy, Cohen and Inesi, 1973) however variations in recording technique, dissection of preparation and temperature adaptation of the animals could easily account for the differences observed. Similarly the temperature dependence of twitch parameters of rat diaphragm muscle can be compared with values from a number of studies on mammalian muscle preparations (Tables A-5, A-6) where intermediate properties between those of fast and slow fibres are again demonstrated. Some general conclusions will be drawn from these tables and specific reference to and explanation of the results of the present study will be discussed below.

Shortening of Tc and $T_{\frac{1}{2}}R$ are common phenomena in all muscles studied as the temperature rises, although there seems to be some variation in the Q_{10}s of the processes underlying these changes. In general there does not appear to be much difference between the temperature dependence of Tc and $T_{\frac{1}{2}}R$ at higher temperatures (Table A-3) but at lower temperatures - below 20°C - $T_{\frac{1}{2}}R$ is prolonged more than Tc. i.e. relaxation of both fast and slow muscle fibres appears to be affected more than contraction over

this temperature range, in general agreement with the findings of Hill (1951) on frog twitch muscle.

As mentioned above however, Pt changes appear to be more complicated than those of Tc and $T\frac{1}{2}R$ even though tension changes would appear to reflect changes in the relative contribution of contraction and relaxation to the twitch. Below 20°C all muscles investigated whether predominantly fast or slow, appear to show a decrease in Pt with decrease in temperature. However in the present study, where twitch tension changes have been monitored continuously while changing temperatures slowly, a peak in the tension is observed between 10-13°C. The only study cited (Isaacson, Hinkes and Taylor, 1970) in which this temperature range has been used with a slow muscle, involved step changes in temperature so that the changes noted in the present study could not have been detected by these workers. Above 20°C fast muscle displays a decrease in Pt with increase in temperature, while muscles composed predominantly of slow fibres consistently show an increase in Pt especially between 30 and 40°C. Even though rat diaphragm contains only approximately 40% slow fibres this is evidently a sufficiently high enough proportion to cause this muscle to demonstrate twitch tension changes with temperature characteristic of slow muscle fibres. On this basis it would be expected that cat TA (Maclagan and Zaimis, 1957) and human AP (Hainaut, 1968) be composed of significant amounts of slow fibres.

An interesting observation on twitch tension changes within the range 20-40°C was observed between approximately 37 and 41°C. This involved a sudden rise in twitch tension (Figs. A-9, A-10) in this region, a change which does not appear to have been described before, perhaps because most of the studies reported in the literature have been performed only up to 38°C. Possible mechanisms of this sudden increase will be discussed in more detail below however it is probable that the slow muscle fibres are particularly involved in contraction at these temperatures and contribute towards this rise in Pt.

An obvious difference in the temperature dependence of fast and slow fibres is seen in the present study, particularly at low temperatures, where below 15°C close examination of traces reveals that a slow component of contraction has its peak twitch tension occurring a considerable length of time after the Pt of a faster component, the time difference increasing as temperature drops further, indicating that the faster component is less temperature dependent than the slower component. (Fig. A-8). A similar separation of fast and slow components in the rat diaphragm has been observed by Westphal and his colleagues (Westphal and Akakpo, 1969; Westphal and Limbourg, 1969). These workers have observed changes in the speed - the first time differential - of twitch tension development of rat diaphragm at 17.5°C and 37.5°C and have shown that there are two stages in this process. An early stage which

always occurred at the same relative time after the onset of contraction was followed by a slower component which demonstrated a maximal rate of rise at a later time after the onset of contraction but which was more temperature dependent, occurring relatively earlier at the higher temperature. In agreement with the present study the implication is that the slower component - slow twitch fibre activity - is more temperature dependent than is the faster component. Westphal's group indicates that these differences can be attributed to the different properties of the two types of muscle fibre in terms of active state parameters, but they give no further elaboration on this argument.

Separation of components of muscle twitches was observed some time ago by Gordon and Phillips (1953), who found a 'hump' in twitch tension traces similar to those depicted in Fig. A-7. By excising part of the cat tibialis anterior and thereby eliminating rapidly contracting superficial muscle, a single trace of slow muscle response could be obtained. It is possible that certain sections or strips of rat diaphragm may be more homogenous than others, explaining why there was some variation observed between preparations in the temperature at which separation of the two components of contraction could first be detected on cooling.

CONTRACTION TIME AND HALF RELAXATION TIME.

It can be argued (Walker, 1960), that onset of contraction and onset

of relaxation during a twitch reflect active state changes that are delayed by the compliance of the SEC within the muscle. Since the temperature dependence of the SEC is small (Walker, 1951; Jewell and Wilkie, 1958) the major contribution to the twitch tension time course temperature dependence must arise from the various electro-chemical processes involved in contraction, as suggested earlier by Bernstein (1908).

The continuous decrease in Tc and $T\frac{1}{2}R$ with temperature increase observed by several groups (Truong, Wall and Walker, 1964; Close and Hoh, 1968) and in the present study suggests that the rate determining processes underlying each of these parameters are continuous functions of temperature. The rates of onset of rise and fall of the active state appear to be implicated here, it being generally agreed that these processes are rate limited by SR-Ca^{++} sequestering and uptake respectively (Ebashi and Endo, 1968). The non continuous relationship observed between Pt and temperature however, indicates that the intensity of the active state, probably related to the Ca^{++} level attained in the sarcoplasm after stimulation, has a more complex basis than merely that determined by a combination of Tc and $T\frac{1}{2}R$.

The changes in the Em and AP that can occur with an increase in temperature - as outlined in the introduction to this section - should contribute towards the observed Tc and $T\frac{1}{2}R$ changes. Although at reduced temperature the muscle cell may be more easily excitable, the kinetics

of ionic excitation would be expected to be slowed (Baker, 1968). Slowing of eg. action currents by cooling causes a lengthening of the mechanical-coupling period (Sandow, 1964) especially since the threshold for mechanical activation is also reduced (Sandow, 1964, 1965). The tension developed therefore at lower temperatures may be higher than normal since the time for liberation of Ca^{++} from SR cisternal sites may be increased sufficiently to overcome any slowing of this process produced by low temperature (see below). A further effect is that relaxation appears to be delayed relatively more than contraction at these temperatures, (Table A-3) allowing increased time for development of tension by Ca^{++} activation of the contractile proteins.

The lack of effect of xylocaine (20ug/ml) on the cold response of the rat diaphragm indicates that plasma membrane effects are not the limiting determinants of the observed slowing of the twitch, or twitch tension changes (Baker, 1968; Bretag, 1970). However the disappearance of the response, without contracture, seen around $3^{o}C$ is probably due to the effect of cold on membrane kinetics, blocking of action currents usually being observed at these temperatures (Douglas and Malcolm, 1955).

In the higher (more physiological) temperature ranges the observed shortening of the twitch is in agreement with all other studies noted (Table A-5). The explanation for this observation usually involves consideration of increased rates of activation of action current processes

(Falk, 1961) together with SR and contractile protein activity changes (see below). Unlike other studies (Walker, 1960; Table A-5) the relaxation and contraction stages display similar temperature dependence in this range (Table A-3), so that Pt is unlikely to be determined largely by one or other of Tc or $T_{\frac{1}{2}R}$ alone at higher temperatures.

Murphy and Hasselbach (1968) have shown that myofibrillar ATPase from rabbit skeletal muscle displays a faster rate of activity at 40°C than at 25°C, which would be expected to increase the speed of the mechanical response at the higher temperature.

The heat contracture that occurred in the present investigations, when rat diaphragm was heated above 40°C, has commonly been observed. The usual explanations for this effect involve consideration of reversible and irreversible structural changes that can occur at membrane sites involved in E-C coupling. These sites could be at the plasma membrane (McFarlane and Meares, 1958), the sarcoplasmic reticulum (Johnson and Inesi, 1969) or, very likely, at the contractile protein sites. The contracture may be partly produced by surface membrane activity which is consequent on metabolic factors that are adversely affected by heat (McFarlane and Meares, 1958); the increase in resting tension however indicates that SR re-accumulation of Ca^{++} may be inhibited gradually as temperature rises above 40°C, thereby maintaining a sufficiently high level of Ca^{++} in the sarcoplasm between stimuli to

continue some activation of contractile proteins so that mechanical activity continues. It is possible that the Ca^{++} requirement for myosin ATPase activity is reduced at high temperatures (Mühlrad and Hegyi, 1965; Murphy and Hasselbach, 1968) - since the enzyme activity is also stimulated by heating, producing tension development at rest. Various structural and functional features of E-C coupling are presumed to break down as protein denaturing and lipid transitions occur with further rise in temperature, eliminating the response to stimulation above about 45°C.

TWITCH TENSION.

The most interesting observations made in this investigation, centre around the twitch tension changes that occur with changing temperature. The pattern: increase in Pt as temperature rises from 0 to 15°C, then a gradual decrease to just above 30°C, followed by a further though smaller increase in tension to around 40°C, has previously been described in this preparation (Jacque, 1970) but does not appear to follow the same pattern seen in other preparations (Table A-6). Contributions to these changes and explanations of the apparent anomolies can be assessed by consideration of the temperature dependence of the underlying processes.

A. Neuromuscular Junction (NMJ)

Curarisation of the preparation had no effect on the twitch response

of the muscle to temperature (Figs. A-9, A-10). This implies that the observed changes are independent of NMJ activity. However, the concentration of curare used (2-5ug/ml) is lower than that used most commonly in other studies (Hutter and Warner, 1967; Isaacson et al, 1970; Bretag, 1970). Hubbard and Wilson (1973) indicate that 4.10^{-7}g/ml curare is sufficient to induce neuromuscular blockade in accord with normally recognised curarisation, which should give the present study a safe margin for intraspecies sensitivity difference. However it is possible that some neuromuscular transmission may have persisted in the apparently curarised preparations.

Jacque (1970) and Creese et al (1953) have also investigated the effects of curare on the temperature dependence of the rat diaphragm twitch. The latter group of workers indicate that curare (5ug/ml) is most effective in reducing the response at 25°C while the mechanical response of Jacque's rat diaphragm-phrenic nerve preparation (Bülbring, 1946) was unaffected by curare (0.2ug/ml) at 19°C yet was reduced by about 1/3 at 15°C. While Jacque tentatively suggests that decreased acetylcholinesterase activity could be responsible for the observed potentiation at 15°C, the lack of description of recording conditions, bathing solutions, times of exposure to temperature etc. suggest other possible explanations. In fact that author refutes his own argument since decreased acetylcholinesterase activity should make the twitch

tension less curare dependent.

Since the effects of curare below 25°C have not been examined in the present study, the results of Jacque cannot be corroborated, however his study suggests that a considerable contribution to the twitch potentiation around 15°C is dependent on the response of the neuro-muscular junction activity to this temperature. It is apparent also from Jacque's work that at this concentration (10ug in a 50ml bath) curare does not completely eliminate mechanical activity and that since an increase in acetylcholinesterase activity would be expected around 36-39°C this mechanism would actually oppose the observed increase in twitch potentiation at this temperature.

Studies on rat diaphragm - phrenic nerve preparations by Straughan (1960) and Hubbard, Jones and Landau (1971), indicate that acetylcholine release reaches a peak around 39°C as a rise in the presynaptic store of acetylcholine increases the size of quantal content. A further peak in acetylcholine release occurs around 20°C. If the NMJ was not completely blocked, the increased store of transmitter released around 39°C would be expected to cause an increase in the size of the EPSP, - since d-tubocurarine would not affect quantal content changes (Fatt and Katz, 1951) - eventually increasing the mechanical activation of the muscle and hence the twitch tension at this temperature. While the twitch tension changes observed at this temperature are more likely to be

coincidental with the reported transmitter release increase, a possible connection cannot be discounted.

B. Plasma Membrane

At increased temperature, ionic current kinetics are increased so that it is possible since the time available for current activation is reduced, that the quantitative effect of drugs on the membrane currents may be different c.f. lower temperatures. Xylocaine (10ug/ml) which does not affect the normal twitch (Juttner and Philpot, 1965; Bretag, 1970), decreased the size of the normal twitch tension response to temperature between 30-40°C. In its role as a stabiliser it probably partially blocks both Na^+ and K^+ channels (Baker, 1968), thereby reducing the current flow and the size of the action potential so that the extent of electro-mechanical coupling is reduced. The same pattern as the normal response is maintained, however unlike the response in the presence of tetrodotoxin (50mM).

In this latter instance the Na^+ current only is reduced and the Pt gradually decreases as temperature rises between 30 and 40°C. This observation suggests that K^+ repolarising currents dominate the action potential increasingly as temperature rises, so that under these conditions action potential height and duration would quickly fall, e.g. perhaps faster than as described by Bretag (1970). The effect of this change would be to decrease the extent of EM coupling with consequent

reduction in twitch tension. These two sets of observations suggest that the normal Pt increase between 30 and 40°C may at least be partly caused by a membrane effect, initiated at the neuromuscular junction.

C. Transverse Tubule

The changes in kinetics of the mobile fixed charge that is proposed to have a possible role in the T tubule - SR linkage mechanism (Schneider and Chandler, 1973) have not as yet been assessed in relation to temperature dependence. Correlation of these changes with experimental observations therefore will not be attempted.

D. Sarcoplasmic Reticulum.

Sarcoplasmic reticular constitution and function have been recently extensively investigated, however limited information on the temperature dependence of SR activity appears to be available (Inesi, 1972).

The essential functions of SR involve release and uptake of Ca^{++} at particular membrane sites, release occurring from cisternal regions while the tubular portions are associated with binding and then uptake - the former process being associated with Ca^{++} activated ATPase activity. A balance between release and uptake determines the Ca^{++} level within the sarcoplasm and hence controls the degree of activation of contractile proteins. An increase in release and/or a decrease in uptake of Ca^{++} will promote increased tension development within the muscle.

Inesi and Watanabe (1967) have shown that Ca^{++} uptake and ATP

hydrolysis demonstrate identical temperature dependence in the range 5 to 20°C in rabbit SR preparations. Hajdu (1970) has used 45 Ca^{++} labelling in rat diaphragm to investigate depletion and repletion of muscle at low temperatures and although sites in addition to SR may have been implicated, these studies showed that both uptake and release of Ca^{++} were inhibited at 0°C compared with room temperature. Taniguchi and Nagai (1970), have investigated the effects of reduced temperature on Ca^{++} - SR activity in frog muscle. This work has shown that both the rate and amount of Ca^{++} uptake is reduced when temperature is lowered from 20 to 0°C and that reversible release of Ca^{++} occurs over the same range. Caputo (1972a,b) has demonstrated a similar phenomenon in frog single muscle fibres, speculating that the slowed and prolonged release of Ca^{++} that occurs in the cold is directly coupled to membrane potential changes. Inactivation of the Ca^{++} release mechanism appears to be slowed as is the reaccumulation of Ca^{++} in the terminal cisternae. Studies by Sreter (1969) and Nishijima et al (1972) on rabbit and toad muscle respectively have shown similar microsomal-Ca^{++} temperature dependence to that described above.

The conclusion from these studies then is that as temperature is reduced from 25 to 0°C, Ca^{++} is released from SR stores and its concentration in the sarcoplasm remains high as uptake is inhibited. The amount of Ca^{++} then required to be released from the SR in response to

stimulation, in order to produce mechanical activity is therefore reduced. i.e. the mechanical threshold appears to be lowered. In the present experiments it is hypothesised that this process continues, despite the slowing of membrane ionic currents, to increase the Pt developed during the mechanically active period. Around 15°C however, critical decreases in ionic current flow may start to reduce the mechanically active period while slowing of myofibrillar ATPase activation may also have a significant effect (Mühlrad and Hegyi, 1965; Murphy and Hasselbach, 1968). A gradual reduction in Pt is thus produced as temperature falls to 0°C despite the fact that active state decay may be slowed more than active state onset probably as Ca^{++} uptake is inhibited more than Ca^{++} release in this range. Close and Hoh (1968) suggest that in fast fibres the "degree of activation" - presumably related to Ca^{++} - myosin ATPase activity - is increased in the cold more than that in slow fibres, so that Pt increase on cooling from 30° to 15°C is mainly a fast fibre phenomenon.

SR activity at higher temperatures has been investigated in rabbit skeletal muscle preparations by a number of workers. Sreter (1969) has shown that Ca^{++} uptake in SR of white (fast) muscle is maximal at 25°C and decreases above this temperature while in red (slow) muscle it is greater at 35 than 25°C although the net amounts are much lower here than in white fibres. Johnson and Inesi (1969) have shown that uptake

and release of Ca^{++} decreases with increase in temperature above 25°C. Nuclear magnetic resonance studies of Davis and Inesi (1971) and Eletr and Inesi (1972) have identified reversible structural transitions that are thermally dependent. These membrane structural changes occurred at 22 and 40°C in the SR studied and involved protein and lipid moieties; they have been implicated in the observed discontinuities in the temperature dependence of SR- Ca^{++} activity, e.g. as described by Scarpa, Baldassare and Inesi (1972) in ionophore treated fragmented SR. Martonosi (1968) and Carvalho (1972) have provided further evidence for the role of the lipid fraction of SR in Ca^{++} uptake since they describe ATPase activity closely associated with SR lipid content.

At increased temperature then, Ca^{++} levels in the sarcoplasm would be maintained at a sufficiently high level to facilitate contractile activity since although the net amount of Ca^{++} released during a twitch may be reduced, the uptake is also reduced. The kinetics of this activity would be expected to be fast as the ionic currents associated with the stimulus would be fast, thereby producing a reduction in the mechanically effective period. Even though the time available for the establishment of a fully developed active state is shortened at these temperatures, an increase in Pt has been observed. A partial explanation for this rise may rely on stimulation of myofibrillar Ca^{++} activated ATPase activity together with contractile activation stimulated by

the increased temperature itself, independent of a Ca^{++} requirement (Murphy and Hasselbach, 1968). These two effects could lower the mechanical threshold for Ca^{++}, thereby allowing fuller development of the active state even when twitch mechanics are faster.

E. Sarcoplasm

Although considerable changes in diffusion rates in intracellular processes occur with temperature variation, another significant effect of cooling and warming, at least in experimental preparations is produced by pH changes within and without the cell. It is well known that temperature changes can produce changes in the pH of bathing fluids, even though they may be buffered. Creese et al (1958) noted a decrease in pH from 7.39 to 7.15 over the range 40-5°C in their carbogen bubbled saline, values which coincide quite closely to those observed in the present study for carbogen bubbled S.I.F. (Table A-1). Certain Tris buffers on the other hand produce a fall in pH as temperature increases, which can affect Ca^{++} levels in SR preparations (Murphy and Hasselbach, 1968).

Although the external pH of a bathing solution can be determined readily, it is more difficult to estimate that of the intracellular environment, in particular in the region of SR and contractile proteins. Even if this latter can be determined, the value may not be representative of the cell as a whole, or a reliable indicator of H^{+} activity

distributed across the cell boundary (Waddell and Bates, 1969).

Conflicting values for intracellular pH in rat muscles appear in the literature, the difference arising from the controversy over whether H^+ is distributed according to a Donnan equilibrium (Conway, 1957; Carter et al, 1967; Stephens, 1969) or whether pCO_2 and extracellular bicarbonate are the critical determinants involved (Adler, Roy and Relman, 1965a,b; Adler, 1970; Roos, 1971; Paillard, 1972).

The reports of Adler, Roy and Relman (1965a,b) indicate that the inside of the rat diaphragm muscle cell is more acidic than the outside, a pH_e change of 7.4 to 7.1 observed in the present experiments with lowering of temperature (Table A-1) - due to increased CO_2 solubility (Albers, Usinger and Spaich, 1971) - would produce only a very small further decrease in pH_i. Such a change would be unlikely to produce large changes in Ca^{++} uptake by SR fragments (Sreter, 1969) although a slight decrease could result producing a rise in Ca^{++} concentration with decrease in temperature. It is unlikely that this pH dependent rise in Ca^{++} would be sufficient to explain completely the suggested effect of reduced temperature on Ca^{++} uptake and release (Inesi and Watanabe, 1967; Taniguchi and Nagai, 1970).

pH effects on membrane conductance (Hutter and Warner, 1967) would not be expected to contribute significantly to twitch potentiation as temperature decreased from 33-15°C in these experiments since the expected

decrease in gCl would only be small. However it would act in the right direction to increase the excitability of the cell as temperature is reduced.

The work of Stephens (1969) and Nakamaru and Schwartz, (1972) suggest important roles for H^+ in excitable tissues. Stephens' hypothesis requires that H^+ - Ca^{++} interactions may be responsible for the ionic currents that flow during activity while Nakamaru and Schwartz produce evidence that SR affinity for Ca^{++} in a dog muscle preparation is directly dependent on pH, increasing as pH falls. While these observations lack substantial supporting claims, Apter and Koketsu (1960) - they amplify the fact that the role of H^+ in excitable tissues is far from clarified, making a precise analysis of its temperature dependence impossible at this stage.

DAC TREATMENT

The temperature dependence of DAC treated diaphragm is displayed in Figs. A-9 and A-10. The significance of these findings; no change in time course, but a decrease in size of the characteristic Pt change between 30 and 40°C will be discussed in Section C in relation to the myotonic phenomena that these preparations can display.

SUMMARY.

The effects of temperature on the isometric twitch response of the isolated rat diaphragm have been determined over the range 0 to 45°C.

The preparation demonstrates a gradual decrease in time course as temperature increases. At lower temperatures the relaxation phase of the twitch is slowed more than the contraction phase while in the higher temperature range there is little difference between their Q_{10} values. Twitch tension displays two peaks in response, the first around 15°C with a further smaller peak near 40°C. These observations have been compared with literature values for similar determinations in other mammalian muscle preparations.

The effects of d-tubocurarine, xylocaine, tetrodotoxin and diazacholesterol treatment on the twitch temperature response have been assessed.

An explanation of the observed temperature dependence has been developed by consideration of the muscle fibre composition of the diaphragm and processes involved in excitation - contraction coupling.

It is suggested that the anomolous rise in twitch tension from 33-40°C is a consequence of the activity of the slow fibres of the diaphragm, while the fast fibres are associated with the increase in twitch tension on cooling from 33 to 15°C.

SECTION B

ARTIFICIAL MYOTONIA I

INTRODUCTION

The naturally occurring myotonias that can afflict man and other animals are characterized by long lasting repetitive muscle activity to single stimuli. This can be recorded experimentally as a "dive bomber" response in the electromyogram and as membrane instability and repetitive firing in intracellular recordings. (Floyd et al, 1955; McComas and Mrozek, 1968). That the cause of this activity can be isolated to the muscle membranes is generally accepted (Brown and Harvey, 1939; McComas and Johns, 1969; cf. Hofmann and Rowe, 1966) and recent attempts have been made to identify the precise membrane abnormality responsible for the myotonia.

These attempts have progressed through three main methods of investigation. The first has involved investigation of membrane electrical constants and ion fluxes in muscles from animals naturally afflicted with myotonia and a comparison of this data with that of muscle from unafflicted animals. The preferred animals have been the "fainting goats" of White and Plaskett, (1904) which demonstrate a congenital myotonia (Kolb, 1938; Bryant, Lipicky and Herzog, 1968) and dystrophic mice of various strains (McIntyre, Bennett and Brodkey, 1959; Michelson, Russell and Harman, 1955). Muscle from humans afflicted by myotonia has also been investigated (Hofmann, Alston and Rowe, 1966; Hofmann and Rowe, 1966; Lipicky and Bryant, 1971; Lipicky, Bryant and Salmon, 1971).

The second line of investigation has involved the induction of myotonic symptoms in experimental animals and man by treatment with drugs which can produce the typical clinical effects of myotonia, i.e. stiffening on initial activity, and dimpling on percussion, followed by similar muscle membrane determinations as outlined above. Myotonic inducers have included 2,4-dichlorphenoxy acetic acid (Ezaguirre et al, 1948; Hofmann et al, 1966), the hypocholesterolemic cholesterol analogues (Winer et al, 1964; Winer et al, 1966; Goodgold and Eberstein, 1968), a humoral substance liberated from contracting muscle of the human forearm (Krull et al, 1966), chloro-substituted benzoic acid derivatives (Edson and Sanderson, 1964; Tang, Schroeder and Keasling, 1968; Bryant and Morales-Aguilera, 1971) and indoleacetic acid and some of its derivatives (Fuller et al, 1971).

The simplest procedure in the investigation of myotonic phenomena, however, involves treatment of in vivo muscle preparations with chemicals designed to affect the excitable membranes in such a way that it will simulate myotonia. Such preparations have been termed "veratrinic" since they display some mechanical and electrical responses similar to those of muscle treated with veratrine (Krayer and Acheson, 1946; Benforado, 1967; Ulbricht, 1969). Recent evidence suggests that while dystrophic muscle may well display a veratrinic response - increased involvement of Na^+ currents during the action potential (Evans and

Schottelius, 1965) - the essential defect involved in human myotonia congenita, in muscle from goats with hereditary myotonia and in muscle from animals treated with 2,4-D or DAC is associated with an increase in specific membrane resistance in particular due to a considerable decrease in resting chloride conductance (Bryant, 1962; Bryant, 1969; Lipicky and Bryant, 1966, 1971; Lipicky, Bryant and Salmon, 1971; Rüdel and Senges, 1972 a,b).

It is well known that a considerable contribution to the resting membrane conductance in mammalian muscle derives from gCl (Hodgkin and Horowicz, 1959a; Rüdel and Senges, 1972b; Bryant and Morales-Aguilera, 1971), and so a ready method of effecting artificial myotonia presents itself. By reducing the concentration of permeant anions in the external environment of the cell, the effective flux of Cl^- will be reduced so that Rm will be increased, thereby making the cell more excitable. It is also suggested that this treatment should, by diminishing the anion contribution to repolarisation, effectively prolong the action potential and lead therefore to an increase in electromechanical coupling as indicated by twitch potentiation.

These kinds of experiments have been performed on both amphibian and mammalian tissues, usually in relation to studies of twitch potentiation effects (Sandow, 1964, 1965; Falk and Landa, 1960a; Juttner and Philpot, 1965; Cheah, 1961; Smith and Roberts, 1966). Until recently,

these studies have suffered from the difficulty of ensuring that the observed effect was due to a change only in gCl, since most of the impermeant anions substituted for Cl^- have been shown to have Ca^{++} binding effects, (Cheah, 1961; Bretag, 1970) and hence would be expected to induce increased excitability via a Ca^{++} deficiency labiliser effect (Adams and Weiss, 1959).

This difficulty has been overcome (Bretag, 1970) and the isolated rat diaphragm has proven to be an ideal preparation for the investigation of treatments which may relieve myotonia (Bretag and Potter, 1969). Bretag (1970) has used sodium 3,5- diacetamido - 2,4,6- triiodobenzoate (Hypaque sodium) as the impermeant anion since it has no apparent Ca^{++} binding effect when substituted for Cl^- in S.I.F. This solution (S.I.F.H.) induces artificial myotonia on the rat diaphragm preparation.

Hitherto, drug treatments of myotonic conditions have been directed at attempts to reduce membrane excitability by the use of membrane stabilisers (Covernton and Draper, 1947; de Jong, 1955) which have effects on specific ionic species current flow during the action potential. Common drug effects may involve limiting the Na^+ depolarising and/or increasing the K^+ repolarising currents associated with electrical excitation, however a more direct treatment would involve effecting a specific increase in gCl at rest and increasing anion contribution to the repolarising currents following activity.

The following experiments were directed at identifying the critical amount of Cl^- required to suppress myotonic activity in the preparation and in an attempt to effect specific increase in g_{Cl} by drug treatment.

RESULTS

The normal mechanical response of the rat diaphragm to S.I.F.H. has been investigated previously (Bretag and Potter, 1969; Bretag, 1970), and its temperature dependence and stabilisation by various treatments have been described. An example of a typical chloride free response (Cheah, 1961; Falk and Landa, 1960a) appears in Fig. B-1. Twitch potentiation is superimposed on an initial contracture which has periodic fluctuations in intensity with time. With prolonged exposure the twitch tension reaches a maximum with delayed relaxation becoming increasingly significant. (Juttner and Philpot, 1965; Smith and Roberts, 1966). This typical response varies a little from one preparation to the next only in the degree of sensitivity to this treatment. The chloride free response can be eliminated by either pretreating or simultaneously adding xylocaine with the S.I.F.H. to the bathing solution (Fig. B-2). Under these conditions some twitch potentiation persists, indicating that the S.I.F.H. response is compounded by at least two processes.

The proportion of Cl^- required in the bathing solution consisting effectively of otherwise impermeant anions, to eliminate the chloride free response, has been established for frog muscle (Falk and Landa, 1960a) and rat muscle (Juttner and Philpot, 1965; Smith and Roberts, 1966) both exposed to a ferro-cyanide Ringer. As a preliminary to the

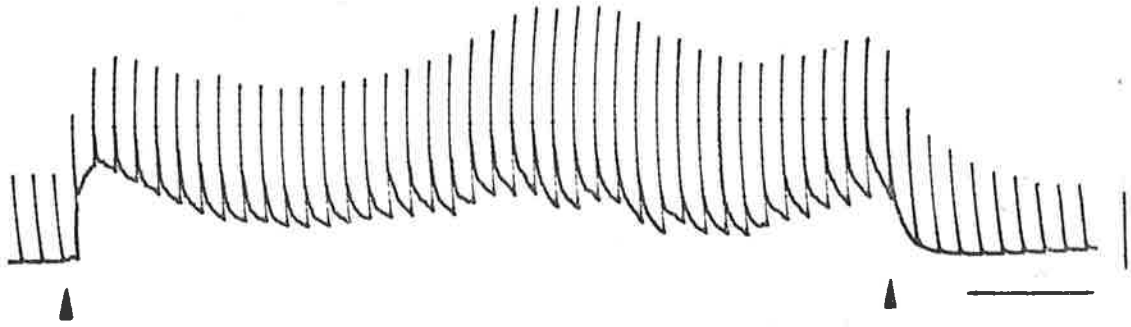


Fig. B-1. The chloride free response. The effect of low chloride solution on isometric twitch tension in the isolated rat diaphragm. Sequence left to right. Preparation bathed in S.I.F.H. between arrows. Temperature 25°C. Calibration: 5 gm-wt, 1 min.

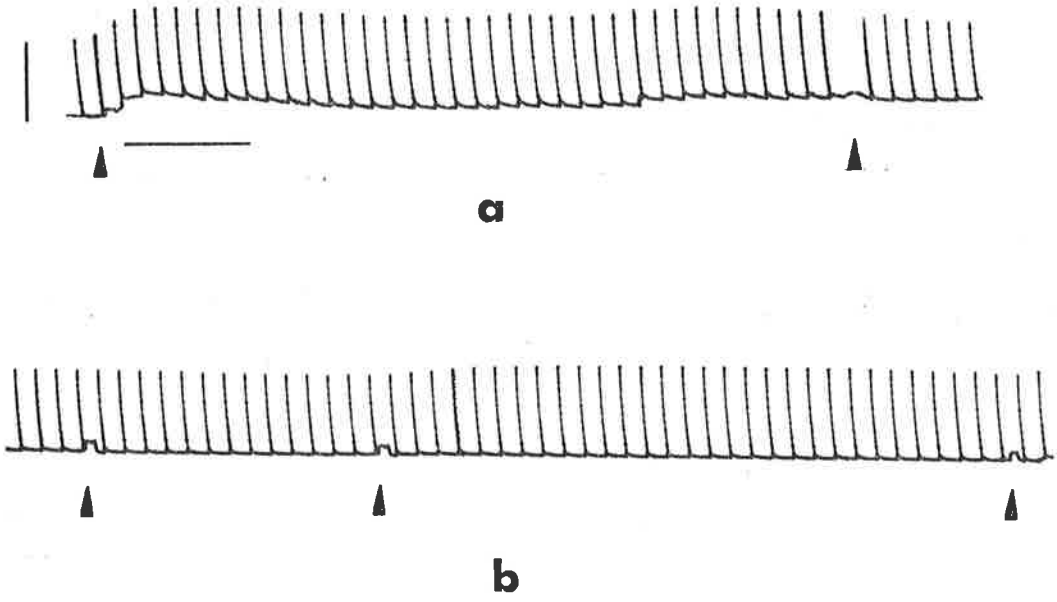


Fig. B-2. Effect of local anaesthetic on S.I.F.H. response.
a: Normal chloride free response to S.I.F.H. added between arrows (note- preparation is less sensitive to S.I.F.H. than Fig. B-1)
b: First arrow xylocaine (10 g/ml.), between 2nd and 3rd arrows XY/ S.I.F.H. was added. Note some twitch potentiation remains. Temperature 25°C. Calibration a,b: 5 gm-wt, 1 min.

next part of the investigation, this concentration was determined for Hypaque anion substitution, by replacing various proportions of normal S.I.F. with S.I.F.H. Responses such as those in Fig. B-3 indicate that only 40% S.I.F. is required to eliminate the typical Cl^- free response. This corresponds to a chloride concentration of just less than 45 mM.

The hypothesis was made that any drug which has a specific ability to increase gCl in the resting membrane would reduce the critical Cl^- concentration necessary to eliminate the S.I.F.H. effect. Accordingly, a preparation treated with a 35% S.I.F. /65% S.I.F.H. bathing mixture would produce a chloride free response, while the addition of a drug to the bath with the above property should reduce the extent of the chloride free response or eliminate it. This criterion on the other hand could be used by corollary to identify drugs which have this specific action on mammalian skeletal muscle.

Two naturally occurring amino acids, γ -amino-butyric acid and glycine, which have been shown to produce increases in membrane Cl^- conductance at various invertebrate inhibitory synapses and invertebrate muscle (GABA) and mammalian inhibitory synapses (GLY and GABA) and possibly other sites (Eccles, 1964, 1969; Ginsborg, 1967; Werman, 1972), were tested for their ability to cause similar changes across the membrane of rat diaphragm muscle. Many attempts were made to detect a consistent effect of these amino acids at concentrations of GABA

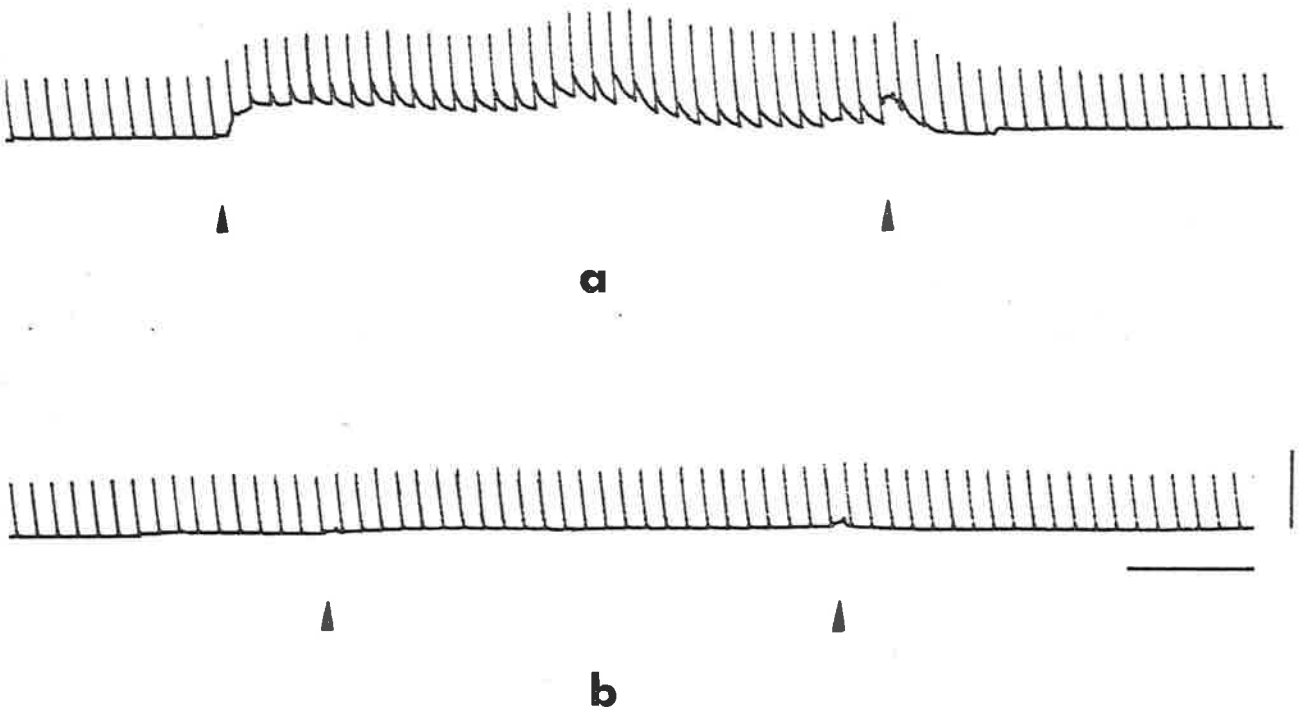


Fig. B-3. Chloride concentration required to eliminate S.I.F.H. response a: Normal chloride free response, S.I.F.H. added between arrows. b: Addition of 40% S.I.F./ 60% S.I.F.H., between arrows, eliminates chloride free response. Temperature 25°C. Calibration a,b: 5 gm-wt, 1 min.

(10^{-3}M) and GLY (10^{-1}-10^{-2}M) on the twitch responses to various proportions of S.I.F./S.I.F.H. from 60 to 100% S.I.F.H. in the bathing solution. Representative responses are shown in Figs. B-4 and B-5, indicating that there is no clearly apparent or consistent effect on the S.I.F.H. response regardless of whether the preparation was pretreated with the amino acid or not, before exposure to the S.I.F.H. solution.

Finally the stabilising effect of high K^+ (Falk and Landa, 1960b; Cheah, 1961) has been reinvestigated on the chloride free response, in this case induced by S.I.F.H. Exposure of the twitching muscle to high K^+ - S.I.F. (4-25 mM KCl) induced the expected twitch potentiation. (Fig. B-9a). When the preparation was pretreated with high K^+ -S.I.F. (2-6x normal) and then exposed to S.I.F.H. with the same K^+ concentration, the chloride free response was antagonised to varying extents in that 18mMK^+ reduced the initial chloride free response a little while 25mM K^+ eliminated it entirely. (Figs. B-6b,c). The twitch tension potentiation was however still apparent, even at the highest K^+ concentration used.

Interestingly, no change to the S.I.F.H. response could be detected unless the muscle had been pretreated with the required K^+ -S.I.F. (Fig. B-7b). In addition, pretreatment with high K^+ -S.I.F. reduced the initial chloride free response to the following normal K^+ -S.I.F.H. treatment (Fig. B-7c). Removal of K^+ from the bathing medium failed to make the preparation more labile to either S.I.F. or S.I.F.H. (Fig.

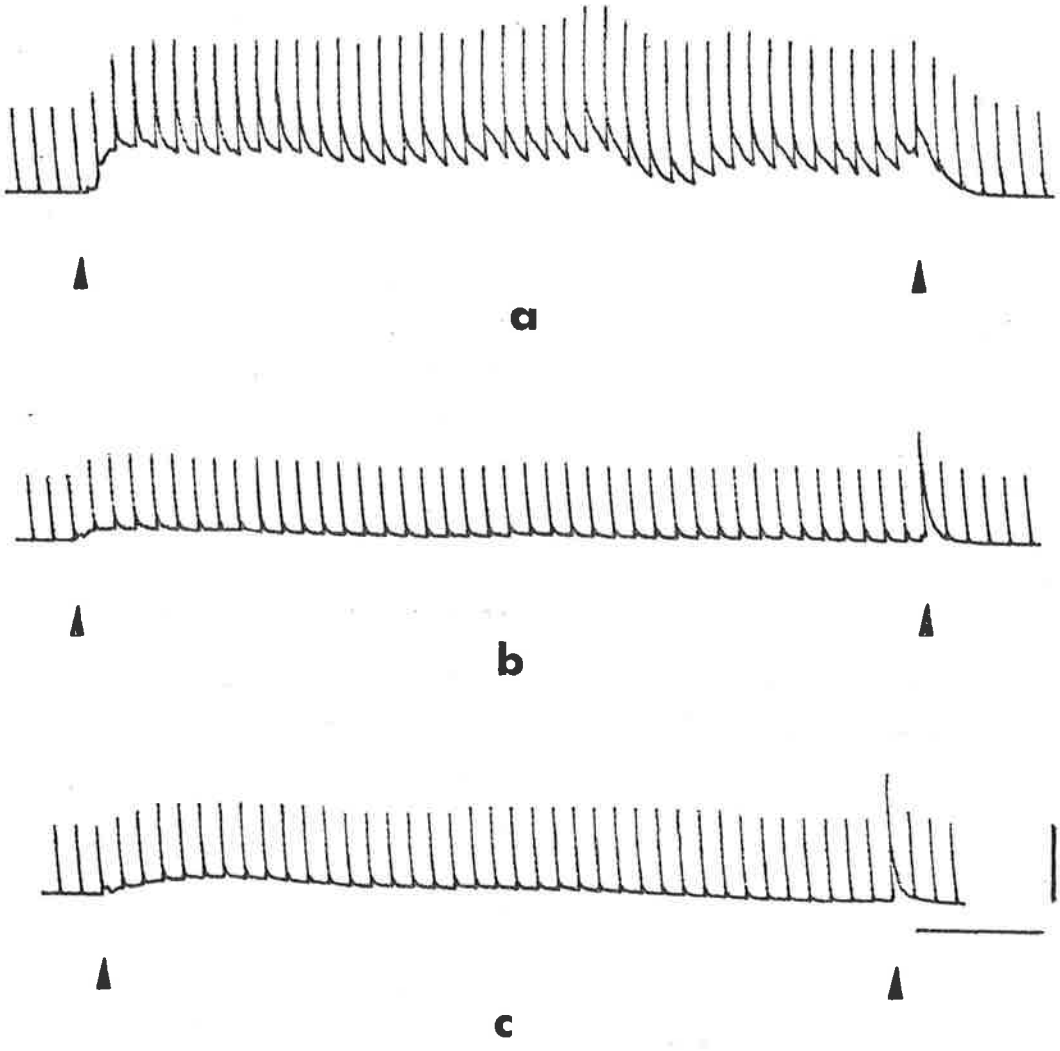


Fig. B-4. Effect of GABA on S.I.F.H. response.
a: Normal S.I.F.H. response. b: Response to 35% S.I.F./65% S.I.F.H. c: GABA (10^{-3}M) added with S.I.F.35%/S.I.F.H.65%. GABA has no obvious effect on the small chloride free response in b. Temperature 25°C. Calibration a,b,c: 5 gm-wt, 1 min.

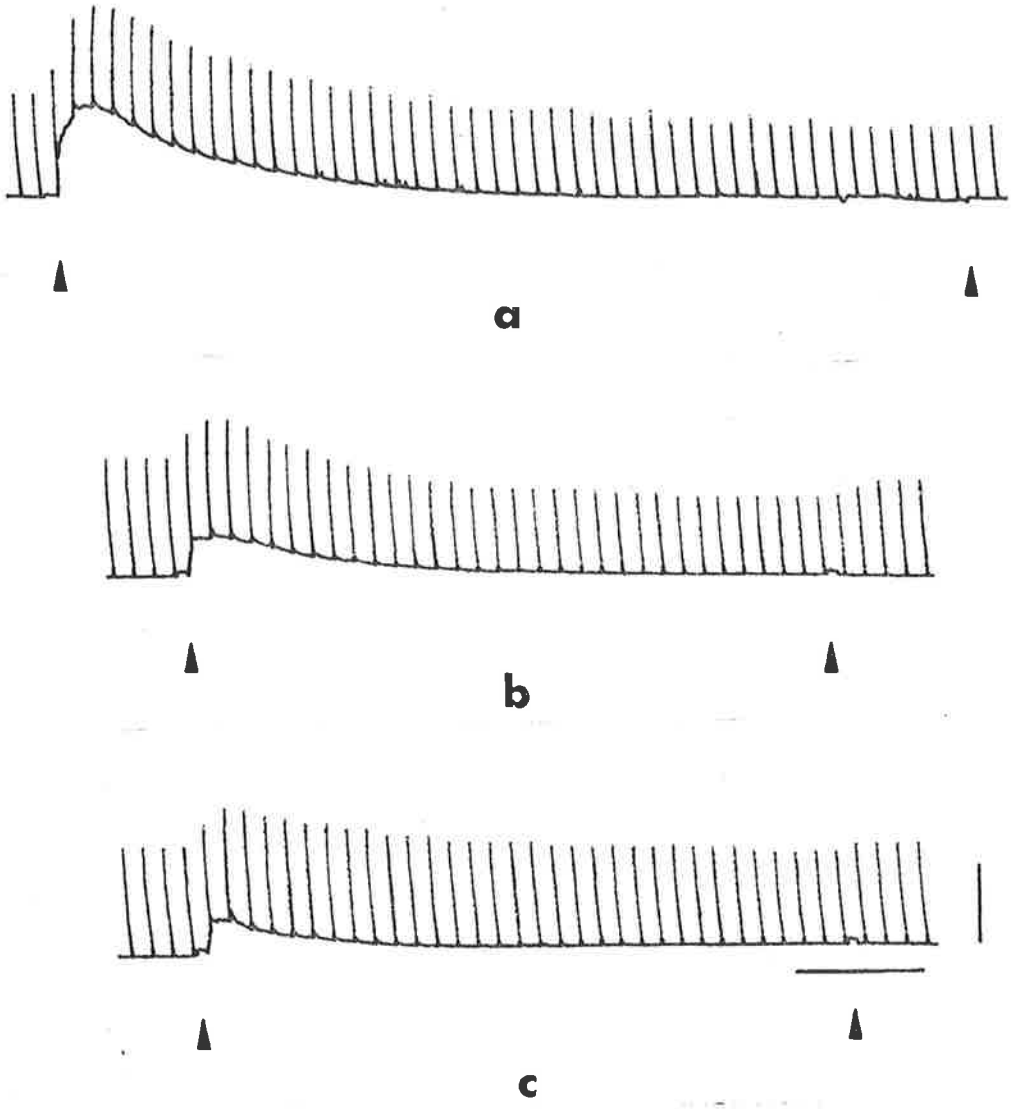


Fig. B-5. Effect of glycine on S.I.F.H. response. a: Normal S.I.F.H. response. b: Response to 35% S.I.F./65% S.I.F.H. c: Glycine (10^{-2}M) added with 35% S.I.F./65% S.I.F.H. Glycine has no obvious effect on the chloride free response seen in b. Temperature 25°C. Calibration a,b,c: 5 gm-wt, 1 min.

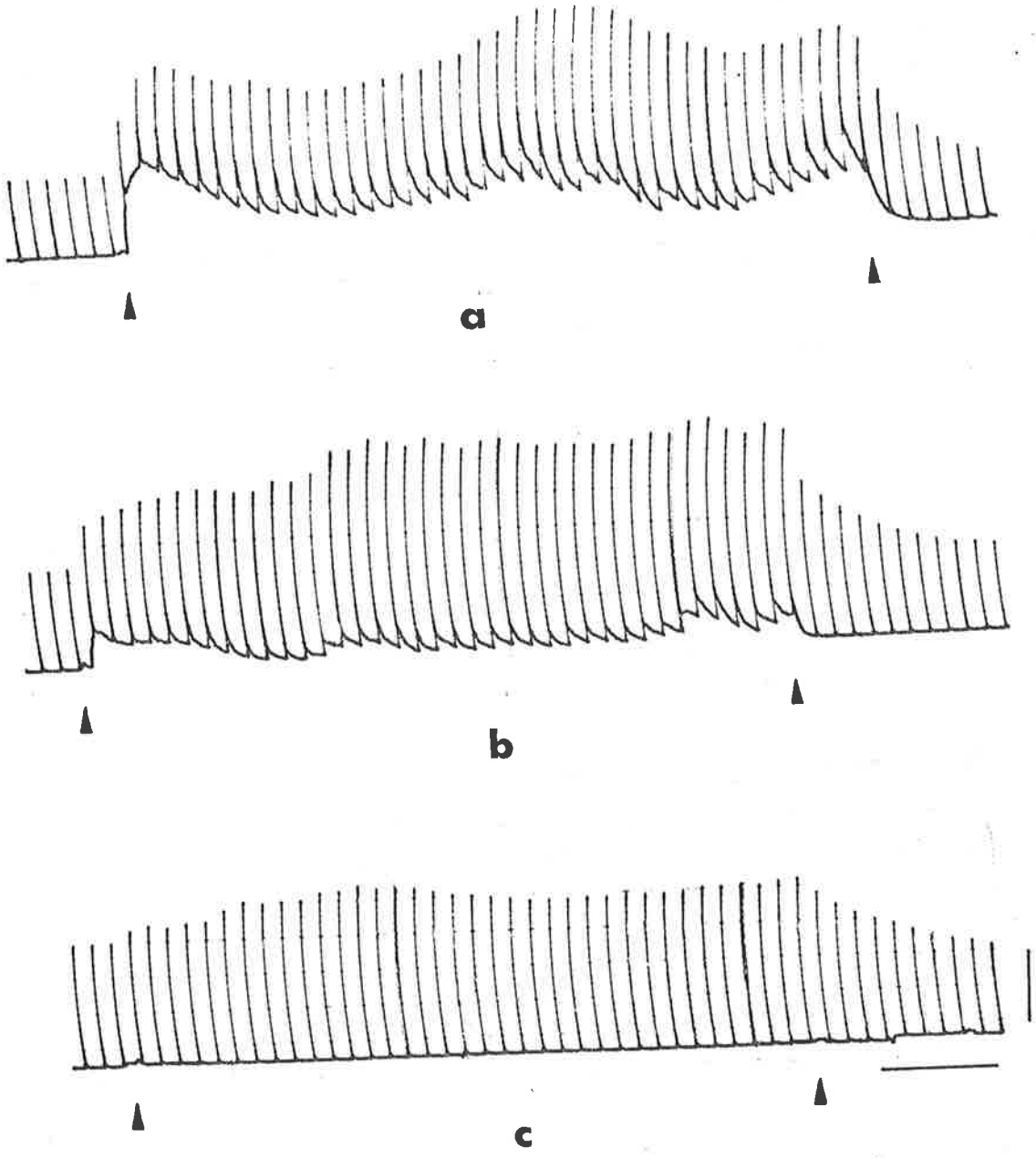


Fig. B-6. Effect of high K^+ on S.I.F.H. response following pretreatment. a: Normal S.I.F.H. response. b: S.I.F.H. (K^+ 18mM) added to S.I.F. (K^+ 18 mM) at first arrow and returned to normal S.I.F. at second arrow. c: As for b but K^+ 25mM. Temperature 25°C. Calibration a,b,c: 5 gm-wt, 1 min.

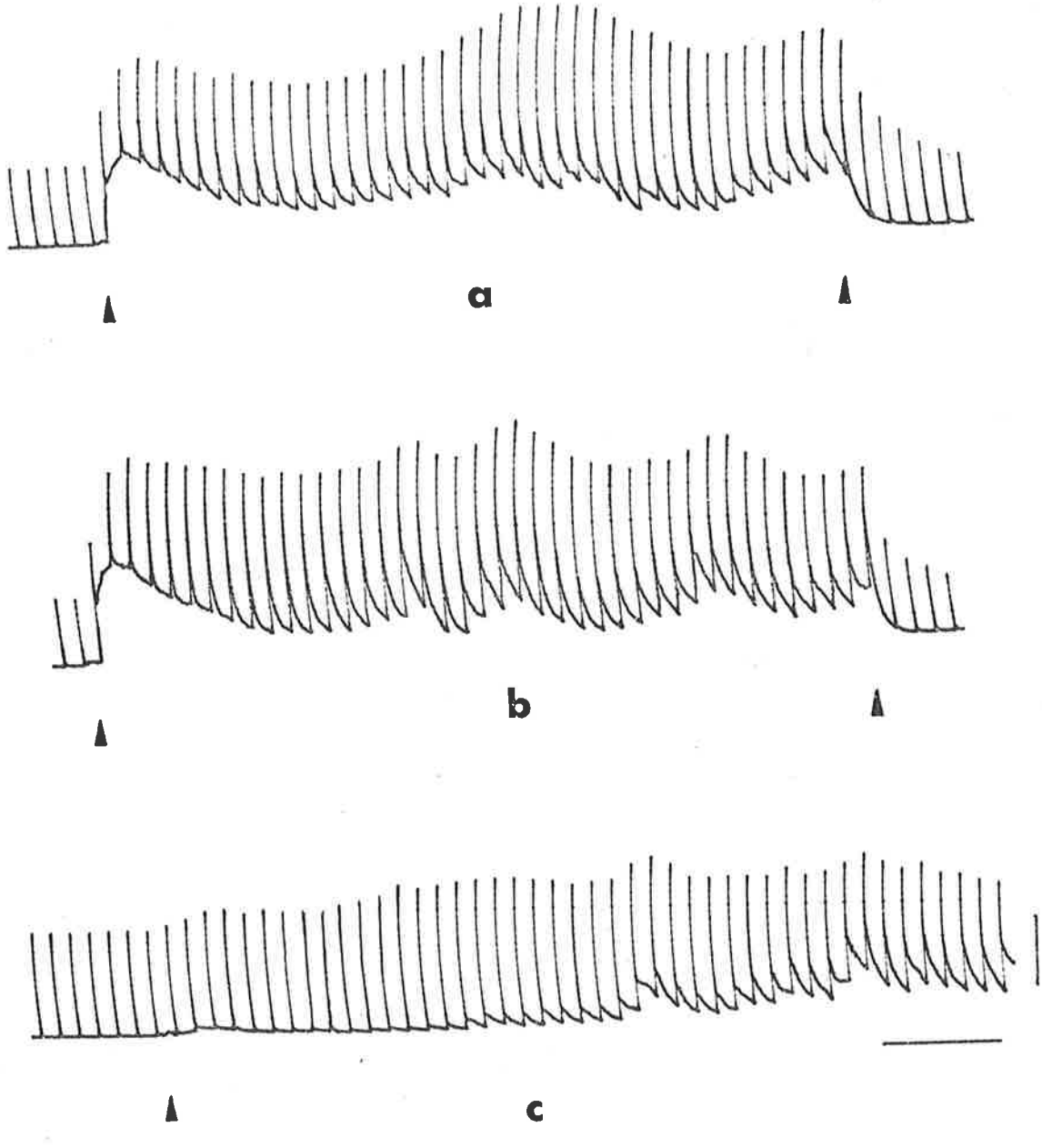
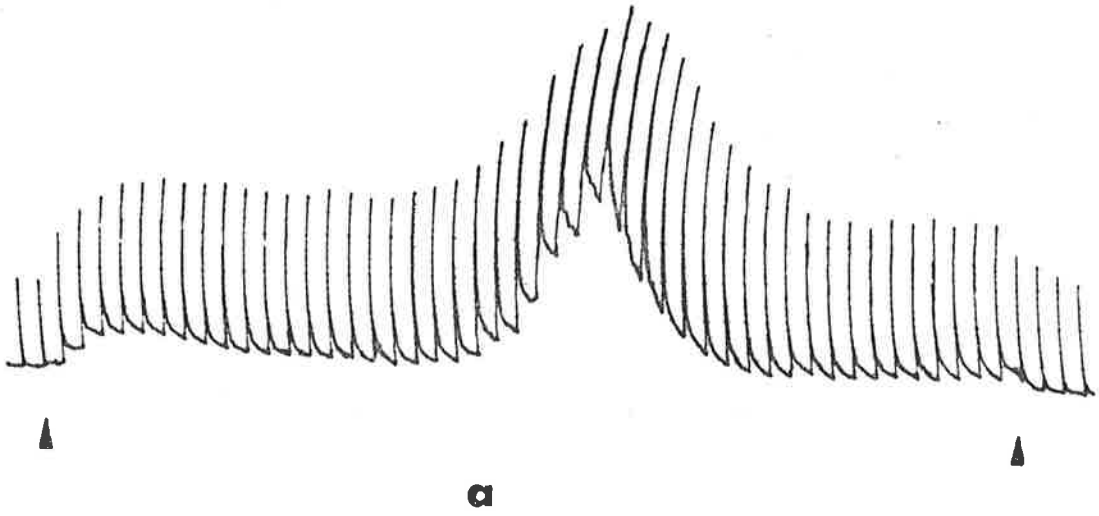


Fig. B-7. S.I.F.H. sensitivity to high K^+.
a: Normal S.I.F.H. response. b: No pretreatment with high K^+, but S.I.F.H. (K^+ 25mM) added between arrows. c: S.I.F.H. added at arrow following pretreatment with S.I.F. (K^+ 25mM) - chloride free response is still antagonised for some minutes. Temperature 25°C.
Calibration a,b,c: 5 gm-wt, 1 min.



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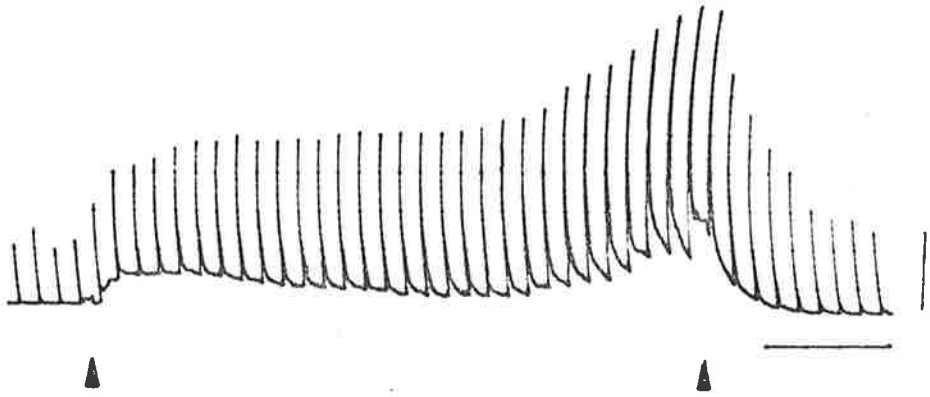

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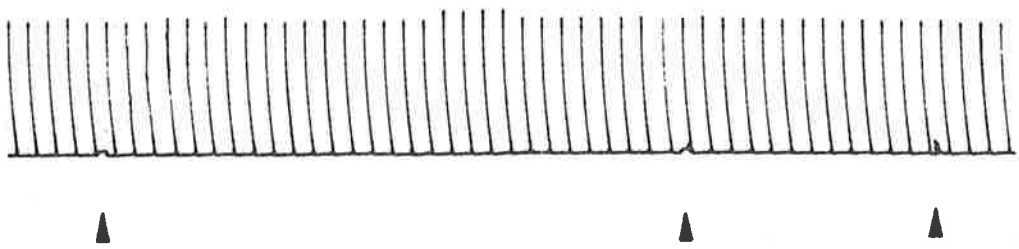


c

Fig. B-8. Sensitivity to low K^+.
a: Normal S.I.F.H. response. b: S.I.F.H. (K^+ 0mM) added between arrows produced no change to chloride free response. c: S.I.F. (K^+ 0mM) added between 2nd and 3rd arrows produced no change to normal S.I.F. twitch. Temperature 25°C. Calibration a,b,c: 5gm-wt, 1 min.

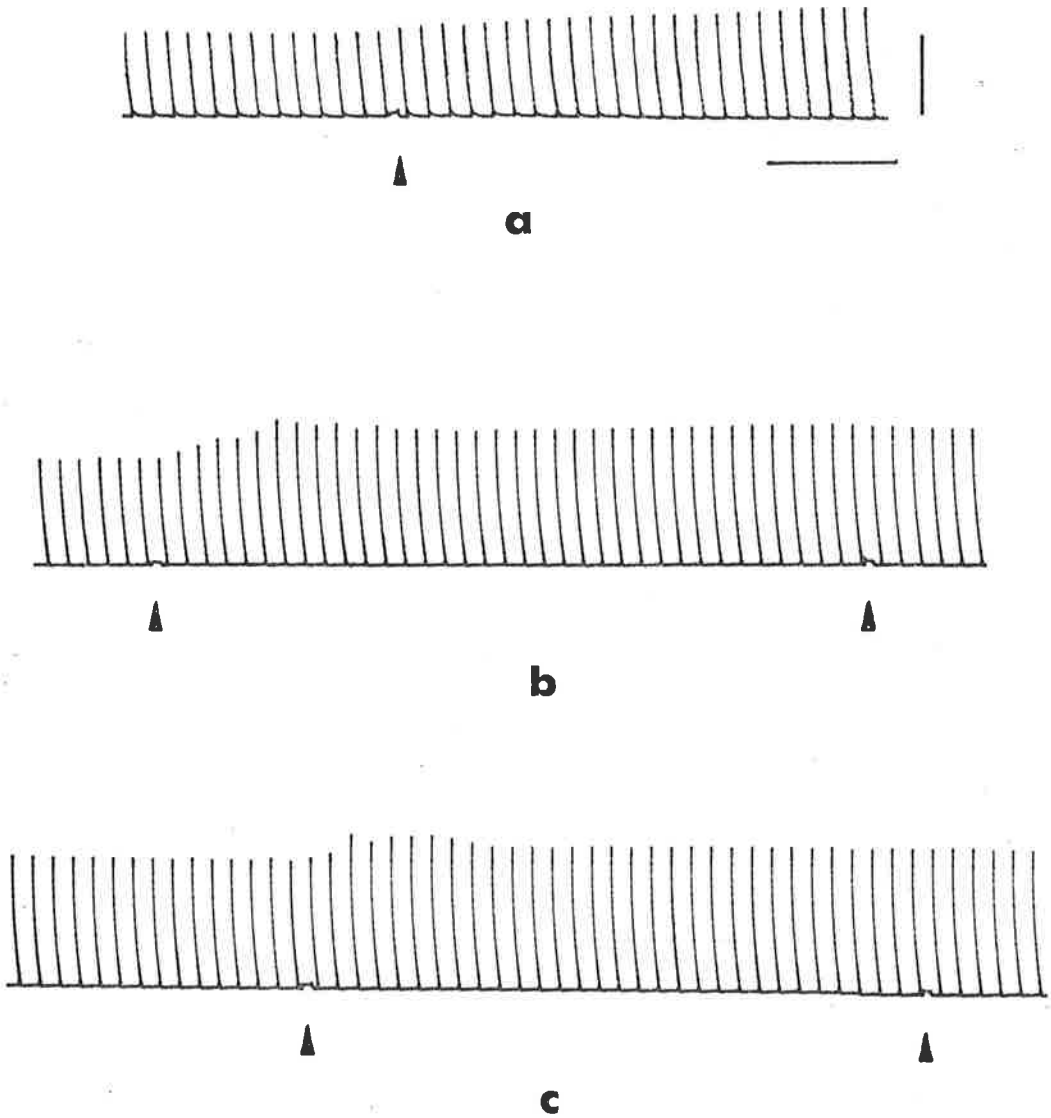


Fig. B-9. Twitch potentiation - K^+ dependent effects.
a: Addition of S.I.F. (K^+ 21mM) at arrow. b: TEA^+(40mM) added between arrows. c: TEA^+(40mM) added to S.I.F. (K^+ 0mM) where potentiation is still apparent. Temperature 25°C.
Calibration a,b,c: 5 gm-wt, 1 min.

B-8). Tetraethylammonium chloride (TEA^+, 50mM) was used in an attempt to examine this effect of high K^+ concentration. However, in the preparation tested TEA^+ had an inexplicable, deleterious effect on the normal twitch in S.I.F., these results have therefore been discarded. In other preparations TEA^+ (40mM) was shown to produce the expected increase in twitch tension potentiation, even in the absence of K^+ from the bathing solution (Fig. B-9b,c).

TABLE B-1

pH OF AMINO ACID SOLUTIONS IN S.I.F. (25°C)

Solution	Concentration	pH
GABA	40mM	7.4
Glycine	26mM	7.4
Hypaque	115mM	7.6

DISCUSSION

The replacement of Cl ions in bathing solutions with less permeant or impermeant anions has become a well recognised method of reducing the Cl^- or anion contribution to resting and action potentials in skeletal muscle (Hutter and Padsha, 1959; Falk and Landa, 1960a; Lüllmann, 1961). This technique has been used extensively to investigate the electro-chemical events associated with E-C coupling, particularly in amphibian fast muscle, as one of a variety of methods of potentiating twitch contractions (Kahn and Sandow, 1950; Hill and Macpherson, 1954; Sandow, 1964, 1965, 1970).

Sandow and his colleagues (Sandow, 1958, 1965, 1971, 1972) have studied the effects of various potentiators on active state kinetics by the standard technique of differentiation of twitch tension records together with a close scrutiny of concurrent action potential changes. These workers describe two types of potentiating effects based on action potential changes which have faithful correlates in the mechanical response. Semi-permeant anions e.g. NO_3^- and the lyotropic series are designated type A potentiators as they produce an increase in the rate of onset of the active state - related to a decrease in the mechanical threshold - the α-transform. A number of other substances, e.g. characterised by large cations, initiate the β-transform by prolonging the

repolarisation phase of the action potential, thereby extending the mechanically effective period and permitting fuller development of the active state. These substances are termed type B potentiators.

Impermeant anions, however, appear to demonstrate characteristics of both A and B types of potentiation since they reduce mechanical threshold (Sandow, 1964, 1965) and also delay repolarisation (Falk and Landa, 1960a). These effects occur presumably at both surface membrane and T-tubule sites, since they have a fairly rapid onset (Hodgkin and Horowicz, 1960a,c), and although impermeant to the cell surface the anions would be able to enter the T-tubules. (Adrian, 1964; Huxley, 1964). While the type B potentiating effects of impermeant anions have been observed (Falk and Landa, 1960a; Bretag, 1970), the type A effects are inferred from those demonstrated for semi-permeant anions (Sandow, 1965).

Since Hypaque substitution (S.I.F.H.) on rat diaphragm (Bretag, 1970) produces similar electrical effects to those of other impermeant anions (Falk and Landa, 1960a) - i.e. a steady state plateau in the repolarisation phase which may show spontaneous depolarisations, this effect being similar to the membrane instability of myotonia congenita and hereditary myotonia in 'fainting goats' - it is assumed that the mechanical responses will also be typical of these preparations. The effects of xylocaine on the S.I.F.H. response can be explained by its

general local anaesthetic action in decreasing the sensitivity of gNa to depolarisation, thereby offsetting the defective repolarisation mechanism, i.e. removing the chloride free response. That there is still some twitch potentiation apparent is an indication of the gK remaining to repolarise effectively the cell and/or to some interaction of the Hypaque anion with cations present without the cell.

The proportion of Cl^- in a bathing solution required to maintain normal action potential shape and twitch response, has been determined by a number of workers. Falk and Landa (1960a) demonstrated a sudden elimination of the chloride free response above 50mM Cl^- concentration in frog sartorius bathed in ferro-cyanide-Ringer. Juttner and Philpot (1965) and Smith and Roberts (1966), working with rat diaphragm also bathed in a ferrocyanide-Ringer found that the required Cl^- concentration was less critical although 48mM Cl^- was always sufficient to eliminate the delayed relaxation characteristic of the chloride free response - some twitch potentiation remained even while only 10% ferrocyanide in Ringer was included in the bathing solution. In the present study slightly less (42 mM Cl^-) was sufficient to eliminate the chloride free response (Fig. B-3). This reduction in Cl^- concentration necessary to produce stabilisation of the membrane probably indicates a real difference between the present study and others cited, since in the latter the low Ca^{++} concentrations caused by anion-Ca^{++} ion pair formation would probably have contributed to the labilising effect of the impermeant

anion by removal of part of the Ca^{++} stabilising action (Shanes, 1958 a,b; Falk and Landa, 1960a; Cheah, 1961).

It is noted however (Rüdel and Senges, 1972b) that at this concentration, although mechanical normality appears to be restored, some fibres would inevitably still demonstrate the electrical instability characteristic of low chloride (Bretag, 1970; Falk and Landa, 1960a).

The action of GABA on mammalian preparations has been reported previously. McLennan (1957), Elliot and Jasper (1959) and Watson (1961) have concluded that it has no action on neuromuscular transmission in either normal or denervated rat diaphragm preparations. Hofmann, Feigen and Genther (1962) however, have shown in rat intercostal muscle that GABA opposes the action of NO_3^- and veratrine at the neuromuscular junction. As the effect of NO_3^- substituted for Cl^- was presumably opposing the synaptic gCl (Hutter and Padsha, 1959), and GABA and glycine have been shown to antagonise this kind of effect at several synaptic sites - in particular at mammalian inhibitory spinal synapses (glycine) and central inhibitory synapses (GABA) (Eccles, 1969) - it seemed reasonable to suggest that some similar action of these amino acids might occur in rat diaphragm muscle, their action having previously gone undetected.

By exposing muscle to a concentration of S.I.F.H. just sufficient to produce a chloride free response after pretreating and/or including either GABA or glycine in the bathing solution it was hoped to observe

a decrease in the extent of the chloride free response or even perhaps complete elimination of it. In a few cases GABA (10^{-3}M) and more often glycine (10^{-1}-10^{-2}M) did appear to decrease the response slightly, however the vast majority of exposures to S.I.F.H. (60-70%) proved ineffective and so other responses are probably accounted for on the basis of inter and intra preparation sensitivity variation. Certainly in no preparation was an obvious inhibition of the chloride free response observed following this treatment. The pH of the test solutions being relatively high (Table B-1), should if anything have tended to increase anion conductance compared with normal S.I.F. (Hutter and Warner, 1967) - provided that this effect of pH dependence on gCl holds for mammalian muscle - so that it should have acted in the same direction hypothesised for the amino acids. Furthermore the concentration of GABA and glycine was quite high at least in the bathing solution. The results, taken with the above considerations argue against any direct effect of these amino acids on the chloride conductance of rat diaphragm muscle. (See Section C).

The effects of high K^+ on the chloride free and twitch potentiation effects of S.I.F.H. are of considerable interest. In this preparation, K^+ (25mM) was not sufficient to produce a potassium contracture - (Sandow, 1955; Hodgkin and Horowicz, 1960b) - probably due to species differences and because extracellular Ca^{++} activity remained sufficiently

high to effect stabilisation of the membrane (Shanes, 1958a,b). In addition the effect of adding K^+ (8-25mM) to S.I.F. in producing a twitch potentiation was also expected since some membrane depolarisation would occur under these circumstances as well as stimulation of the Ca^{++}-SR release mechanism during the action potential (Hodgkin and Horowicz, 1960c; Chapman, 1969) - both effects tending to lower the mechanical threshold thereby prolonging the mechanically effective period and the active state. TEA^+, in that it limits the K^+ repolarisation current by blocking of the K^+ pores (Hagiwara and Watanabe, 1955; Hille, 1967; Volle, 1970) also produced an expected response either in the presence or absence of K^+ in the bathing fluid, i.e. an increase in the twitch tension (Edwards, Ritchie and Wilkie, 1956).

High K^+ on the S.I.F.H. response did not produce the expected changes (Falk and Landa, 1960b). No effect was observed on the chloride free response unless the muscle had been pretreated with high K^+- S.I.F. prior to the addition of high K^+- S.I.F.H. Under these conditions however, when seven times normal K^+ was used (25 mM KCl) the chloride free response was abolished though some twitch tension potentiation remained. Falk and Landa (1960b) and Cheah (1961), while observing the stabilising effect of high K^+ on the chloride free response required lower concentrations of this cation and no pretreatment to obtain the effect.

In the present investigation it should be noted also that some inhibition of the S.I.F.H. response remained even when after pretreatment

with high K^+ - S.I.F., normal S.I.F.H. was added. There was no apparent change either to the normal response to S.I.F. or S.I.F.H. when K^+ was eliminated entirely from the bathing fluid - an action that usually causes instability of the resting membrane and which would be expected to have an additive effect to the S.I.F.H. response (Falk and Landa, 1960b).

These observations on the effects of variable K^+ concentrations on the mechanical responses of the diaphragm can be attributed to at least three causes. Firstly it has been shown by functional disconnection of the T tubules from the surface membrane in frog muscle (Gage and Eisenberg, 1967; Eisenberg and Gage, 1969) that K^+ conductance occurs in the T tubules and the surface membrane in the proportions 2:1 while all the gCl changes occur on the surface membrane. This finding is in agreement with Hodgkin and Horowicz, (1960a), who suggested that because external chloride concentration changes affect the cell more rapidly than external K^+ alterations, - the sites sensitive to K^+ may be less accessible than the Cl^- sites. The conclusion then is that unless K^+ can pass into the T tubules unhindered by large impermeant anions in the solution it cannot counteract the chloride free effect. i.e. to have its best stabilising effect K^+ must be exposed to the preparation before S.I.F.H. is added. Apparently the Hypaque anion restricts either the passage of K^+ into the T tubules or prevents it from exerting its membrane stabilising effect, perhaps

by interacting with it.

Next, an explanation of the generally increased stability of this preparation when compared with those of other workers, (Falk and Landa, 1960b; Hodgkin and Horowicz, 1960c; Juttner and Philpot, 1966; Rüdel and Senges, 1972b) probably resides in the high Ca^{++} activity maintained in the solution, since hypaque does not form ion pairs with Ca^{++} in solution (Bretag, 1970). Ca^{++} itself can act as a stabiliser in these preparations possibly by acting to increase the electrical threshold by adsorption to the outer cell membrane (Hodgkin and Horowicz, 1960c) where it may cause constriction of ion pores either by lipid expansion or electrostatic interaction (Shanes, 1958a,b).

Finally, it is suggested that high K^+ reduces the chloride free effect by causing a redistribution of ions across the muscle cell membranes so that more KCl enters the cell prior to S.I.F.H. treatment. On exposure to S.I.F.H. - high K^+ a further redistribution of ion species occurs, with the eventual concentration of Cl^- outside the cell sufficient to maintain a high enough gCl to provide for a fairly normal repolarisation current. The critical amount of additional Cl^- required to effect this stabilisation appears to be about 25mM. Stabilisation under these conditions of KCl addition is not surprising being more apparent in the present investigation than other workers have shown only because an impermeant anion is usually substituted with the high K^+ (Falk and Landa,

1960b). It is interesting however that a clear indication of K^+ action in the T tubules is obtained by the present method.

Contrary to this stabilising effect of high K^+ is the suggestion of Chapman (1969) that interaction between K^+ and sarcoplasmic reticular Ca^{++} releasing mechanisms may be responsible for the decrease in mechanical threshold usually seen in K^+ depolarisation contracture. These effects if they occur in this muscle would oppose the stabilising influence of high K^+ in S.I.F.H.

From the foregoing study it is suggested that Hypaque potentiates the twitch response in a number of ways. Addition of S.I.F.H. to the external environment of the cell will cause redistribution of K^+ since it will limit surface anion conductance - K^+ should move out of the cell - and membrane resistance will rise. This redistribution may affect the kinetics of Na^+ entry during depolarisation possibly producing an increase in the rate of rise of the action potential. Some preliminary release of Ca^{++} from the SR may also occur - perhaps linked with the K^+ and Cl^- redistribution within the cell and across the cell boundaries. These last two effects would lower the mechanical threshold. The most obvious effect of the impermeant Hypaque anion is however seen in its prolongation of repolarisation (Bretag, 1970), since the anion contribution to the repolarising current is severely reduced and the K^+ current is probably also affected (Harris, 1958; Hutter and Padsha, 1959), thereby prolonging electro-mechanical coupling further and allow-

ing greater development of the active state.

This schema is to some extent hypothetical since explanation of the mechanical threshold lowering effects of anions have not yet been attempted while the extent and contribution of anion - cation interactions can only be surmised at this stage (Horowicz, 1964; Diamond and Wright, 1969; Sandow, 1970; Wieth, 1970).

SUMMARY.

Considerable recent evidence suggests that the particular membrane defect in human myotonia congenita, hereditary myotonia in goats and a number of other induced myotonic states, involves an increase in membrane resistance mediated by a decrease in gCl which leaves the muscle cell unstable.

This condition can be simulated artificially in vitro by the replacement of Cl^- ions by non permeant anions in the bathing solution (S.I.F.H.), producing prolonged mechanical twitch responses.

Attempts have been made to stabilise this myotonic model by directly opposing its gCl limiting characteristics.

The preparation requires less than 42mM Cl^- concentration to overcome the myotonic response of isotonic S.I.F.H.

The amino acids GABA and Glycine do not appear to have any action on gCl in this preparation since they appear not to affect the S.I.F.H. response.

Pretreatment with 25mM KCl abolishes the myotonic response to high K^+- S.I.F.H.

These modifications are discussed in relation to the present understanding of excitation-contraction coupling.

SECTION C

ARTIFICIAL MYOTONIA II

(Some of the experiments in this section were carried out in collaboration with Dr. A. H. Bretag.)

INTRODUCTION

Myotonic symptoms can be induced in experimental animals and man by treatment with a number of drugs (Section B). Such artificially myotonic muscle can then be examined - usually in vitro - in an attempt to elucidate the causative defect and if this should prove to be the same as that in a natural disorder, the experimental preparations should prove useful in the assessment of therapeutic treatments for the disease.

Two kinds of myotonia inducing chemicals have been used recently with the above aim.

The first group - plant growth stimulant/insecticides - characterised by 2,4-dichlorophenoxyacetate (2,4-D), has been known to have myotonia inducing ability,for some time. The clinical symptoms are produced only a matter of minutes after ingestion of the required dose of the drug (Bucher, 1946), electric and mechanical confirmation of the myotonia being usually effected (Eyzaguirre, et al, 1948). Since Hofmann et al, (1966) were unable to show any effect of this drug in vitro on human intercostal or rat diaphragm muscle preparations, it took some time for the potential of this drug to be realised in this area. Eventually, however, Stein and Kuhn (1968) observed its effect on the mechanical response of the isolated rat diaphragm preparation. They noted in particular the delayed relaxation produced by 2,4-D and the

conditions under which this response could be enhanced or eliminated.

Several groups of workers have attempted to elucidate the basis of 2,4-D action, concentrating on membrane property changes either at the sarcolemma (Seiler, 1971) or sarcoplasmic reticular levels (Kuhn and Stein, 1966). The suggestion was made (Bretag, 1970; Rüdel and Senges, 1971) that 2,4-D myotonia may involve a similar surface membrane defect to that of myotonia congenita and hereditary myotonia in goats (Bryant, 1962; Lipicky and Bryant, 1966; Bryant, 1969): i.e. a large increase in resting membrane resistance in particular associated with a decrease in specific chloride conductance. Rüdel and Senges (1972a) following up these suggestions determined Rm in rat diaphragm treated with 2,4-D. Their results confirmed that an increase in Rm is indeed produced by 2, 4-D and that decreased gCl is responsible for the change (Senges and Rüdel, 1972). It seems reasonable then to assume that 2,4-D myotonia does artificially mimic the effects of myotonia congenita and represents a useful model for the characterisation of this disease.

Another group of drugs which has been shown to induce myotonia is the hypocholesterolemic - azacholesterols. Various azacholesterol analogues have been shown to block the desmosterol to cholesterol conversion step of cholesterol biosynthesis, suggesting a therapeutic use forthese drugs in the treatment of patients with high blood serum chol-

esterol levels (Counsell, Klimstra, and Ranney, 1962; Martt, Talbert and Lee, 1964). In the course of trials of 20,25-Diazacholesterol (DAC) Winer et al, (1964) and Winer, Klachko etal, (1965) discovered that apart from the expected decrease in cholesterol levels, some patients receiving the therapy started to display myotonic symptoms including all the usual mechanical and electrical expressions of the myotonias which were excaberrated by cold and relieved by warm-up. The symptoms disappeared soon after discontinuation of the DAC treatment.

Further investigations of DAC drugs on rats (Winer et al, 1966; Goodgold and Eberstein, 1968) and goats (Winer, Martt et al, 1965) have confirmed the myotonic inducing ability of this group of drugs and have characterised the precise electrical and mechanical changes that occur in muscle after a prolonged period of treatment. There is a striking similarity between the response of muscle treated in this way and in vitro muscle responses to low chloride solution. Both demonstrate repetitive electrical activity to single stimuli,resulting in prolonged mechanical responses, instead of the normal twitch. Recently Rüdel and Senges (1972a,b) have confirmed this similarity on the basis of electrical measurements in the diaphragm muscle of rats which had been bred on a DAC regime. These muscles demonstrated an increased Rm which was directly related to decrease in gCl, the same findings being apparent in muscle bathed in only 7.5% normal chloride concentration Tyrode. As

mentioned previously these are the same characteristics displayed by muscle from 'fainting goats' and patients suffering from myotonia congenita.

The DAC treated animal therefore offers another suitable preparation for the investigation of these diseases.

Although attempts at opposing myotonic (chloride free) phenomena with known gCl stimulants - GABA and glycine - had proved ineffective (Section B), the K^+ sensitivity of the 2,4-D preparation (Stein and Kuhn, 1968) suggested that under these conditions the muscle may react differently to the amino acids than it did in chloride free S.I.F. The mechanical effects of GABA and glycine on the 2,4-D treated diaphragm therefore have also been investigated in this section.

No previous observations on DAC - 2,4-D mechanical interaction appear to have been reported. It was expected that diaphragm muscle from a DAC animal, if treated in vitro with 2,4-D would demonstrate extreme myotonic symptoms; results of such an interaction are also discussed in this section. Finally, some effects of temperature on the mechanical response of the diaphragm from previously treated DAC rats (Section A) are discussed.

EXPERIMENTAL DESIGN

A. 2,4-D Treatment

Stein and Kuhn (1968) demonstrated prolonged mechanical responses to single stimuli of rat diaphragm muscle that had been treated with 2.5mM 2,4-D, the phenomenon taking some minutes to become apparent and being progressively eliminated by following stimuli. Attempts to reproduce these effects under the same conditions were almost entirely unsuccessful (see below Results), however discussions with Rüdel and Senges (Bretag, personal communication) indicated that the conditions of our experimental preparation were not entirely similar to those of Stein and Kuhn.

In particular these workers used stimulus durations of much greater length than ours (5-25 m.sec. cf. 0.5-1 m.sec.) and bathed their preparation in a Tyrode with lower Ca^{++} activity than S.I.F. Both of these factors would be expected to increase the response of the diaphragm considerably. Senges and Rüdel (1972) have shown that provided the Ca^{++} activity of the bathing solution was low enough, the effects of reduced stimulus duration could be overcome, permitting the development of myotonia after 0.5 m.sec. duration stimulus.

To ensure that any effect observed was directly due to 2,4-D in the bathing solution, S.I.F. was retained unchanged, but the

stimulus arrangement was altered from single 0.5-1 m.sec. pulses to a train of 3-4 impulses spread over a total duration of up to 10 m.sec. 2,4-D treatment (Stein and Kuhn, 1968) involved addition of the required concentration of the drug to the bath in S.I.F. (usually 0.5-5mM) and maintaining the preparation quiescent for 15-30 mins. After this time the original rate of stimulation (usually 10^{-1} sec.$^{-1}$) was continued for 30-40 secs. then the solution was changed and the process repeated as required.

B. DAC Treatment

Rats were treated as described in Techniques and Materials. It had been hoped to demonstrate clinical myotonia in these animals and intramuscular recording of the 'dive bomber' phenomenon (Winer, Klachko et al, 1965; Winer et al, 1966), however attempts to do so were abandoned when it was found impossible to continue the treatment for longer than about two weeks at a time, without the formation of extensive keratoderma at the sites of injection (cf. Anderson and Martt, 1965) and deterioration in the animals' general condition.

Since these attempts were made, several workers have succeeded in overcoming this difficulty, using a variety of methods. Goodgold and Eberstein (1968) injected 0.1mg./kg./day of 25 azacholesterol - Winer et al (1966) showed this drug to be at least ten times more potent a myotonic inducer than DAC. An oesophageal tube has been

used to admit the chemical in solution (Kuhn et al, 1968; Hofer et al, 1971) while Rudel and Senges (1971; 1972 a,b) included the chemical in the animal's normal diet.

Diaphragms from rats which had been treated intermittently with DAC, when their condition permitted, over a period of three months, were later used as experimental preparations. Since these investigations were carried out within two months of the animals' last treatment with DAC it is probable that some effect of the drug was still present (Burns, Dale and Langley, 1965; Eberstein and Goodgold, 1969).

RESULTS

In five preparations tested for 2,4-D myotonia in response to single stimuli three were entirely unaffected, one demonstrated a small response and the other - from a DAC treated rat - showed a clear myotonic mechanical effect. This variability in sensitivity to 2,4-D is similar to that described by Senges and Rüdel (1972).

Significantly the only preparation to show a clear cut myotonia was the DAC rat diaphragm (Fig. C-1). This preparation displayed the typical response described by Stein and Kuhn (1968) - a delayed relaxation, sometimes associated with an increase in twitch tension, which comes on only gradually, being obvious after 20-30 mins of quiescence and which is readily eliminated by successive stimuli.

The preparation that showed a slight 2,4-D response was tested for K^+ sensitivity - as were the other preparations. None of these, contrary to the findings of Stein and Kuhn (1968), showed any semblance of increased sensitivity to 2,4-D in the presence of increased K^+ concentration in the bathing solution. In fact the sensitivity if anything was reduced (Fig. C-2).

In the experiments where trains of impulses were employed to stimulate the diaphragm, 2,4-D myotonia was easily produced with low concentrations of the drug (0.5mM) (Fig. C-3). This response displayed all

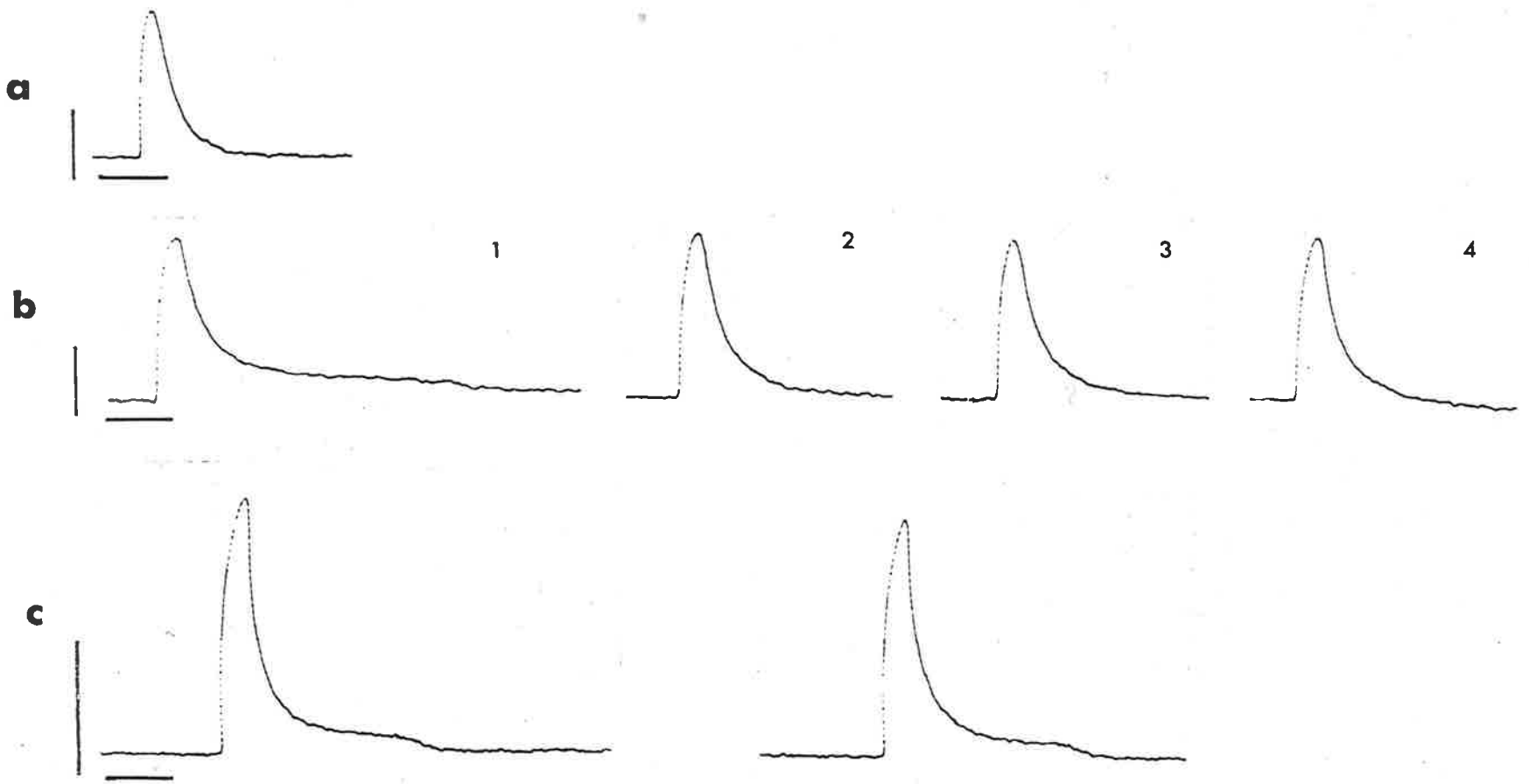


Fig. C-1 : DAC - 2,4-D Interaction
Diaphragm from rat treated with DAC. a: Twitch in S.I.F. b: First response, after 25 mins in 2,4-D (2.5 mM) without stimulation. 2-4 = successive stimuli at 10 sec. intervals. c: A further 2,4-D effect showing delayed relaxation in successive responses. Stimulation: single pulses, 1.m.sec. duration. Calibration: 2gm-wt, 200 m.sec.

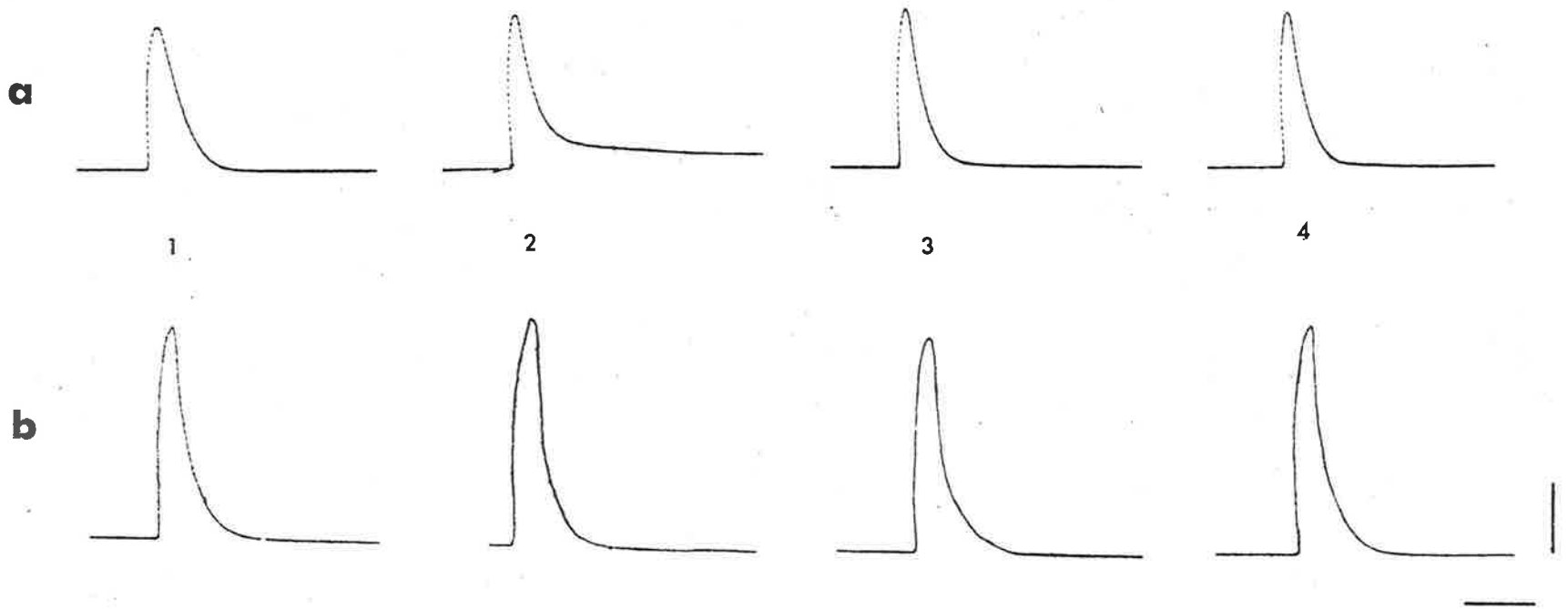


Fig. C-2 : 2,4-D - K^+ Interaction
Diaphragm from normal rat, treated in vitro with 2,4-D. a: 1 = S.I.F.; 2-4 = After 40 mins. in 2,4-D (2.5 mM). Myotonic response in 2 is eliminated by successive responses at 10 sec. intervals (3-4). b: 1= S.I.F. (K^+ 7 mM); 2-4 = After 40 mins. in 2,4-D (2.5 mM, K^+ 7 mM). Twitch height increases (K^+ effect) but myotonic signs are absent. Trace retouched. Stimulation: single pulses, 1 m.sec. duration. Calibration: 2 gm-wt, 200 m.sec.

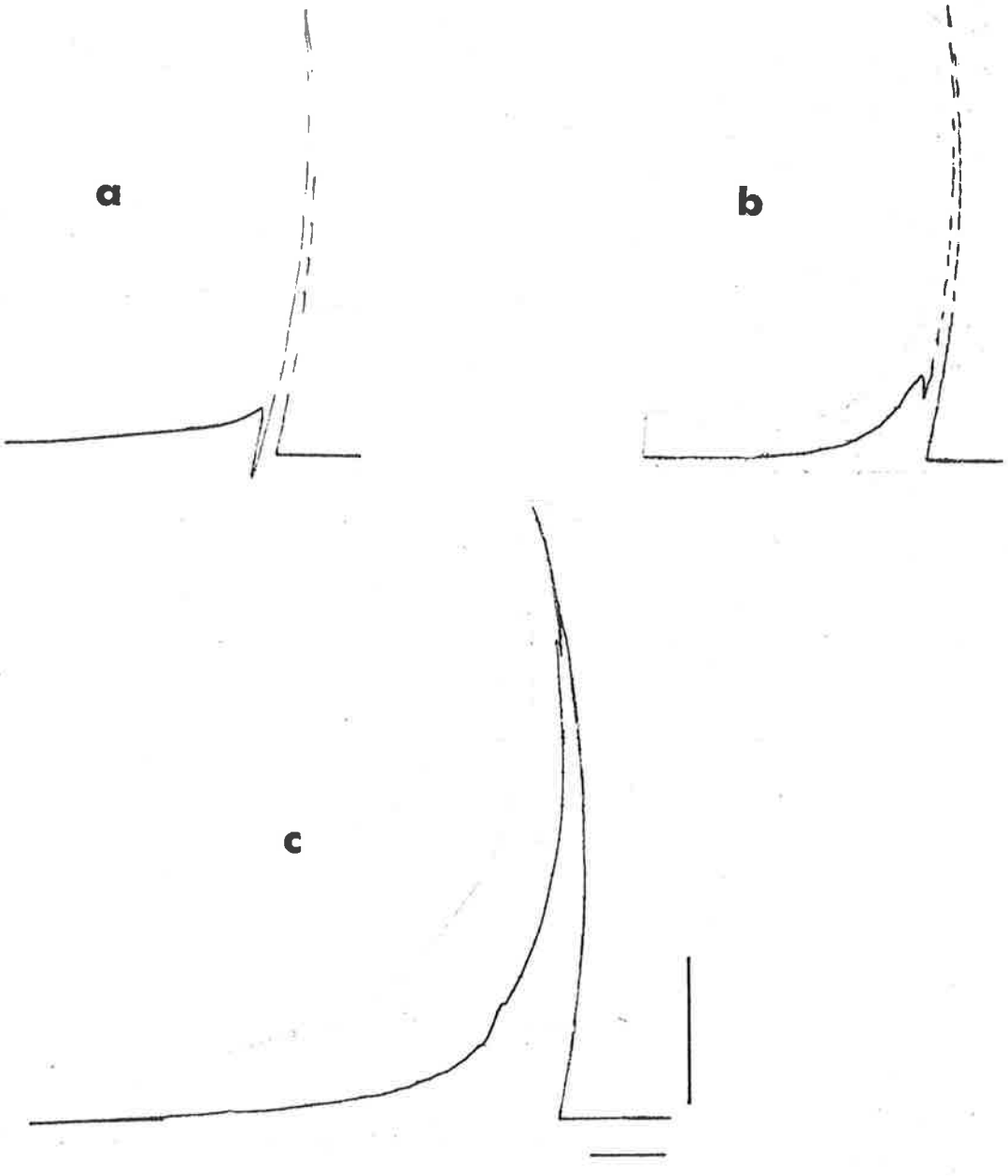


Fig. C-3: Development of 2,4-D Myotonia to trains of stimuli. a, b; successive stages in development of delayed relaxation in response to 2,4-D (0.5mM) and: another preparation in 2,4-D (3mM). For explanation of experimental conditions, see Text. Traces read from right to left. Calibration: 5 gm-wt, 1 sec. Figure retouched.

the characteristics of the 2,4-D - DAC preparation and in addition demonstrated an increase in relaxation time after only a few minutes (Senges and Rüdel, 1972).

The nature of the 2,4-D response was investigated by the addition of drugs expected to stabilise the muscle cell membrane. Unfortunately the recording apparatus did not permit a quantitative evaluation of twitch mechanics under 2,4-D treatment or while influenced by these drugs. The effects were most readily observed when the preparation was only slightly myotonic, ie. affected by only low doses of 2,4-D (0.5mM).

Xylocaine (2-10ug/ml) if added with 2,4-D prevents the onset of myotonic twitches and eliminates (already established) myotonia in the preparation (Fig. C-4).

GABA (10^{-2} M) and glycine (2.10^{-2}M) have a slight depressant effect on the 2,4-D response (Figs. C-5, C-6) seen as a shortening of the relaxation time.

The pH values of the bathing solutions were determined (Table C-1) since the concentrations of acidic components was increased cf. S.I.F. Only 2,4-D S.I.F. appears to have any potentially significant effect on the pH of the bathing fluid.

The effects of temperature on the twitch response of diaphragm muscle from DAC treated rats are shown in Section A, Figs. A-9 and A-10 and are described in more detail below.

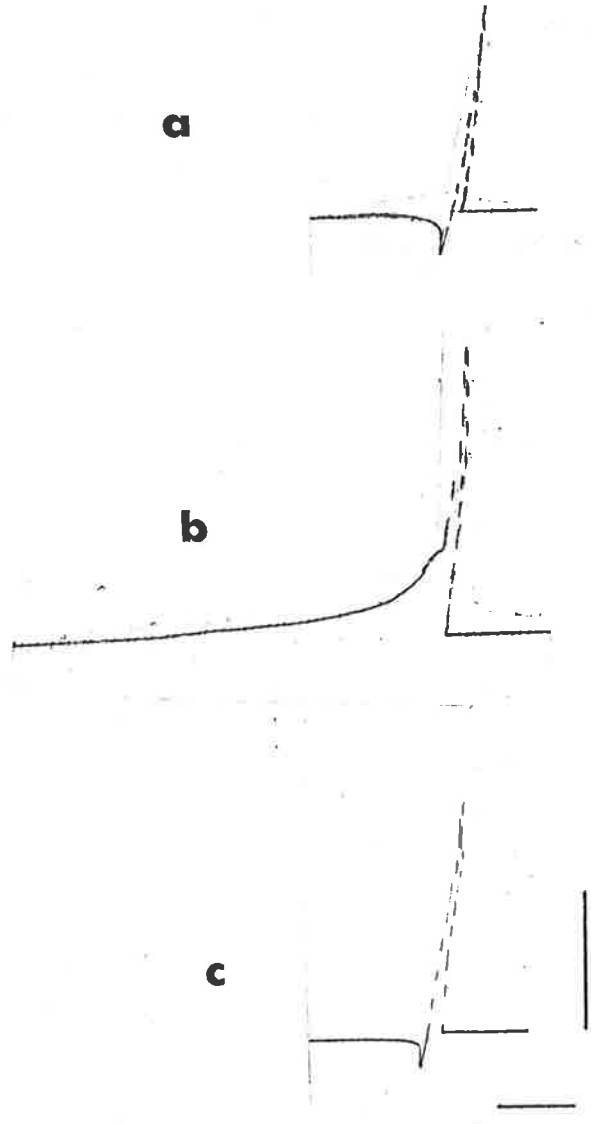


Fig. C-4: Effect of xylocaine on 2,4-D Myotonia.
a: Response of rat diaphragm in S.I.F. b: After 30 mins. quiescent in 2,4-D (3 mM). c: After a further 18 mins. quiescent in 2,4-D (3 mM) + xylocaine (10 ug/ml). Note elimination of myotonic response. Traces read from right to left. Stimulus conditions: Trains - see text. Calibration: 10 gm-wt, 1 sec. Figure retouched.

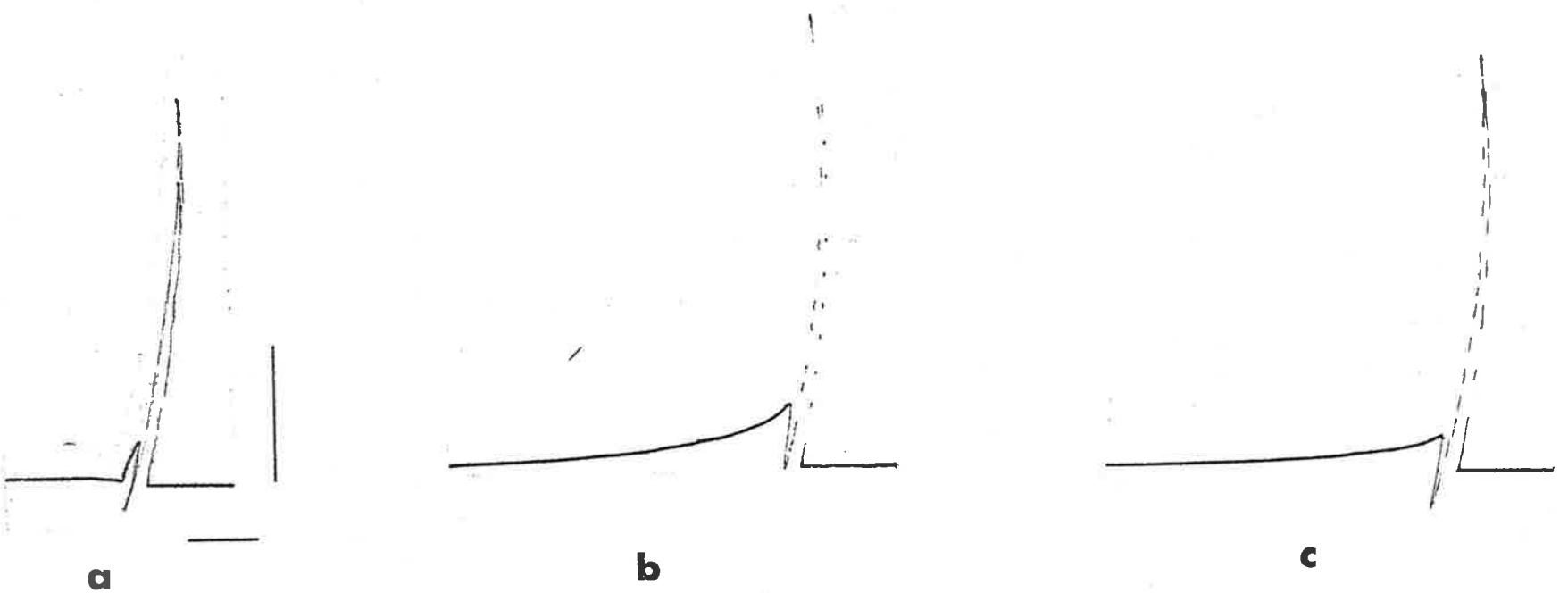
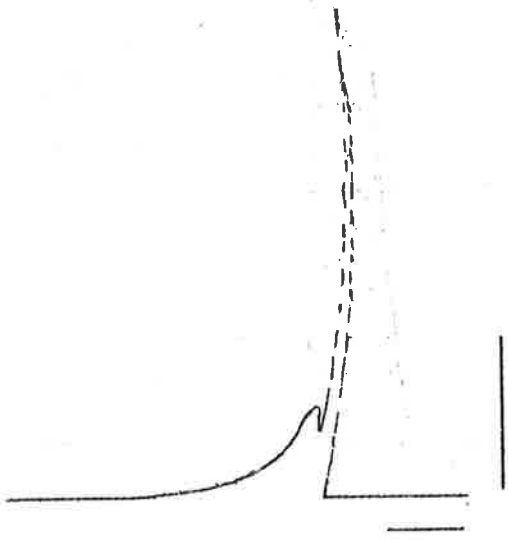
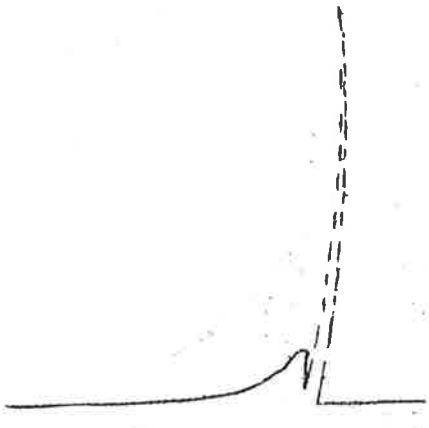


Fig. C-5: Effect of GABA on 2,4-D Myotonia.
a: Response in S.I.F. b: Response to 2,4-D (0.5 mM) after 25 mins. quiescent.
c: After a further 30 mins. quiescent in 2,4-D (0.5 mM) + GABA (4.10^{-2}M). Note slight decrease in height and delayed relaxation. Traces read from right to left.
Calibration: 5 gm-wt, 1 sec. Stimulus conditions: Trains. Figure retouched.



a



b

Fig. C-6: Effect of glycine on 2,4-D Myotonia.
a: Response of preparation to 2,4-D (3 mM) after 30 mins. quiescent.
b: Addition of glycine (10^{-1} M) with 2,4-D (3 mM) after a further 25 mins. quiescent. Note reduction in myotonic response. Traces read from right to left. Calibration: 5 gm-wt, 1 sec. Stimulus conditions: Trains. Figure retouched.

TABLE C-1

pH OF BATHING SOLUTIONS AT 25°C

Solution	Concentration of component added	pH
S.I.F.	-	7.3
2,4-D	5mM	7.06
GABA + 2,4-D (5mM)	40mM	7.3
Glycine + 2,4-D (5mM)	26mM	7.26

DISCUSSION

The actions of both 2,4-D and DAC have been shown to be entirely similar in at least one effect on muscle membrane. This involves an increase in specific membrane resistance due to a decreased chloride ion conductance, which has little effect on Em but leaves the cell more excitable than normal since gK must make an increased contribution to the resting membrane conductance, thereby reducing the current required to depolarise to the threshold potential. In addition the slow repolarisation phase - normally due to chloride ion movement into the cell - is slowed, thereby increasing the mechanically effective period and active state development so that an increase in twitch tension results, together with a slowed rate of recovery to the resting tension (Bretag, 1971; Senges and Rüdel, 1971; 1972; Rüdel and Senges, 1972 a,b). These effects appear to be qualitatively the same as those occurring in natural myotonia congenita and hereditary myotonia in goats (Lipicky and Bryant, 1966, 1971; Bryant, 1969).

Other possible actions of these drugs must also be considered in order to predict that these myotonic models will demonstrate faithfully the potential effects of a therapeutic agent on the natural disease. Some of the effects of other agents on 2,4-D and DAC treated muscle can be explained simply on the basis of the surface membrane change described above.

The response from the DAC preparation treated with 2,4-D confirms the established facts. As expected, both agents since they have the same membrane gCl effect, enhance each other's separate action. DAC treatment per se did not produce any myotonic symptoms under normal stimulating conditions (Figs. A-10, C-1). Eberstein and Goodgold (1969) have shown that the clinical indications of myotonia induced by DAC are observed only after prolonged treatment with the drug and so would not be apparent in the preparations used in this investigation. 2,4-D treatment involving single stimuli likewise produced little if no myotonic response, however when acting together evidently the combined effect - presumably in part of increasing Rm - was sufficiently large to produce increased excitability and enhanced mechanical activity.

The stabilising effect of the local anaesthetic xylocaine was apparent on 2,4-D induced myotonia. This effect does not necessarily indicate an involvement of Na^+ in the membrane abnormality that established the increased membrane excitability. Xylocaine inhibits the action of the lyotropic anions, and impermeant anions (Section B) by reducing the excitatory action currents flowing during the action potential thereby indirectly opposing the 'chloride free' myotonia.

The mechanism of action of 2,4-D on the surface membrane gCl remains unexplained at the present time. The H^+ releasing properties of the acid would probably contribute to the observed effect since decrease in

pH is associated with decreased gCl in frog muscle (Hutter and Warner, 1967). Any effect of this kind would be expected to be small however since the pH change observed was small (Table C-1).

The small but clear effect of GABA and to a lesser extent glycine on the 2,4-D myotonia suggests that these amino acids may have some effect on mammalian muscle, possibly related to gCl. Addition of GABA and/or glycine to the bathing solution tended to increase pH slightly, an effect that would be expected to increase gCl according to the evidence of Hutter and Warner. This change would then oppose the myotonic response by itself, but a direct effect of the amino acids on the membrane gCl may occur also.

The precise nature of this proposed amino acid - gCl interaction is not clear. The two residues would not be expected to have identical actions on mammalian muscle since they have been shown to act at other sites - Central Nervous System inhibitory synapses - with differential specificity (Eccles, 1969). A specific action of GABA on gCl might involve combination with a membrane component or drug added (eg. 2,4-D) that is preventing passage of Cl^-, thereby weakening its gCl inhibitory action. Such interactions may occur on the surface membrane to affect resting gCl (Eisenberg and Gage, 1969) or within the T tubule to help restore the contribution of Cl^- to the repolarisation phase of activity (Bretag, 1970).

The proposed effects of these amino acids on gCl is only inferred however; other possible specific membrane interactions with these residues may occur and must be possible since some mammalian membranes display specific carrier mechanisms for these amino acids (Schoffeniels, 1967). Confirmation of a specific gCl-GABA/glycine interaction in rat diaphragm under these conditions may be derived from an examination of the membrane properties of the muscle. It is of interest that some amino acid effect was observed in this section of the investigation, but not on the impermeant anion response (Section B).

The nature of the stimulus - a train of 3-4 impulses cf. a single pulse used in Section B - appears to make the preparation more sensitive to agents which could affect the myotonic response. This last observation may relate to the observed unresponsiveness of the singly stimulated 2,4-D preparation to increased K^+ in the bathing fluid. The results obtained agreed with those from Section B and Falk and Landa (1960 b) whereby a decrease in the excitability was observed with increased K^+ concentrations. Stein and Kuhn (1968) on the contrary found that K^+ increase enhanced the myotonic response of the rat diaphragm to 2,4-D in accord with the general observations on the effects of high K^+ on the naturally occurring myotonias (Brown and Harvey, 1939; de Jong, 1966). Evidently the increased stimulus duration in vitro, represents a more faithful copy of the naturally occurring stimulating conditions.

The long time course of myotonic induction by 2,4-D and DAC suggests that the basis for the observed effects may lie with structural alterations to muscle membranes and possibly to alterations of intracellular metabolism. Both in vivo and in vitro, the onset of 2,4-D myotonia is gradual, while the decrease in the extent of delayed relaxation seen on continuing stimulation (Fig. C-3) suggests that perhaps diffusion barriers are involved in 2,4-D activity and/or that depletion of some factor(s) occurs rapidly on stimulation.

Several studies have examined factors related to muscle cell membrane structure and function in patients with one of the naturally occurring myotonias.

Wakamatsu et al (1970) have observed lower levels of total serum lipids - desmosterol, cholesterol and triglycerides - in patients with m. congenita cf. normal subjects. Since constancy of lipid composition is important for maintenance of the efficiency of membrane function, these changes would be expected to have an effect on the sarcolemma and other sarcoplasmic membranes. Since the lipid content of plasma membrane is rather more than in other membranes of the rat muscle cell (Ashworth and Green, 1966; Kidwai et al, 1973) the effect of serum lipid changes should be greatest at this site. Kuhn and Seiler (1970) and Kuhn et al (1968) have detected a means of differentiation of muscle from patients with the recessive and dominant forms of m. congenita -

the recessive form of the disease having only recently been described (Becker, 1961) - the normal form involving a structural membrane change induced by a considerable alteration in the fatty acid pattern of muscle phosphatides. Hofer et al (1971) have noted disturbances to the energy supplying system of the muscle cell involving alteration to the normally constant hexokinase/citrate synthase activity ratio in muscle from patients with m. congenita and rats with DAC induced myotonia. They suggest that alterations in hexokinase activity could represent a basic membrane lesion of myotonia.

DAC induced myotonia appears to rely on the loss of cholesterol from membrane sites and its gradual structural but not functional replacement with DAC and desmosterol (Seiler and Kuhn, 1969; Peter and Fiehn, 1973). The greater the amount of cholesterol circulating in the animal or present in the membrane the longer it takes for myotonic symptoms to be induced (Eberstein and Goodgold, 1969). Peter and Fiehn, (1973) have followed the accumulation of desmosterol in the sarcolemma and fragmented SR of DAC treated rats, and although unlike Seiler and Kuhn (1971) they did not detect any significant alteration in the fatty acid pattern they noted that the activities of Na^+, K^+ and Ca^{++} stimulated ATPases of sarcolemma are increased during this treatment, cf. Seiler (1971 a,b). This increased activity would compensate for the increase in Na^+ influx and K^+ efflux occurring during repetitive activity in the myotonic

state associated with decreased gCl.

2,4-D has been shown to cause uncoupling of oxidative phosphorylation in rat muscle (Kuhn and Stein, 1965), producing an increase in energy turnover associated with increased ATP breakdown. This process would probably involve a change in the permeability of the cell to glucose, perhaps similar to that in DAC induced myotonia (Hofer et al, 1971).

Myotonic muscle also displays other membrane abnormalities. Although Samaha et al (1967) and Radu et al (1970) did not observe any change in actomyosin ATPase activity or Ca^{++} uptake ability of the SR in muscle homogenates from patients with m. congenita, Seiler and Kuhn (1970) have shown that the rate and total quantity of Ca^{++} uptake by SR vesicles is increased, while Ca^{++} efflux is decreased in this kind of muscle.

Both DAC and 2,4-D treated muscle demonstrate changes in SR activity. Seiler et al (1970) have shown that in muscle from DAC treated rats the SR membrane permeability to Ca^{++} is increased above normal, thereby reducing SR-Ca^{++} concentrating ability which would be expected to delay inhibition of myofibrillar ATPases and hence prolong relaxation. 2,4-D slows Ca^{++} uptake by the SR and increases the free Ca^{++} concentration in the sarcoplasm. This effect would also be expected to prolong relaxation and lead to a decrease in mechanical threshold (Kuhn and Stein, 1966). Enhancement of this effect as observed by these workers could be brought about by a direct action of K^{+} on the SR-Ca^{++} activity during

stimulation (Stein and Kühn, 1968; Chapman, 1969). Inability to demonstrate this K^+ effect in the present investigation was probability due to the differences in experimental conditions, in particular the normal Ca^{++} activity of S.I.F. (Bretag, 1969) would oppose the increased excitability due to 2,4-D. The apparent anomolous stabilising effect of high K^+ on 'chloride free' effects cf. its labilising action in myotonia, requires further investigation.

It is difficult to see how the action of 2,4-D on Ca^{++} concentrating ability of the SR can explain the apparent washout effect, similar to warm up in naturally occurring myotonia, observed in continued stimulation of the preparation, unless Ca^{++} leaks out of the SR during inactivity so that although the initial stimulus produces a very high Ca^{++} level in the sarcoplasm, successive stimuli following at 10^{-1} sec.$^{-1}$ stimulate Ca^{++} uptake to reduce the free Ca^{++} faster than it is raised by leakage from the SR.

It has been observed that on occasion single stimuli do not produce spasmic reactions in myotonic patients e.g. in the elicitation of reflexes (Senges and Rüdel, 1972). This may be connected with the present observation that 2,4-D myotonia is much more readily seen when trains of stimuli are used instead of single pulses. It is probable that there is a particular level of Ca^{++} that can be adequately taken up by the SR in response to a stimulus within the normal time of twitch

(Ebashi, Endo and Ohtsuki, 1969). 2,4-D lowers this threshold - the rate of Ca^{++} uptake is decreased - so that although one stimulus may not cause Ca^{++} level to exceed this threshold, successive stimuli may do thereby producing myotonia to a train of impulses but not to the single stimulus.

Twitch responses from DAC treated animals did not display any myotonic effects when bathed in S.I.F. of either a spontaneous nature or when stimulated. The response on heating although similar to that of normal muscle was only slight showing very little change in twitch tension between 30 and 40°C. These two preparations may have been 'inherently' less sensitive to the factors affecting the twitch in this temperature range (Section A). Since the Ca^{++} accumulating ability of DAC treated muscle SR is reportedly reduced compared with normal and reduced also by heating (Seiler et al, 1970; Johnson and Inesi, 1969) the increase in twitch response between 30 and 40°C might have been expected to have been greater than in the normal preparation. However the increase in kinetics of membrane current flow associated with activity at increased temperature may to some extent offset this effect in part explanation of the small changes recorded. Further experiments on the effects of temperature on the twitch response of DAC treated muscle are needed to clarify the situation.

The slight inhibitory effect of GABA and glycine on 2,4-D myotonia

provides some stimulus for the search and testing of agents which may have a specific effect on increasing excitable membrane chloride conductance, since such agents could prove to be potential anti-myotonia therapeutics.

SUMMARY.

In vitro myotonia has been observed in isometric twitches of diaphragm muscle from a rat treated with 20,25- Diazacholesterol (DAC) under the influence of 2,4-Dichlorophenoxyacetic acid (2,4-D) in response to single stimuli.

A similar effect has been observed in normal diaphragm preparations treated with low concentrations of 2,4-D when stimulated by a short train of 1 msec duration impulses.

The effects of temperature, 25-40°C, have also been examined on the isometric twitch response of the preparation from a formerly DAC treated rat.

The effects of high K^+ concentration, xylocaine, GABA and glycine have been demonstrated on 2,4-D myotonia.

The basic mechanism of 2,4-D and DAC myotonia appears to follow the natural disorders of the autosomal dominant variety of myotonia congenita and the hereditary myotonia of 'fainting goats'. These defects include increased specific membrane resistance related to a decrease in membrane chloride conductance, alterations in plasmalemma structure including enzyme and lipid dysfunction and decrease in Ca^{++} uptake ability of sarcoplasmic reticular membranes.

The treatments outlined above of DAC and 2,4-D affected muscle have

been discussed in terms of the membrane effects described and where appropriate, comparison has been made with similar treatments of 'chloride free' myotonic preparations.

B I B L I O G R A P H Y

(Abbreviations as listed
Index Medicus - January
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