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THE POTASSIUM INDUCED INCREASE IN METABOLISM
AND THE MECHANICAL THRESHOLD
IN FROG SKELETAL MUSCLE.

by



EVERT CORNELIS VOS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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OF DOCTOR OF PHILOSOPHY

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled The Potassium Induced Increase in Metabolism and the Mechanical Threshold in Frog Skeletal Muscle submitted by Evert Cornelis Vos in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Raising the potassium concentration of the Ringer's solution bathing frog skeletal muscle to just below the mechanical threshold resulted in an increase in the elasticity (resistance to stretch) of the muscle. The increase in the resistance to stretch of the muscle preparation reached a maximum in about 30 sec, with longer exposures it declined. The relation between the potassium concentration below the mechanical threshold and the resistance to stretch appears to hold only below the mechanical threshold. When a sub-maximal K^+ -contracture was elicited following a short exposure to submechanical threshold concentrations of potassium it was found to be potentiated with respect to the control which was elicited by the same potassium concentration without a pre-exposure to elevated potassium. The potentiation was characterized by an increase in the rate of tension development, a small increase in the maximum tension developed, an earlier and faster relaxation, and an increased resistance to stretch in the first few seconds of the contracture. In general potentiation was at a maximum following a 30 sec pre-exposure, with prolonged pre-exposures potentiation diminished and finally the contracture became inhibited. Potentiation was found to persist when following an exposure to a submechanical threshold potassium concentration the muscle was returned to Ringer's solution and then exposed to a contracture concentration of potassium

(Washout effect). This effect persisted for up to about 2 min if the initial period of exposure to elevated potassium was kept to 15-45 sec. Short, 10 sec, pre-exposures to a subthreshold concentration of caffeine or isotonic sucrose also resulted in potentiated submaximal K^+ -contractures; when the electrolyte concentration of the isotonic sucrose solution was progressively increased potentiation did not occur.

From experiments in which the membrane potential of peripheral fibers of toe muscles was continuously monitored while the potassium concentration was quickly raised either in one or two steps, it was found that potentiation could not be attributed to either an increased rate of depolarization or an increased depolarization. Also, the potentiation seen in the Washout effect could not be due to continued depolarization of the membrane because the resting membrane potential was restored in about 15 sec.

The oxygen consumption of a pair of toe muscles does not begin to increase till about 30 sec after the potassium concentration was raised to below the mechanical threshold; following the initial delay the oxygen consumption increased to a steady level with a half time of about 60 sec. Thus, a potentiated contracture was obtained during a period when the oxygen consumption was not yet increasing, while inhibition occurred during the increase in and increased level of oxygen consumption.

It is concluded that early during an exposure of frog's striated muscle to submechanical threshold potassium concentrations a condition exists which is similar to the mechanical latent period preceding the twitch. This mechanical threshold condition is brought about by a small increase in the internal free Ca^{++} concentration which will stimulate the sarcoplasmic reticular Ca^{++} -uptake resulting in an increase in metabolism. Once the metabolic rate is increasing the counterpart of the mechanical latent period is over and a condition similar to relaxation prevails; the latter constitutes the Solandt effect proper.

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To my wife, Rudolphine,
and my sons, Arnold and Aston.

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INTRODUCTION

The potassium contracture produced by elevating the potassium concentration in the medium bathing striated muscle has been of great importance in helping towards an understanding of the process of excitation-contraction coupling (Frank, 1958, 1960; Hodgkin and Horowicz, 1959, 1960a and b; for review Sandow, 1965). Over the past two decades a vast amount of information has accumulated which tells us how excitation-contraction coupling may be modified, which structures are involved in the process of activation and relaxation, the interplay of the contractile proteins, etc.

Although it was known since the early twenties (Rauh, 1922) that contraction followed excitation after a definite delay, this period of delay - the mechanical latent period - has received comparatively little attention since the early 1950's. It was known that increasing the potassium concentration to below the mechanical threshold caused marked changes in resting metabolism (review, Hill and Howarth, 1957), this response - the Solandt effect - was treated more as a curiosity than a phenomenon which could be related to events occurring during the mechanical latent period, i.e.

those events involved in producing the subsequent contraction.

The investigation reported in this thesis has dealt with the possibility that the Solandt effect could be identified with events occurring in the muscle when it goes from rest to activity at the mechanical threshold.

CHAPTER I

LITERATURE REVIEW

I(a). General Comments.

The work in this thesis is concerned with the Solandt Effect (Hill and Howarth, 1957) and the events at or near the mechanical threshold, thus by definition within the general area of excitation-contraction coupling. The latter term was coined by Sandow in 1952 and was defined as "the function of the muscle fiber in which an electrical depolarization of the plasma membrane initiates a sequence of reactions that causes mechanical activation of the contractile myofibrils lying within the membrane."

The general field of muscle physiology has been regularly reviewed particularly in the last decade (Gelfan, 1958; Mommaerts, Brady and Abbott, 1961; Huxley, 1964; Sandow, 1965; Wilkie, 1966; Peachey, 1968; Hoyle, 1969), furthermore, numerous symposia and books (e.g. Bourne, 1958; Ernst, 1963; Ebashi et al, 1965; Paul et al, 1965) have dealt exhaustively with historical and recent developments of the physiology of frog striated muscle in particular and comparative muscle physiology in general (Peachey, 1968; Hoyle, 1969; Comparative Aspects of Muscle Contraction, 1967). Because of the regular

reviews and in particular Sandow's (1965) exhaustive treatment of excitation-contraction coupling, the survey made here will be of a general nature. The Solandt effect will be dealt with thoroughly and material of relevance to it will be drawn upon in order that an understanding of this interesting effect may result.

I(b). The Mechanical Latent Period.

Following a single stimulus of sufficient strength to a twitch-type (fast) striated muscle there is a period of quiescence before tension development or shortening may be observed. This period, known as the mechanical latent period or simply latent period, has at room temperature a duration of about 3 msec. (frog) and at 0°C about 20 msec. The period varied depending on the source of the muscle, for example, in tortoise muscle the latent period at 0°C is 60-70 msec. (Hill, 1950). The latent period may be subdivided into two halves, during the first half nothing has been observed to happen; whereas during the second half a number of changes in the properties of the muscle occur which will be discussed below.

It was first observed by Rauh (1922) that a small drop in tension occurred just before the onset of tension increase. This observation was confirmed by Schaeffer and Göbfert (1937), and Sandow (1944, 1957). Sandow (1944) and Abbott and Ritchie (1951a, b) extended the original findings.

They were able to modify both the amplitude and the duration of latency relaxation. The duration and the amplitude may be decreased when a muscle is relaxed at a shorter length than its normal resting length, and conversely, the amplitude and duration is increased when the muscle is slightly stretched beyond the resting length. As Hill (1951) pointed out the latter finding appears rather paradoxical because slightly stretching the muscle and presumably also the series elastic elements which due to their stiffness limit the rate of tension development, would mean that the rate of rise of tension should increase and the moment the tension curve leaves the baseline should occur at the same moment as in the muscle at resting length. A. F. Huxley (1957) speculated that latency relaxation may be due to a momentary lengthening of the actin filaments should these be joined by the postulated S-filaments (Hanson and H. E. Huxley, 1955). Of more interest in explaining latency relaxation may be the finding of Baskin and Paolini (1966) and Baskin (1967) that in that period there is a small increase in volume of the muscle. Depending how this volume increase changes the length and diameter of the fiber an apparent lengthening could result, i.e. if the fiber were more extensible lengthwise than across its breadth. This explanation which as far as I am aware had not been proposed elsewhere, would also account for a decrease in the amplitude of latency relaxation at shorter than resting lengths because then the volume increase could

be taken up across the fiber assuming that the sarcolemma does not shorten as do the contractile filaments in the process of relaxing the fiber at the shorter length. Likewise, stretching the muscle beyond the resting length would make the sarcolemma less extensible across and the volume increase would be expressed as a greater increase in the length of the fiber proper.

The reason for the early increase in fiber volume is obscure. Baskin (1967) discussed his findings in the light of a report by Ikkai, Ooi, and Noguchi (1966) that the G to F transition in actin is accompanied by an increase in volume which is sufficient if 10% of the actin participates in the twitch. However, since the volume increase occurs at about the same time as the release of internal free Ca^{++} (Jöbsis and O'Connor, 1966; Ridgway and Ashley, 1967; Ashley and Ridgway, 1968), it is perhaps not unreasonable to suggest that the volume increase could at least in part be the result of a movement of water accompanying the Ca^{++} . This would probably be a release of osmotically inactive (bound) water because the increase in volume would not be measured if it were transferred across the sarcolemma.

Gilles (1969) offered an explanation for the duration of the mechanical latent period, he postulated that the decrease and increase of this period produced by varying the length of the muscle may be due to decreasing and increasing the diffusional pathway for Ca^{++} between the postulated site

of release at the terminal cisternae (Winegrad, 1965, 1967) and the A-I junction.

Latency relaxation may also be modified by increasing the potassium concentration in the bathing medium (Sandow and Kahn, 1952). These workers showed that increasing the potassium concentration to 17.6 mM led to an increase in the amplitude of latency relaxation, a decrease in duration of the latent period and twitch potentiation. On the other hand, prolonged exposure to elevated potassium caused a decrease in the amplitude of latency relaxation to below normal values, and eventually to zero while the duration of the latent period increased. Concomitant with these changes, the action potential height showed a steady decrease eventually reaching zero. Presumably by the time that the action potential disappeared (14-60 minutes) the extracellular space of the sartorius had become completely equilibrated with the bathing medium. The changes in the mechanical parameters could have been related to the Solandt Effect because the threshold for the metabolic effects of raised external potassium is at about 8-12 mM K^+ (Hegnauer, Fenn and Cobb, 1934).

Clinch (1968) also observed potentiation of the twitch at concentrations of potassium which would definitely have caused the Solandt Effect. He was able to maintain an excitable preparation by replacement of the Cl^- by NO_3^- which results in a shift of the Solandt Effect threshold

and the mechanical threshold to lower concentrations of potassium. Clinch (1968) did not, however, relate the potentiation of the twitch to the latent period and the time of exposure.

A. V. Hill (1949, 1950) determined that the heat production could be detected before mechanical activity was recorded. This heat of activation precedes tension development (in frog and toad sartori (Hill, 1949) and in the extensor iliobtibialis of the tortoise (Testudo mauretunica). In the latter, the onset of the heat of activation could be detected quite accurately and was placed at about 60 msec with tension development taking place about 40 msec later (at 0°C). The time of onset of the heat of activation agrees remarkably well with the onset of the increase in the resistance to stretch (Hill, 1950). The early increase in the resistance to stretch, i.e. before the end of the latent period, had been shown by Hill (1949) using the technique of applying quick stretches during the latent period of frog and toad sartorii. This finding was really confirmation of Gasser and Hill's (1924) experiments in which by means of quick releases they showed that the muscle was activated to the same extent at the moment that tension development began as it was on the plateau of tension in a tetanus. (but see, Jewell and Wilkie, 1958)

Sten-Knudsen (1953) did an extensive study on the torsional elasticity of single striated fibers. He found

that the torsional rigidity started to increase about 10 msec before the earliest increase in tension development. Since the experiments were performed at 0°C, this would place the beginning of the torsional rigidity increase at approximately halfway through the latent period and possibly at or at the beginning of latency relaxation (from data of Abbott and Ritchie (1948) initial negative phase at 7 msec, positive phase at 18 msec after the stimulus).

Finally, D. K. Hill (1949) observed that the transparency of the muscle increased reaching a maximum at the time that tension abruptly increased. The change in transparency coincided with latency relaxation which was measured simultaneously.

It is evident from the survey presented above that during the latent period and particularly during the latter half, definite mechanical and chemical changes do occur. These changes in the status of the muscle fiber must be considered as manifestations of the processes which constitute excitation-contraction coupling. It seems essential that to come to an understanding of these processes that changes which have been described will need to be explained. It is therefore of some importance that the investigator be permitted more time to study these than the few tenths of milliseconds at his disposal following an electrical stimulus.

I(c). The Role of Calcium in Excitation-Contraction Coupling.

That calcium is of major importance to sustain contractile activity was realized nearly a century ago by Ringer (1883) who reported that frog heart would cease to contract when Ca^{++} was omitted from the perfusing medium. This finding became even more significant when Locke and Rosenheim (1907) and Mines (1913) were able to show that omission of Ca^{++} from the perfusion medium abolished contractile activity in the rabbit and frog heart but not electrical activity. Locke and Rosenheim (1907) concluded that Ca^{++} was necessary for the "production of the wave of contraction out of the wave of excitation." Mines (1913) also showed that Sr^{++} in place of Ca^{++} prolonged both the mechanical and electrical responses of the frog heart.

Until recently very little was added to the findings made by the above mentioned workers, only confirmatory evidence was obtained (Daly and Clark, 1921; Bojue and Mendez, 1930). In addition Bay, McLean, and Hastings reported in 1933 that in the rabbit heart the strength of contraction was proportional to the Ca^{++} concentration in the bathing medium.

In skeletal muscle a different situation exists. Electrical activity and mechanical activity are both abolished when Ca^{++} is removed from the bathing medium (Ishiko and Sato, 1957; Edman and Grieve, 1964). Thus, the requirement of Ca^{++} for generation of the action potential would obscure

an involvement of Ca^{++} in skeletal muscle similar to that originally seen by Ringer (1883) in cardiac muscle.

It was not until 1958 that the requirement of external Ca^{++} in E-C coupling was established when Frank (1958) showed that the potassium contracture of the extensor digitorum longus IV (toe muscle) of the frog could be abolished after removal of external Ca^{++} for as short a period as 1 minute without a change in the degree of depolarization. In a subsequent paper, Frank (1960a) enlarged upon his earlier findings and showed that the rate of abolition of the potassium contracture in a medium not containing Ca^{++} was dependent on the rate at which Ca^{++} was removed from the extracellular space and concluded that Ca^{++} was superficially and loosely bound to the membrane. Of equal importance was his finding that although the potassium contracture was abolished, caffeine was still able to elicit a contracture. Hence, removal of loosely bound Ca^{++} was necessary for excitation-contraction coupling, i.e. necessary to elicit a contracture in response to depolarization of the membrane, but another source of Ca^{++} appeared to be needed for contraction itself. The latter fraction subsequently termed "the firmly bound Ca^{++} " (Frank, 1962) was releasable by caffeine. The firmly bound fraction of Ca^{++} was probably identical with that described by Harris (1957), Gilbert and Fenn (1957), and Shanes and Bianchi (1959) on the basis of flux studies. Also this would be the fraction of Ca^{++} postulated by Heilbrunn

(1937, 1940, and 1943) as that Ca^{++} present in the "cortex" of the cell which is necessary for activation of the myosin adenosine triphosphatase.

Frank (1962) furthermore showed the importance of the superficially located Ca^{++} in excitation-contraction coupling and the firmly bound Ca^{++} fraction in contraction when he showed that certain multivalent cations, Sr^{++} , Be^{++} , Mg^{++} , Ni^{++} , and Co^{++} , could support a contracture in response to depolarization by high K^+ after the superficially located Ca^{++} had been removed and K^+ -contracture had been abolished. Of these cations only Sr^{++} , Be^{++} , and Mg^{++} are related to Ca^{++} , but Ni^{++} and Co^{++} are in no way similar to Ca^{++} . Although these cations could replace Ca^{++} in excitation-contraction coupling they could only do this for a limited number of contractures and once the contracture had been abolished caffeine was ineffective in producing activity. Conversely, contractures could not be restored using the 'foreign' cations once caffeine contractures had been abolished in a Ca^{++} -free medium. Edwards, Lorković and Weber (1966) showed furthermore that Sr^{++} is the only cation among the group mentioned above which could in a limited way replace Ca^{++} in activating myofibrillar ATPase and be taken up by isolated sarcoplasmic vesicles. Also Sr^{++} was taken up by the muscle during activity whereas Co^{++} was not.

Thus it is clear from these results that Ca^{++} in the tightly bound fraction is intimately associated with

contraction while the superficially located Ca^{++} is essential in excitation-contraction coupling where it probably plays a permissive role as judged by the Ca^{++} replacement studies.

Winegrad (1961) and Frank (1961) calculated on the basis of previous flux studies (Bianchi, 1959) that a rather large discrepancy existed between the amount of Ca^{++} that moved into the muscle as a result of electrical stimulation or K^{+} -depolarization, and that which was required for contraction. That the discrepancy existed was confirmed by the findings and calculations of Weber, Herz and Reiss (1963) and Portzehl, Caldwell and Ruegg (1964). The Ca^{++} influx was at least 10X too small to obtain threshold effects and at least 100X too small for maximal activation of the contractile apparatus (Sandow, 1965).

The survey of the role of Ca^{++} in excitation-contraction coupling may be concluded by stating that Ca^{++} is the only physiological divalent cation which is necessary to permit the coupling of excitation to contraction, and this Ca^{++} is superficially located. The above survey was limited to fast, twitch type striated muscle of the frog and it may be concluded that in at least this type of muscle the superficially located Ca^{++} does not directly participate in contraction. The same general conditions pertain to other fast striated muscles (for review see Sandow, 1965; Peachey, 1968; Hoyle, 1969).

I(d). Regulation of Calcium Movements in Contraction and Relaxation.

In the previous section evidence was discussed which strongly indicated that Ca^{++} is involved in the contractile process proper. The evidence that Ca^{++} is necessary for stimulation of myosin adenosine triphosphatase goes back to Bailey (1942) following the discovery of adenosine triphosphatase associated with myosin by Engelhardt and Ljubimowa (1939). However, it was pointed out (Sandow, 1965) that Bailey's conclusions would be regarded as fortuitous because he dealt only with myosin and not actomyosin. Nevertheless, Bailey (1942) postulated that upon excitation Ca^{++} is liberated close to myosin and that the consequent splitting of ATP provided the free energy for contraction. Heilbrunn (1937) had made a similar postulate and in later work he (Heilbrunn, 1940) provided additional evidence. More direct evidence was provided by Heilbrunn and Wiercinsky (1947) who showed that Ca^{++} was the only physiological ion which could produce shortening. They injected by micropipette various ions into frog skeletal muscle fibers and followed the contraction microscopically. They observed that in addition to CaCl_2 in concentrations as small as 0.2 mM, BaCl_2 and SrCl_2 also caused shortening but that injected Mg^{++} , Na^+ , and K^+ did not. The fact that K^+ when injected did not cause shortening was rather decisive evidence against Szent-Györgyi's (1945) theory in which he proposed that

movements of K^+ governed contraction and relaxation because Ca^{++} and Mg^{++} were both strongly bound by myosin and therefore could not participate in the regulation of contraction.

The participation of Ca^{++} in contraction or superprecipitation (isolated contractile proteins) had been a continuing problem since it was shown that highly purified actomyosin preparations undergo superprecipitation in the presence of Mg^{++} and ATP. This occurred even in the presence of EGTA, a specific Ca^{++} chelating agent, in sufficient amounts to reduce the Ca^{++} concentration to negligible levels (Perry, 1967). However, crude actomyosin (natural actomyosin) or glycerinated muscle fibers showed a requirement for Ca^{++} (e.g. Bozler, 1954). Ebashi (1963) showed that the difference in behavior of the model contractile system and the natural actomyosin complex was due to the absence in the former of a protein fraction termed the EGTA sensitizing factor. Ebashi and Kodama (1966) suggested that the EGTA sensitizing factor consisted of tropomyosin which had been identified earlier (Bailey, 1948) and a second protein called troponin which are associated with actin. Recently, Ebashi, Kodama and Ebashi (1968) reported further on their earlier observations and concluded that troponin in the presence of tropomyosin profoundly affected the physico-chemical properties of F-actin. The Ca^{++} sensitivity of the acto-myosin system is conferred by troponin through tropomyosin. These workers concluded furthermore that "in the absence of Ca^{++} troponin

exerts a depressing action on the interaction of actin and myosin and this inhibition is abolished by the action of Ca^{++} on troponin."

At about the time that the discrepancy of Ca^{++} requirement of natural actomyosin and model (purified) actomyosin was being resolved, A. Weber and her co-workers (Weber and Winicur, 1961; Weber and Herz, 1962, 1963; Weber, Herz and Reiss, 1963, 1964) made highly significant contributions to understanding the Ca^{++} participation in contraction. The use of EGTA greatly aided in quantitating the Ca^{++} requirements (Weber and Winicur, 1961). It was found that in their 'natural' actomyosin preparations contractile-like activity could only occur when Ca^{++} was present. The minimum, threshold, concentration was found to be about 2×10^{-7} M and maximum activity was reached at Ca^{++} concentrations of $5 - 10 \times 10^{-6}$ M. These results agreed remarkably well with those obtained by Portzehl, Caldwell and Ruegg (1964) who injected Ca^{++} -EGTA buffered solutions into living fibers of the crab Maya squinado and found threshold effects at a free Ca^{++} concentration of $3 - 15 \times 10^{-7}$ M and full activation at a concentration $7 - 14 \times 10^{-6}$ M.

Recently Gilles (1967, 1969) reported on a series of elegant experiments conducted on glycerinated myofibrils of the crab (Cancer). The advantage of this preparation is that the sarcomere length is $10-15 \mu$ which allows application of Ca^{++} by means of a micropipette at discrete points

along the sarcomere. The glycerinated myofibrils were suspended in a relaxing medium containing Mg-ATP and EGTA, Ca^{++} was applied iontophoretically and the current needed to release sufficient Ca^{++} to observe contraction was measured. It was found that the threshold current increased with the distance from the A-I junction along the I band. Along the A-band the threshold current also increased provided that the A-I overlap was small, so that when there was a great deal of overlap of the filaments the threshold current did not increase. The conclusions drawn from these results were that in order for Ca^{++} to initiate contraction it has to reach at least the A-I junction and that the region of the A-I junction is sensitive to Ca^{++} but I and A bands where no overlap is present are not.

In recent years Podolsky and his associates have conducted a series of experiments using the so-called Natori preparation (Natori, 1954). This preparation consists of a single fiber of the frog's semitendinosus from which a 1-2 mm length of sarcolemma has been removed by careful dissection thereby giving direct access to the interior of the muscle fiber. Costantin, Franzini-Armstrong and Podolsky (1964) applied drops of KCl (1×10^{-1} M) with diameters similar to that of the fibers (40 - 100 μ) to the denuded preparation and found that in about half the trials contractions were elicited which often began after a definite delay some distance away from the point of application. The

contractions were reversible and lasted 1-2 seconds, they could only be elicited when Cl^- was present; replacement of Cl^- by methyl-sulfate or proprionate resulted in an inability to elicit the response. Reducing the diameter of the drop to less than 40μ resulted in a loss of the chloride response but contraction could then be obtained if Ca^{++} or Sr^{++} were present (Podolsky and Hubert, 1961). The chloride response was not repeatable, a second application resulted in only a reduced contraction or none at all (Costantin et al., 1964). It was concluded that the application of Cl^- led to a potential change across the membranes of the sarcoplasmic reticulum which in turn causes a release of Ca^{++} from these structures. Costantin and Podolsky (1965, 1967) added weight to their earlier conclusions when they found that short (0.2 - 1 sec) direct current pulses of $1-10\mu$ amp led to similar responses to those observed as a result of Cl^- application. It was also concluded that application of Ca^{++} led to contraction which resulted from direct activation of the contractile apparatus. More recently Hellam and Podolsky (1966, 1969) were able to report on force measurements of contractions elicited by application of Ca^{++} to the Natori preparation. Controlling the Ca^{++} concentration with EGTA they found that the threshold for contraction was at a Ca^{++} concentration of about 10^{-7} with maximum force developed at 10^{-6} . They also showed that above threshold, the rate of isometric force development and the isometric force was related to the concentration of Ca^{++} .

The relationship between Ca^{++} concentration and force development was S-shaped similar to that found by Edwards, Lorkovic and Weber (1966) who showed a S-shaped relationship between frog myofibrillar ATPase activity and Ca^{++} concentration.

Weise (1934) comparing the Ca^{++} content of filtrates of rat skeletal muscle before and after exercise demonstrated that activity caused a change of the muscle Ca^{++} from a bound to a free form. In the following year Lánzos (1935) demonstrated release of Ca^{++} from stimulated frog hearts. Following the introduction of Ca^{45} , Woodward (1949) was able to report on the release of this isotope during contraction of frog skeletal muscle, and some years later Shanes and Bianchi (1960) showed release of Ca^{45} during electrical stimulation and potassium contractures of frog sartorius muscles. Thus it became clear that in contraction, which needed Ca^{++} , this cation was released and some of it could be detected extracellularly.

Of great significance were the findings by Hasselbach (1964) using glycerinated fibers of the frog and Costantin et al (1965) using the Natori preparation that in relaxation Ca^{++} is accumulated by the lateral sacs (terminal cisternae) of the triads. This was confirmed by Winegrad's (1965) autoradiographic studies of Ca deposition in frog sartorius. He found that at rest virtually all the Ca^{++} was localized at the centre of the I band where the terminal cisternae are located, but when the muscle was fixed during contraction

it was found that the Ca^{++} was located predominantly in the region of A-I overlap. In a further paper Winegrad (1968) reported on a more extensive investigation of internal Ca^{++} translocation. At rest as was found previously, Ca^{++} was almost exclusively located in the terminal cisternae of muscles exposed to Ca^{45} for 5 minutes. After prolonged soaking (5 hours) in Ca^{45} -Ringers Ca^{45} was present in the A band portion of the myofibrils, and in the intermediate cisternae and longitudinal tubules. Following brief tetani he found that the amount of Ca^{45} in the longitudinal tubules and intermediate cisternae decreased with time and the Ca^{45} accumulated in the terminal cisternae. Winegrad proposed that during relaxation Ca^{++} is accumulated by the intermediate cisternae and longitudinal tubules, i.e. the structures closest to the region of A-I overlap, and that this Ca^{++} is cycled then to the terminal cisternae from where it could be released again.

Direct evidence of Ca^{++} release which could be correlated with contractile activity was provided by Jöbsis and O'Connor (1966, 1968). These workers used murexide which forms a complex with Ca^{++} the appearance of which may be followed spectrophotometrically. Using toad sartorius it was possible to follow Ca^{++} -murexide changes and tension simultaneously for both the twitch and tetani. At about 10°C free calcium levels rise in 1-5 msec after the stimulus and well within the latent period. The peak of release is

reached at 55-75 msec and is half gone at 110-150 msec. The appearance of free Ca^{++} and disappearance closely parallel the active state curve. Unfortunately, however, the murexide technique is extremely difficult and resists quantitative measurements to an extreme degree (Ridgway, personal communication; see also Hoyle, 1969).

Ridgway and Ashley (1967) and Ashley and Ridgway (1968) have developed a method for following Ca^{++} transients (i.e. the intracellular level of free Ca^{++} ions) which has been described as extraordinarily pretty (Hoyle, 1969). This method employs the Ca^{++} -sensitive bioluminescent protein, aequorin which was injected into single muscle fibers from the barnacle Balanus nubilus. Using this technique, it was possible to measure simultaneously the membrane potential, tension, and the Ca^{++} transients. The Ca^{++} transient begins approximately 1 msec. after depolarization above threshold while tension development begins 2-6 msec after the stimulus. Thus taking the lower limit at which tension begins to be registered would place the beginning of the free Ca^{++} increase halfway through the latent period, presumably coincident with the beginning of latency relaxation. However, taking the average, i.e. 4 msec., then Ca^{++} release would start very early in the latent period. Ashley and Ridgway (1968) also showed that following an above threshold stimulus the time of appearance of Ca^{++} depended on the stimulus strength. Also, the amount of Ca^{++} released, the

tension developed and the rate at which it is developed all were determined by the stimulus strength. Similarly, at a constant stimulus strength varying the stimulus duration resulted in graded releases of Ca^{++} but the time of release and the initial rate of release were coincident. Using the latter procedure tension development was directly related to the amount of Ca^{++} released. It appears, therefore that a relationship exists between the active state and the Ca^{++} released. It would be interesting to see whether a definite relationship exists between the rate at which Ca^{++} is released and the rate of tension development particularly in the early phase of tension generation, as is suggested in the figures of Ashley and Ridgway (1968). It was seen in the previous sections that full activation of the contractile apparatus required that the internal free Ca^{++} concentration be raised approximately 50-fold over the mechanical threshold concentration. It was also pointed out that Ca^{++} release appears to be very rapid and of short duration. Further, the duration of the twitch in a fast muscle, (i.e. the frog sartorius) is about 250 msec. at room temperature; it is therefore evident that special mechanisms must exist which will rapidly reduce the free internal Ca^{++} concentration to below mechanical threshold levels.

The rate at which Ca^{++} is reduced to below mechanical threshold concentration is rapid indeed: Podolsky and Costantin (1966) using the Natori-preparation estimated the

half-time of inactivation to be about 25 msec. at room temperature, a similar half-time was estimated by Portzehl, Caldwell and Ruegg (1964) using muscle fibers of the crab, and by Hodgkin and Horowicz (1960) from their study of K^+ -contractures of single twitch fibers of the frog.

Marsh (1952) reported on the presence in the supernatant of muscle homogenates of a factor - Marsh factor - which had a relaxing effect. This factor was closer examined by Bendall (1953, 1958) who found that the Marsh factor could be identified with granules in the supernatant of muscle homogenates, while Portzehl (1957) reported that the relaxing factor may be isolated in the microsomal fraction.

The relaxing factor was definitely ascribed to granules or vesicles which were derived from the sarcoplasmic reticulum of striated muscle by Nagai, Makinose and Hasselbach (1961), Muscatello, Andersson-Cedergren and Azzone (1962), and Ebashi and Lipmann (1962). The latter workers, and Hasselbach and Makinose (1961) showed that the sarcoplasmic vesicles were able to accumulate Ca^{++} from the medium against high electrochemical gradients and that the energy required for transport is derived from ATP. Costantin, Franzini-Armstrong and Podolsky (1965) using denuded frog muscle fibers (Natori preparation) were able to show Ca^{++} accumulation, precipitated as the Ca^{++} -oxalate after perfusion with Na-oxalate, in the lateral sacs of the sarcoplasmic reticulum. Winegrad (1965, 1968) showed by autoradiographic studies

of Ca^{45} uptake that Ca^{45} was accumulated in the lateral sacs (terminal cisternae), moreover, Winegrad (1968) by fixing the muscle at various times during and after contraction observed that Ca^{++} is accumulated in the intermediate cisternae and the longitudinal tubules early in relaxation; these structures are closer to the region of A-I overlap than the lateral cisternae.

Hasselbach (1968) in summary of mostly his own work discussed the evidence which led him and his co-workers to conclude that the hydrolysis of ATP is catalysed by Ca^{++} on the outer surface of the sarcoplasmic vesicles. Also that, when the free Ca^{++} concentration approaches the minimum to which the reticulum can reduce the Ca^{++} concentration (2×10^{-9} M) the efficiency of the system drops, whereas at high Ca^{++} concentration 2 molecules of Ca^{++} are transported for every ATP split, at lower Ca^{++} concentrations only 1 Ca^{++} is transported per ATP split. A. Weber, Herz and Reiss (1966) took exception to Hasselbach's findings, they reported that the $\text{Ca}^{++}/\text{ATP}$ ratio is usually 2 and only very occasionally may drop to 1, however, not until 90% filling had occurred.

It is clear, that differences in experimentation and interpretation exist between different workers in this area. It is also evident that relaxation can be considered a result of Ca^{++} uptake by the sarcoplasmic reticulum. Biochemical studies and the results obtained using the Natori

preparation indicate that the process is rapid enough to account for the time course of relaxation. Further evidence for the rapidity of the process was given by Mommaerts and Wallner (1967) who could find no ATP splitting during relaxation. This latter finding appears to correlate well with the disappearance of Ca^{++} transients obtained by Jöbsis and O'Conner (1966), Ridgway and Ashley (1967), and Ashley and Ridgway (1968).

Although relaxation may be attributed to the special properties of the sarcoplasmic reticulum it is clear from the work of Luttgau (1963) and Foulks and Perry (1966) that the process is not independent of the membrane potential, as was originally assumed by e.g. Hodgkin and Horowicz (1960) and Podolsky and Costantin (1966). Also, agents which cause potentiation of the twitch and K^{+} -contractures appear to influence the rate of relaxation. The factors which influence relaxation will be discussed under potentiation (Section I(f)).

I(e). The Transverse Tubular System and the Sarcoplasmic Reticulum.

Although it has become generally accepted that the transverse tubular system (T-system) is the path along which activation is conducted into fast, striated muscle, it is nevertheless of interest to briefly refer to its history.

The anatomists and histologists during the last century

and early years of this century published enormous studies on the structure of muscle obtained from a wide variety of species of vertebrates and invertebrates. Of particular interest in this survey is the work of Krause (1869), Retzius (1880), and Verratti (1902). Krause appears to be the one who defined the (anatomical) unit of muscle as the structures limited on either side by Krause's membrane later named the Z-line (or disc). Retzius and Veratti were among the first to describe the sarcoplasmic reticulum including the transverse tubules. The importance of these findings was, however, not realized at that time. Although it may be argued (a posteriori) that "the stage had been set for a truly 'modern' interpretation of muscle" (Hoyle, 1967), and that muscle physiology became dominated by a physico-chemical approach (e.g. by Hill and Meyerhof) it is nevertheless true that when the mechanical and energetic properties of muscle became better understood together with improvements in biochemical and electrophysiological techniques, attention was increasingly brought to bear on the problem of activation and excitation-contraction coupling.

When in 1952 Sandow formally defined the problem of excitation-contraction coupling no mention was made of the T-tubular system and associated structures.

A. F. Huxley and Taylor (1955, 1956, 1958) using microelectrodes with a tip diameter of $1-2\mu$, were able to stimulate at fairly discrete points along a sarcomere. It

was found that local contractions to minimal depolarizing currents could only be elicited when the microelectrode was opposite or very slightly off the Z-line (frog's skeletal muscle fibers). With the electrode in these positions contraction manifested itself as shortening of the two half I-bands on either side of the Z-line. It was therefore suggested that the Z-line was the structure which conducted the stimulus inward. A. F. Huxley and Taylor (1956) and A. F. Huxley and Straub (1958) were able to pinpoint the structures responsible. Using crab and lizard muscle, they found that the most sensitive spot along the sarcomere was on the sarcolemma opposite the A-I junction and that shortening took place only in the I-band adjacent to that particular junction. This was correlated with the presence of the T-tubules in that region. So, it was evident that the Z-line was not necessarily involved or that frog striated muscle constituted a special case - depending on one's point of view.

At the time when A. F. Huxley and co-workers were conducting their local activation experiments the study of the ultrastructure of muscle was revived (e.g. Porter, 1953, 1956, 1957, and 1961; Bennett and Porter, 1953; Bennett, 1955; Andersson-Cedergren, 1959). It was found that in close apposition to the T-tubule there were elements of the sarcoplasmic reticulum, belonging to the sarcomeres on either side of the Z-line, these elements called the lateral sacs

or terminal cisternae together with the portion of the T-tubule was termed the triad. In crab muscle where the T-tubules are offset to the A-I junction, only the lateral sac of one sarcomere makes a close connection with the T-tubule, the combined structure is then called the dyad. Tubules may nevertheless be present at the level of the Z-line in these muscles (Peachey, 1966).

It was also observed that the extent of the sarcoplasmic reticulum appeared closely related to the rapidity with which a muscle was able to contract. (Peachey and Porter, 1959; Peachey and A. F. Huxley, 1962; Porter, 1961). Thus the T-tubular system and associated sarcoplasmic reticulum appear to be a specialization of muscle which permits rapid excitation-contraction coupling.

Following the discoveries surveyed above it became evident that some sort of connection between T-tubules and sarcolemma must exist if as had been shown the stimulus was conducted inwards along this system. The experiments conducted by Hodgkin and Horowicz (1960b) showed the way in this regard. In these experiments employing single fibers of the frog semitendinosus the fiber membrane was depolarized by raising the external potassium concentration. It was found that depolarization was very rapid, however, repolarization, i.e. when the potassium concentration was suddenly lowered, was much slower. The off-effect of K^+ was much slower than that produced by an alteration of the Cl^-

concentration in either direction, it was independent of Cl^- and it was slower in large diameter fibers than in fibers with smaller diameters. Hodgkin and Horowicz suggested that at least a partial explanation was that "K ions can be retained for a short time in a special region whose volume is tentatively estimated as 1/500 to 1.200 of the fiber." Shortly thereafter Adrian and Freigang (1962) explained their findings that during the passage of a hyperpolarizing current the resistance of a muscle fiber membrane slowly increased, on the basis of a special space or region with a volume of 0.2 to 0.4 per cent of the fiber volume. The increase in resistance would be due to a decrease of current-carrying K^+ from this special space.

In 1964 a number of papers appeared which added greatly to the understanding of the T-tubule. Franzini-Armstrong and Porter (1964) published electron-micrographs which showed a connection between the T-tubule and the cell surface in skeletal muscle of a fish and tadpole, i.e. the lumen of the T-tubule is continuous with the extracellular space. H. E. Huxley (1964) and Page (1964) showed using ferretin as a marker of the extracellular space that the lumen of T-tubules was in connection with the extracellular space. In the area of electrophysiology, Freigang, Goldstein, and Hellam (1964) observed that the special space could be charged up with K^+ as a result of an action potential. They predicted and found that a large amount of K^+ would

accumulate in this space after a train of action potentials and upon cessation a long lasting depolarization is produced which decayed as K^+ diffused out of this space (Hodgkin and Horowicz, 1960a). They named the depolarization following an action potential or a series of them the "late after potential." In the same year, Freigang, Goldstein, Hellam and Peachey (1964) showed that the 'late after potential' could be prolonged if the muscle was exposed to Cl^- deficient Ringers or Ringers made hypertonic with sucrose. The prolongation of the late after potential could be explained if the special space had undergone an increase in volume and this they were able to show by electronmicroscopy. "Thus we can say that during depolarization of a frog muscle fiber, outward ionic current probably flows from the sarcoplasm into the transverse tubules and then to the external medium, and this current is probably carried into the tubules by potassium ions" (Peachey, 1966).

Since 1964 much work has been done in this area and by the end of 1964 the T-tubular system was fairly well characterized and its relationship to excitation-contraction coupling established. The (exact) mechanism by which the positive current out of the tubules triggers the release of Ca^{++} is, however, to this day unknown.

In closing this section the dramatic demonstration of the importance of the T-tubular system and its connection with the outer surface of the muscle fiber should be mentioned.

Fujino, Yamaguchi and Suzuki (1961) reported on their "Glycerol effect" which was, that immediately following exposure of a single muscle fiber to Ringers made hypertonic with glycerol (400 mM) a loss of the twitch would occur while the action potential remained. However, 6-10 minutes later the twitch partially (50-70% of control) returned. They also observed that when the muscle is subsequently returned to Ringer's solution the development of tension was initially reduced and in a few minutes abolished whereas action potentials could still be elicited. At that time Fujino et al (1961) could not offer an explanation for this effect. Howell and Jenden (1967) presented morphological evidence for the last mentioned effect (Fujino et al., 1961) of uncoupling of excitation from contraction. These workers observed in the toe muscle of the frog that whereas there was no morphological change while the muscle was in 400 mM Glycerol Ringers for 1 hour, marked ultrastructural alterations resulted following the return of the muscle to Ringers solution. Electron microscopic studies showed that the T-tubular system was generally disrupted and no longer appeared as the intermediate element of the triad. Also the sarcoplasmic reticulum was broken or disorganized in the region of the A-band (fenestrated collar) and the intermediate cisternae were absent or severely disrupted. In addition, Howell and Jenden confirmed the loss of the mechanical response to electrical stimulation (Fujino et al., 1961)

and it was also observed that K^+ -contractures (35 mM K^+) could not be obtained, however, the muscle responded normally to caffeine.

Shortly after Howell and Jenden's (1967) abstract Eisenberg and Gage (1967) and Gage and Eisenberg (1967) observed that the electrical properties of frog muscle fibers with disrupted T-tubules were strikingly different. They found that the early after potential and late after potential so characteristic of frog skeletal muscle were abolished, instead the early after potential was replaced by a small hyperpolarization of short duration and the later after potential could not be observed after a train of action potentials. Also it was found that the membrane capacitance was markedly reduced.

Eisenberg and Eisenberg (1968a and b) reported on a quantitative study of T-tubular disruption in frog sartorius muscle. They used horseradish peroxidase as an extracellular marker and showed that the T-tubular system following disruption was no longer part of the extracellular space. Whereas in normal muscle 98.5% of the tubules are filled with horseradish peroxidase, following treatment with glycerol only about 2% of tubules were part of the extracellular space - it was also found that 84% of the sarcomeres in normal muscles possess T-tubules. It could be concluded that by means of glycerol treatment the properties of the sarcolemma and the T-tubular could be studied

independently.

In a series of recent papers Gage and Eisenberg (1969a and b), and Eisenberg and Gage (1969) showed that the glycerol treatment method can be exploited to separate the electrical characteristics of both the sarcolemma and the T-tubules. It was found that although the membrane and internal resistivities were about the same for both normal and T-tubular disrupted muscle, the capacitance of fibers in which the T-tubules were disrupted was about $2.2 \mu\text{F}/\text{cm}^2$ while the capacitance of normal and in glycerol fibers was about $6.1 \mu\text{F}/\text{cm}^2$. The capacitance attributed to the T-tubules is then about $3.9 \mu\text{F}/\text{cm}^2$ (Gage and Eisenberg, 1969). These figures are similar to those obtained by Falk and Fatt (1964) who analysed potential changes produced by sinusoidal currents of different frequencies. Their analysis was based on an equivalent circuit of a parallel capacitance and resistance which is in parallel with a capacitance and resistance in series. The first arm, i.e. a capacitance in parallel with a resistance was attributed to the surface membrane, and the second arm to the T-tubules. This analysis yielded a capacitance for the surface membrane of $2.6 \mu\text{F}/\text{cm}^2$ and a T-tubular capacitance of $4.6 \mu\text{F}/\text{cm}^2$. Eisenberg and Gage (1969a) determined the resting ionic conductances of K^+ and Cl^- for both the surface and T-tubular membranes and found that the chloride conductance of the normal muscle fiber is located exclusively in the surface membrane, i.e.

the chloride conductance of the T-tubular membrane is zero, the potassium conductances are distributed differently with about 60% of the total located in the T-tubules ($55 \mu\text{mho}/\text{cm}^2$) and the remainder $28 \mu\text{mho}/\text{cm}^2$) in the surface membrane.

The distribution of potassium conductances fits in well with that postulated by Hodgkin and Horowicz (1960) and Freigang and co-workers (e.g. 1964) and the observation of the absence of the early and later after potential in fibers with disrupted T-tubules (Gage and Eisenberg, 1967, 1969). Indeed, the slow repolarization of single muscle fibers when the external K^+ is suddenly reduced (Hodgkin and Horowicz, 1959, 1960), is no longer present in fibers with disrupted tubules (Nakajima, Nakajima and Peachey, 1968). The distribution of the chloride conductance presents a problem in view of previous observations. Girardier, Reuben, Brandt and Grundfest (1963) observed that the transverse tubules of crayfish striated muscle become enlarged as a result of Cl^- withdrawal, Foulks, Pacey and Perry (1965) observed the same in frog toe muscle. This swelling was thought to be a result of movement of Cl^- accompanied by K^+ and H_2O into the tubular lumen which being a 'restricted' extracellular space would swell. Further Endo (1964, 1966) using the dye Lissamine Rhodamine B200 which has a net negative charge showed that it moved freely and quickly into the T-tubular space. Endo (1966) concluded that if anything the fixed charges in the lumen of the tubules should be positive and

not negative as proposed by Fatt (1964) and Eisenberg and Gage (1969). Thus there is a discrepancy which appears to be difficult to resolve. However, it may be pointed out that Eisenberg and Gage (1969) used "a highly selected sample consisting of fibers [following glycerol treatment] with resting potentials of magnitude greater than 70 mV." However, in all their calculations they implicitly assumed that the population of fibers was that which only had about 2% of their tubules intact. Since there is no evidence that the fibers with the higher membrane potential belong to that population (Eisenberg and Eisenberg, 1968) the chloride conductance of the T-tubules may be underestimated. It should, however, be pointed out that their experiments showing the alteration of the action potential (Gage and Eisenberg, 1969) were done on the 'high membrane potential population' as well, this population may have longer stumps of the T-tubules left after treatment and may as a result be less leaky. An explanation which may partially account for the divergent results is that the K^+ and Cl^- conductances are located in anatomically different sites of the T-tubule. The K^+ conductance could be located at the level of the triads whereas the Cl^- conductance is located closer to the mouth of the tubule, i.e. closer to the surface membrane.

Nevertheless, the studies cited above may subsequently lead to a better understanding of this highly

interesting specialization of fast fibers and perhaps the mechanism by which activation moves into the muscle fiber. That the T-tubular system is a specialization of fast muscle fibers and necessary for normal excitation contraction coupling while it is poorly developed in slow fibers (Peachey and A. F. Huxley, 1962) was underlined by Stefani and Steinbach (1968) who showed that glycerol treatment leads to the abolition of excitation-contraction coupling in fast muscle but not in slow muscle fibers of the frog.

I(f). Potentialiation.

Potentialiation of the twitch or a submaximal contracture of frog skeletal muscle may be accomplished by a variety of chemicals. None of these potentiators act directly on the contractile proteins and the maximum tension the muscle is capable of producing is not increased by these agents (Sandow, 1965; for potentialiation of insect muscle see Huddart, 1968, 1969a,b).

Potentialiation may be affected by replacing all or part of the Cl^- in Ringer's solution by one of the lyotropic anions, NO_3^- , Br^- , I^- or SCN^- ; and CH_3SO_3^- , or by the addition of Zn^{++} , UO_2^{++} and a number of other metallic cations discussed below. Caffeine in concentrations which are insufficient to produce contracture (1 mM or less), as well as quinine and quinidine also can potentiate the twitch or submaximal K^+ -contractures (Sandow, 1964, 1965).

Whether twitch potentiation is caused solely by an increase in the duration of the active state plateau or whether the active state also may be increased is still a matter of dispute. It has been known for many years that the lyotropic anions, some divalent cations, and caffeine cause an increase in the rate of isometric tension development, an increase in the rate of isotonic shortening and a prolongation of the twitch (e.g. Ritchie, 1954; Hill and McPherson, 1955; Kahn and Sandow, 1955; Sandow and Seaman, 1964; Johnson and Loomis, 1965; Sandow and Brust, 1966; Isaacson and Sandow, 1966). However, whether the increase in the rate of tension development is a reflection of an increase in the rate at which the active state develops, an increase in duration of the plateau phase or an increase in the final intensity is not known. According to the Hill school of thought (Hill, 1950) the active state is maximally developed even in a twitch at the moment that tension may be recorded while the initial rate of tension development is determined by the series elastic elements; this view would only permit an increased duration of the plateau phase as an explanation for potentiation (Hill and McPherson, 1955). Desmedt and Hainaut (1968) in a study of potentiation of human skeletal muscle during the staircase phenomenon presented evidence which was interpreted by them to mean that potentiation was a result of an increase in the magnitude of the active state during a twitch. Therefore, their

interpretation would be that the maximum active state is not necessarily or usually reached in a normal twitch (also see Jewell and Wilkie, 1958). Rosenfalck (1968) a short time later hotly disputed the interpretation of Desmedt and Hainaut (1968), he argued that their findings could be explained along 'Hillian' lines; potentiation was due to a slight prolongation of the plateau phase following which the active state declined more rapidly than in a normal twitch, thereby Rosenfalck explained the greater but quicker twitch seen by Desmedt and Hainaut during the positive phase of the staircase effect. The staircase (or treppe) effect which results after a series of rapidly delivered stimuli (e.g. 2 per second) and then stimulating at approximately 1 per minute (Isaacson, 1969) is obscure. Isaacson (1969) offered two possibilities, it may be a result of an internal translocation of activator Ca^{++} which is more slowly reversed than that Ca^{++} involved in excitation-contraction coupling, or it may be due to the release of a potentiating substance which he postulated could be Zn^{++} .

Sandow (1965) classified the potentiators as type A and type B. To the type A potentiators belong the lyotropic anions and caffeine, their actions are typified by:

- a) quick on and offset of their action (1.5 - 2.0 seconds, Hodgkin and Horowicz, 1960b).
- b) reduction of the mechanical threshold to higher values of the membrane potential or lower values of K^+ in the case of contractures.

- c) insignificant changes of the action potential.
- d) prolongation of the active state.
- e) increase in the rate of tension development.

The mechanism of action of the type A potentiators is largely obscure. The lyotropic anions increase the resistance of the muscle membrane (Hutter and Padsha, 1959) and the effect on the T-tubular membrane may be similar (Sandow, 1965). The prolongation of the active state caused by the lyotropic anions could presumably be explained by their interference with Ca^{++} binding by isolated sarcoplasmic reticulum (Ebashi, 1965; Carvalho, 1968a) and caffeine by its ability to mobilize Ca^{++} which is either passively or actively bound by the sarcoplasmic reticulum and by interference with the active uptake of Ca^{++} by the sarcoplasmic reticulum (Weber and Herz, 1965, 1968; Carvalho, 1968b). These effects on Ca^{++} mobilization may cause a lowering of the mechanical threshold as well, however, the effect of e.g. CH_3SO_3^- to which the sarcolemma is impermeable could not be explained in that way.

Type B potentiators include not only Zn^{++} and UO_2^{++} but also Be^{++} , Ba^{++} , Cd^{++} , Ni^{++} , Cu^{++} , and Pt^{++} acting in concentrations of about 0.5 mM (Sandow, 1965; Sandow and Isaacson, 1966). Their effects are in general similar to those of the type A group, however, they do not cause a lowering of the mechanical threshold and they do affect the shape of the action potential. They cause a prolongation

of the action potential, specifically an increase in the duration of the after potential. It would be interesting to see whether these effects on the action potential will still be present in muscles in which the T-tubular system has been disrupted. The quick on-and offset of the effects caused by the metal cations suggest that they are bound to readily accessible membrane sites, the finding that EDTA, PO_4 and cysteine can rapidly reverse the potentiating effects with the metal cations still present in medium lends support to this view (Sandow and Isaacson, 1966).

There is a third type of potentiation, briefly discussed in the section on the latent period, which results from a slight elevation of the potassium concentration of the Ringer's solution (Sandow and Kahn, 1952; Clinch, 1968). Clinch (1968) has shown clearly that potentiation of the twitch which occurred following elevation of K^+ in NO_3^- -Ringer could be explained by a prolongation of the active state as measured by the first derivative of the rate of tension development. The rate of tension development was, however, not increased. The effect of elevated potassium on the twitch may be related to the Solandt effect since in NO_3^- -Ringers used by Clinch (1968) the potassium concentration was probably above the threshold of the Solandt effect (Hill and Howarth, 1957).

CHAPTER II

THE SOLANDT EFFECT

The Solandt effect which is an increase in the resting metabolism of frog skeletal muscle when it is exposed to potassium concentrations lower than required to produce a contracture, was first discovered as a phenomenon accompanying loss of excitability. Embden and Lange (1923) reported that a freshly dissected frog sartorius when left unwashed would show a slow increase in oxygen consumption accompanied by a loss of muscle phosphate and excitability. These changes in the muscle could be reversed or prevented by washing the muscle in Ringer's solution. Embden and Lange (1923) found as well that exposing the muscle to isotonic sucrose also resulted in an increase in oxygen consumption which could be reversed by placing the muscle in Ringer's solution. A few years later Dulière and Horton (1929) and Horton (1930) showed that muscles not washed or not washed long enough after dissection become inexcitable because of the loss of potassium from the fibers into the extracellular spaces. Fenn (1930) confirmed the earlier findings of Embden and Lange (1923) and he found furthermore that in addition to isotonic sucrose, isotonic glucose, lactose, or maltose or potassium chloride could cause an increase

in muscle oxygen consumption. In 1931 Fenn enlarged upon his earlier findings, he found that exposing the muscle to 0.2 % KCl (27 mM) resulted in an increase in oxygen consumption. Moreover, the increase in the oxygen consumption of the muscle resulting from exposure to isotonic sucrose could be reversed by adding 0.8 % CaCl_2 or 0.65 % NaCl or KCl to the sucrose medium and alternatively the increase in the oxygen consumption could be prevented when the muscle was exposed to isotonic sucrose with the electrolytes added. Although isotonic sucrose caused a slight contracture in many experiments (probably due to chloride withdrawal), this had no bearing on the oxygen consumption because muscles which did not show a contracture nevertheless increased their oxygen consumption. Fenn (1931) also investigated membrane potential changes, this was done by measuring the potential between two calomel electrodes one of which was connected to that half of the muscle which was not exposed to the test solution while the second electrode was in contact with that part of the bath containing the other half of the muscle exposed to the test solution. In isotonic sucrose the potential change was initially about -10 mV which lasted for 15-30 minutes at which time the potential reversed itself reading +30 to +40 mV during the following 30 minutes. The potential dropped slowly in the next 3 hours to +20 mV. In isotonic KCl on the other hand the potential dropped to -30 mV and remained at that level

with a very slow rise to -20 mV over the next 3-4 hours. The oxygen consumption showed, however, no direct relationship to the potential changes: in isotonic sucrose the increased oxygen consumption was maintained whereas in isotonic KCl it would fall off. That in isotonic KCl the oxygen consumption is not maintained has been amply confirmed; in general the oxygen consumption and the heat production are not maintained during or following a phasic contracture (e.g. Smith and Solandt, 1938; Van der Kloot, 1967a). But when the depolarization of frog sartorius muscle is below that required to produce a contracture, a maintained increase in the oxygen consumption results as was first shown by Hegnauer, Fenn and Cobb (1934). These workers reported furthermore, that muscles poisoned with bromoacetate showed the normal increase in oxygen consumption when exposed to isotonic sucrose, maltose, lactose, or KCl. Muscles in isotonic sucrose showed an increase in lactic acid production which was not reversed by adding NaCl, whereas noted earlier, the oxygen consumption was decreased. However, muscles whose oxygen consumption was raised by increasing the potassium concentration in the Ringers to 30-60 mg % (7.7 - 15.4 mM) showed no increase in lactic acid production but a decrease in creatine phosphate which after 5 hours of exposure would rise and be higher than in control muscles.

They also found that contractures could occur at 35-45 mg % (9 - 11 mM) potassium; normally contractures do not occur till the potassium reaches 18 - 20 mM. Tipton (1936) also found an increase in oxygen consumption when the potassium concentration was more than 10 mM. More recently, Muller and Simon (1960) observed in muscles from the toad (Bufo marinus) that the maximum oxygen consumption occurred at about 18 mM K^+ but the lactic acid production did not increase till the potassium concentration was 40 mM or higher (i.e. at concentrations which induce contractures). Contrary findings were reported by Kaye and Mommaerts (1961) who using sartori of the frog, Rana pipiens, showed that lactic acid production increased with potassium concentrations to 20 mM and this stimulation of glycolysis was dependent on the presence of Ca^{++} in the bathing medium; in zero Ca^{++} Ringer's, stimulation of glycolysis did not take place. Furthermore, the potassium effects were enhanced when the sodium was replaced by choline, suggesting a competition between sodium and calcium ions. These results seem to be at variance with those of Muller and Simon (1960), however, this may be due simply to differences in the species of amphibian used.

Solandt (1936) demonstrated that increasing the external potassium to 16 - 20 mM KCl causes an increase in heat production of the frog sartorius 20X the resting heat production in Rana temporaria and to 10X the resting heat

in Rana esculanta. The maximum heat production was reached in about two hours after which it slowly declined. He also found that Rb^+ could replace K^+ in increasing the resting metabolism. Calcium as well as Strontium could oppose the effect of elevated potassium when the calcium/potassium ratio was kept the same as in normal Ringer's, barium could also oppose the increase, although it was toxic. Dextrose and sucrose (isotonic) also causes a 10 - 20X increase in the resting heat production. Thus the oxygen measurements by Fenn were now correlated with heat measurements. Smith and Solandt (1938) further resolved the relation of contracture to the increased heat production; whereas Solandt (1936) reported that there was no heat production associated with a high potassium induced contracture, they reported that this was incorrect. The rate of development and rate of fall of heat production both increased with increasing concentration of potassium; thus with high K^+ concentrations there was a large but transient increase in heat production. Hill and Howarth (1957) reinvestigated and enlarged upon the findings of Solandt (1936). They also reported that NO_3^- and I^- reduced the threshold for this effect, these ions also reduce the mechanical threshold (Hodgkin and Horowicz, 1960b). The effects of varying the Ca^{++} concentration was similar to that reported by Solandt (1936) thus, increasing the concentration of Ca^{++} at a fixed K^+ which would cause a rise in heat production, would lead to a decrease in the

heat production and if sufficient Ca^{++} was added the heat production could be brought back to basal levels. The effect of Ca^{++} was the same when NO_3^- or I^- Ringer was used. The effects of the impermeant SO_4^{--} anion were rather interesting. In Ringer's where all the Cl^- was replaced with SO_4^{--} the results were the same as with isotonic sucrose, that is, the potassium concentration need not be raised in SO_4^{--} -Ringer in order to obtain the increase in the heat production. And like isotonic sucrose, adding a small amount (10%) of Cl^- -Ringers brought the heat production back to normal. However, if the SO_4^{--} -Ringer was saturated with CaSO_4 no spontaneous increase in the heat production occurred but then raising the potassium concentration the heat production would increase although the concentration of potassium needed was about twice that required in Cl^- -Ringers. These results are rather puzzling, the level of ionized Ca^{++} in SO_4^{--} -Ringer was about 0.15 mM and in SO_4 saturated SO_4^{--} -Ringer was approximately 0.7 mM, however, this would not explain the results. Hill and Howarth (1957) also found that iodoacetate (0.06 - 0.3 mM) did not effect the increase in heat production due to elevated potassium, however, the increment was less (50%) and short-lived, the muscle going into rigor after about 1 hour. Acetylcholine could not cause an increase in the heat production and it did not alter the increment due to potassium. The Solandt effect was, however, virtually abolished under strict anaerobic conditions.

New light was shed on the Solandt effect commencing with the studies on Ca^{++} -fluxes by Bianchi and Shanes (1959). These workers reported on Ca^{++} influx in the frog sartorius during rest, activity (twitches), and potassium contractures. They found that exposing the muscle to 20 mM KCl caused an increase in Ca^{++} influx, an increase was also observed in a high potassium contracture. In the latter the influx was brief because exposing the muscle to the high potassium and Ca^{45} simultaneously did not result in a demonstrable increased influx but preincubation for about 2 - 3 minutes with Ca^{45} in normal Ringer's always resulted in an increased Ca^{45} influx following a subsequent exposure to high potassium with Ca^{45} . The effect of different potassium concentrations in the bathing medium on Ca^{45} uptake by the frog sartorius was further investigated by Weiss, and Bianchi (1965). They found that Ca^{45} uptake by the muscle increased when the external potassium concentration was raised from 1.6 to 32 mM, above that concentration no further increase in Ca^{45} uptake could be observed. However, it was noted that the time course of Ca^{45} uptake depended on the external potassium concentration. Thus, it was found that at potassium concentrations of less than about 30 mM the Ca^{45} uptake is prolonged whereas above 30 mM the Ca^{45} uptake is very brief; the total amount taken up being about the same for both conditions. These findings correlate reasonably well with the myogram as well as with the rates of heat development in muscles

made to contract by raising the external potassium concentration. Thus, the myogram of a 30 mM K or lower contracture shows a very slow development of tension whereas above that concentration of potassium the contracture becomes increasingly more phasic, i.e. faster rate of tension development and faster rate of relaxation. Likewise, as mentioned earlier (Smith and Solandt, 1938) when the external potassium concentration is raised the rate of heat production and the rate of decline progressively increase while the duration becomes progressively shorter. Weiss and Bianchi (1965) also investigated the effect of replacing chloride by nitrate. It is known that the replacement of Cl^- by NO_3^- reduced the concentration of potassium needed to obtain tension development (e.g. Hodgkin and Horowicz, 1960b) and increased the heat production (Hill and Howarth, 1957). The effect of the nitrate ion on Ca^{45} uptake is similar, that is, uptake is increased at lower external potassium concentrations. This increase in Ca^{45} uptake occurs, however, only up to about 30 mM KCl; above that concentration no increase in Ca^{45} influx was observed. Novotny and Vyskocil (1966) using the method of Bianchi and Shanes (1959) reported essentially the same results. These authors chose the term of calcium exchangeability instead of calcium influx or uptake as used by Bianchi and co-workers, they felt that the method employed did not yield results representing net fluxes but rather an increased release of calcium from its

bound form. Of some interest were their results showing that procaine (1mM), physostigmine (0.1 mM) or phenobarbital (0.1 mM) not only prevented the increase in oxygen consumption due to subthreshold potassium (Novotny et al, 1962) but also prevented the increase in calcium exchangeability (also Novotny and Vyscocol, 1963). CaCl_2 in high concentrations (20 mM) acted like procaine on the 20 mM K^+ induced increase in oxygen consumption and calcium exchangeability but on 40 mM K^+ induced responses it acted in the opposite direction. These results are in keeping with the results of Solandt (1936) and Hill and Howarth (1957) who found that as long as the $\text{Ca}^{++}/\text{K}^+$ ratio was kept as in normal Ringer's an increase in heat production could still be elicited by an additional increase of K^+ of 18 - 20 mM.

The effect of caffeine on Ca^{++} fluxes is slightly different from that seen with 20 mM K^+ . Caffeine in low concentrations (approx. 1 mM) will cause an increased influx followed by an increased efflux, presumably reflecting an increased Ca^{++} -turnover or exchangeability (Bianchi, 1961; Novotny and Vyscocol, 1966). The increased Ca^{++} influx caused by caffeine (5 mM) does not prevent an additional transient influx due to potassium depolarization. Feinstein (1963) reported that local anesthetics prevent the caffeine induced contracture, increases in Ca^{++} influx, the increased net uptake of sodium, loss of potassium, and the increase in oxygen consumption. Novotny and Vyscocol

(1966) have confirmed Feinstein's results with respect to calcium exchange and oxygen consumption. They, moreover, reported that under some conditions procaine and physostigmine but not phenobarbital can prevent or inhibit the increased oxygen consumption due to isotonic sucrose or isotonic sucrose with phosphate buffer (3 mM, pH 7.6). It was found that procaine and physostigmine blocked the increase in oxygen consumption due to isotonic sucrose with phosphate buffer but not in isotonic sucrose alone. Unfortunately, they did not investigate the effect of these sucrose solutions on calcium fluxes.

The latest contribution, albeit minor, to the Solandt effect was made by Van der Kloot (1967a, b). Unfortunately, tension was not measured in parallel experiments so that it is not known whether at 20 mM and 25 mM K^+ the Solandt effect was estimated or that oxygen consumption was measured during and after small contractures. It was found that Mg^{++} , Mn^{++} and Co^{++} in concentrations of 5 - 20, 5 and 5 mM respectively were able to antagonize the increase in oxygen consumption due to exposure to 20 mM K^+ . Further, Van der Kloot showed that external Ca^{++} is necessary for at least a maintained increase in oxygen consumption. Without external Ca^{++} , exposure to 20 mM K^+ led to a phasic burst response in oxygen consumption, restoration of external Ca^{++} in the presence of 20 mM K^+ caused the oxygen consumption to increase once more. From his and already known findings Van der Kloot

(1967a) constructed a model which appears to account for the time course of the stimulation of oxygen consumption and of the potassium contracture. The model consists of three compartments for Ca^{++} ; 1) the extracellular space, 2) a compartment B from which Ca^{++} is released into the sarcoplasm (from the lateral cisternae) and 3) the sarcoplasm. He depicts a fourth compartment (Van der Kloot, 1967b, fig. 11) which is the longitudinal reticulum; it enters into consideration only as a Ca^{++} -sink. The muscle according to this model is proposed to act as follows: as the membrane is depolarized by potassium above 10 mM the rate of release of Ca^{++} from compartment B is increased while the rate at which compartment B is replenished from the extracellular space decreases progressively as the external potassium concentration increases above 2.5 mM. The rate at which Ca^{++} leaves the sarcoplasm into the Ca^{++} sink is however invariant and thus independent of the membrane potential. Below the mechanical threshold the release of Ca^{++} would be sufficiently high to keep the sarcoplasmic Ca^{++} concentration elevated resulting in continuous activity of the sarcoplasmic reticulum above resting levels hence increased metabolism. Above the mechanical threshold the sarcoplasmic free Ca^{++} concentration would be sufficient to cause mechanical activity and the rate of relaxation would primarily be governed by the rate at which compartment B becomes depleted. Although Van der Kloot's model appears to account

for the evidence, a few discrepancies remain, e.g. the rate at which Ca^{++} is pumped into the sarcoplasmic reticulum depends on the Ca^{++} gradient across the reticular membrane e.g. (Weber, Herz and Reiss, 1966), in the model the rate was held constant; Ca^{++} appears to be returned from the longitudinal reticulum to the lateral cisternae (Winegrad, 1968) in the model the lateral cisternae (compartment B) is replenished only by Ca^{++} coming from the extracellular space.

In summary, the Solandt effect is the increase in metabolism resulting from partial depolarization of the membrane of frog skeletal muscle without accompanying tension development. The threshold for the effect is at about 10 mM K^+ with the mechanical threshold at about 20 mM K^+ . The threshold for the Solandt effect may be lowered by NO_3^- or I^- replacing Cl^- which also results in a reduction of the mechanical threshold. The threshold for both the increase in metabolism and tension development may be increased by increasing the external concentration of Ca^{++} or Sr^{++} ; as well, it may be affected by the addition of Mg^{++} , Mn^{++} or Co^{++} . The increase in metabolism can be inhibited by procaine, phenobarbital and physostigmine and the increase in respiration cannot be maintained in the absence of external Ca^{++} . Depolarization causes an increase in the exchangeability of Ca^{++} across the membrane, this may be a reflection of an increase in the internal free Ca^{++}

concentration which in turn would stimulate the sarcoplasmic reticulum causing an increase in the turnover of ATP, liberation of ADP and hence stimulation of respiration.

CHAPTER III
METHODS AND MATERIALS

III(a). Muscle Preparations.

Both the extensor digitorum longus IV (toe muscle) and the dorsal semitendinosus of the frog, Rana pipiens, were used for experiments measuring tension development. In a few experiments the extensor digitorum longus IV of the frog, Rana temporaria, was also used. Only semitendinosus preparations were used when the resistance to stretch before or during contractures was estimated. The extensor digitorum longus IV was used in experiments in which membrane potential changes were followed and in which the oxygen consumption was estimated.

The toe muscle is a long, slender bundle of small diameter (approx. 30μ) fibers which when dissected from a small frog is approximately 15 to 20 mm long with the greatest cross-sectional diameter rarely exceeding $250-300\mu$. This muscle is extremely suitable for work on excitation-contraction coupling (Frank, 1960a, b). Since, the muscle will respond well for more than 5 hours with tests every 10 minutes, it is possible to obtain the 'optimal' length by eliciting a few contractures at the beginning of an experiment. The muscle is removed together with surrounding

muscles and connective tissue and placed in a dissecting dish. With the aid of a dissecting microscope the surrounding muscle debris is dissected away and the bundle freed from its connective tissue sheath. The distal tendon is large and can be used as is but the proximal tendon is very small, it merges with a larger tendon which was retained for support. The tendons were tied with 6-0 surgical silk and the muscle transferred to the appropriate bath as described below.

The dorsal head of the semitendinosus was carefully removed from the frog and placed in a dissecting dish. Using a dissecting microscope, small dissecting knives, and jewellers forceps the muscle was reduced to a bundle of usually 8 to 12 fibers while some contained as few as 4 or as many as 15 fibers. Cross-sectional diameters were estimated using a calibrated disc micrometer in the dissecting microscope. In calculating the cross-sectional areas the bundles were assumed to be elliptical in cross-section. The cross-sectional diameters are given in the figure legends with each experiment in which a bundle of the semitendinosus was used.

III(b). Solutions.

All solutions were made up in distilled water which had passed through a filter to remove organic material (Barstead 0812, Barstead Still & Sterilizer Co) and a deionizer (ILLCO-WAY, Research Model Ion Exchanger, Illinois Water Treatment Co.)

In early experiments a choline - Ringer's solution was used which had the following composition (Frank, 1956),

Choline Chloride	111.8 mM
KCl	2.47 mM
CaCl ₂	1.08 mM
NaHCO ₃	2.38 mM
Glucose	11.1 mM

During dissection of the muscle it was kept in Ringer's solution having the above composition except that the choline chloride was replaced with an equivalent amount of NaCl and 5×10^{-5} d-tubocurarine chloride (Burroughs Wellcome) was added. Experiments illustrated in this thesis in which the above solution was used are clearly labelled 'choline - Ringer's'.

For most of the experiments which were done Ringer's solution (Adrian, 1956) was used. The reasons for the switch in solutions although it did not affect the results in any way was that the semitendinosus preparation remained viable for a longer period in Ringer's solution but mainly because the pH of the Ringer's (Adrian, 1956) remains stable. Also, in the oxygen consumption experiments the presence of both choline and glucose gave rise to a 'stagnation' artifact; i.e. solutions kept stagnant for a while showed a decrease in pO_2 which was prevented by removing glucose from the solutions. The composition of Ringer's solution (Adrian, 1956) was as follows.

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NaCl	115 mM
KCl	2.5 mM
CaCl ₂	1.8 mM
Na ₂ HPO ₄	2.15 mM
NaH ₂ PO ₄	0.85 mM

to which was added d-tubocurarine chloride, 5×10^{-5} , and 5×10^{-6} tetrodotoxin (Sankyo Co.) was added in order to eliminate twitching when the potassium concentration was raised. The pH of this Ringer's solution was 7.2.

The isotonic KCl solution contained 123 mM KCl, 1.8 mM CaCl₂, and 5×10^{-6} tetrodotoxin.

The isotonic sucrose solution contained 222 mM sucrose (Novotny and Vyskocil, 1966), and when PO₄-buffer or CaCl₂ was added, it was in the same concentrations as in the Ringer's solution (Adrian, 1956).

Solutions in which the potassium concentration was above 2.5 mM were prepared by mixing the appropriate amounts of the Ringer's solution with an aliquot from a stock solution containing 494 mM KCl (Frank, 1965b).

Experiments with caffeine were done using the choline-Ringer's solution. Caffeine in the appropriate amount was dissolved in a few drops of concentrated HCl and then transferred to the choline - Ringer's solution. The pH was adjusted to 7.2 using solid NaHCO₃.

III(c). Tension Recording.

Following dissection, the toe muscle was mounted vertically in 2 ml. syringe bath which could be filled from the bottom and drained by suction from the top. One tendon was tied to a post in the bath and the other tendon was connected to the anode of a RCA 5734 transducer tube. Following a 30 minute equilibration period, testing was started. Tests were carried out every 10 minutes. Isometric tension was recorded on an Esterline Angus, speed servo, Model S-601-S00 a Bausch and Lomb potentiometric recorder (Model V.O.M. 5).

Following dissection, the tendons of the semi-tendinosus preparations were tied to the hooks attached to small, 1 X 2 mm, platinum plates in which 2 small holes had been drilled (Fig. 1, insert). The preparation was then transferred to a horizontal bath similar to the one in Fig. 1. During the transfer the bundle was kept in Ringer's solution because bringing these small preparations through the fluid-air interphase usually result in damage to the fibers. With the aid of a binocular microscope, the plates to which the tendons were attached were placed on other small platinum plates which had 2 pins soldered on so that the plates attached to the tendons fitted snugly. One of the small platinum plates with pins was permanently attached to the anode of a RCA 5734 transducer which was fixed to a Prior micromanipulator, and the other to the movable arm of the muscle puller. Since, the platinum plates were

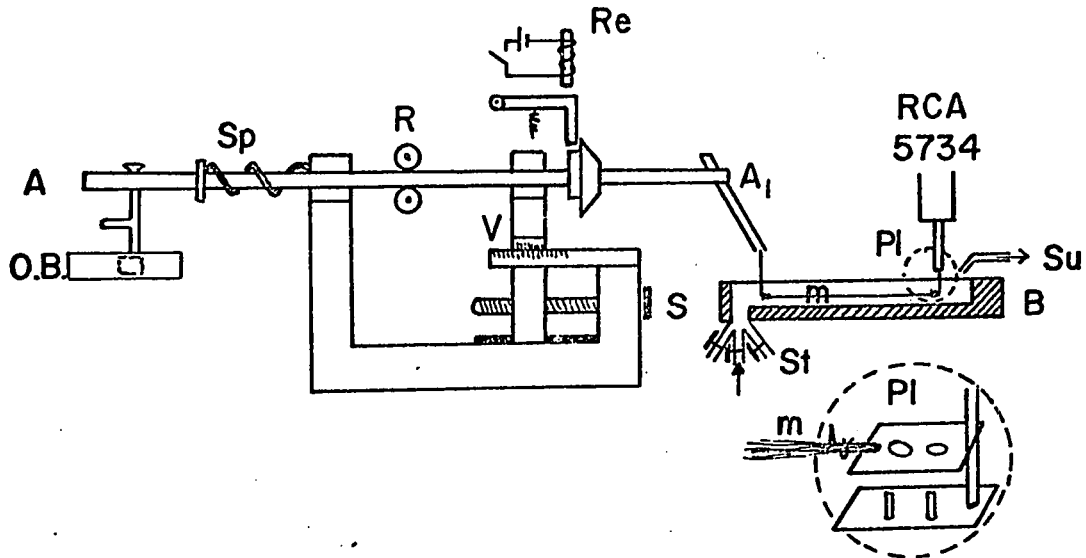


Fig.1. Diagram of apparatus used to apply quick stretches to small bundles of the semitendinosus (m), and experimental bath (B). The bath was filled through one of three stopcocks (St) at the bottom and drained by suction (Su) at the top. Arrangement of Pt plates (Pl) to which the muscle was tied and hooked to the RCA 5734 transducer and the arm of the puller is shown enlarged. A---A₁, movable arm. A keying arrangement to prevent angular rotation is not shown. S, lead screw to move post, V, vernier scale to set position of the post and thus the amount of stretch. Re, relay used to release the catch at the desired instant. R, one of two sets of roller bearings to provide for smooth movement of the arm. Sp, spring which provided the force to move A---A₁ to the left. O.B., oilbath with silicone fluid containing adjustable vane for damping. Except for Pl exactly as used by Frank (1965b).

attached horizontally, the bundle of fibers was suspended freely, i.e. the weight of the plates was not supported in any way by the bundle of fibers. The muscle puller (Fig. 1) was the same one used by Frank (1965a; b), the amount of stretch could be set with an accuracy of 0.1 mm with a vernier. The amount of stretch was usually in the order of 2.5 mm, the duration of the stretch was approximately 20 msec.

The preparation was allowed to rest for $\frac{1}{2}$ to 1 hour in the bath prior to testing, tests were carried out at 10 minute intervals. The '0' length was determined to be that length at which the muscle was just taut, i.e. at that length a reduction by 0.1 to 0.2 mm would just produce a sag in the bundle. When the muscle was stretched, the amount of stretch was 15% of the '0' length. This would extend the muscle to $1.15 \times$ '0' length which is slightly less than the 1.20 to $1.35 \times$ the '0' length used for single fibers by Hodgkin and Horowicz (1960b) and Lüttgau (1963). Contractures elicited at '0' length and $1.15 \times$ '0' length or stretched from '0' length by 15% were therefore limited to the rising phase or early plateau region of the isometric length - tension diagram (Ramsey and Street, 1940). This choice of working lengths ensured that the phenomenon of 'slip' (Hill, 1949d) which takes place when the muscle is stretched more than 10 - 15 of the in situ length (l_0), did not occur.

III (d). O₂ Consumption Measurements.

The method employed for measuring the O₂ consumption of toe muscles exposed to elevated K⁺-Ringer's solutions was an adaptation of the method used by Ritchie (1967).

Since the O₂ consumption of small toe muscles (dry weight 0.35 - 0.5 mg for 2 toe muscles) had to be followed it was necessary to use a material which was relatively impermeable to oxygen. That is, if a rather permeable material was used the small loss of oxygen from the perfusing Ringer's solution could be made up by oxygen coming from the material of which the bath was constructed. Nylon (Ertalon 6SA^(R), Erta Plastics, Belgium) which was readily available as rods, and which could be handled fairly easily was chosen. Nylon has a very low oxygen permeability of $0.38 \times 10^{-10} \text{ cc}_{\text{STP}}\text{O}_2/\text{cm}^2/\text{mm}/\text{sec}/\text{cm Hg}$ in the temperature range of 20 - 30° C (A. Lebovits, 1966), only polyvinylidene chloride (Saran^(R)) is less permeable than Nylon, the permeability of Saran^(R) being $0.05 \times 10^{-10} \text{ cc}_{\text{STP}}\text{O}_2/\text{cm}^2/\text{mm}/\text{sec}/\text{cm Hg}$ at 20 - 30° C.

The perfusion chamber was constructed of two 6 cm long halves of 1 3/4 inch Nylon (Ertalon 6SA^(R)) rods, the two halves were milled to a smooth surface after cooling in liquid N₂. In one half rod a small groove was milled which measured 6 cm long X 1.0 wide and 0.8 deep (Fig. 2). Three small platinum wires were inserted in the groove, to which two muscles were tied. Further details are shown in Fig. 2.

The chamber was perfused with a motor-driven

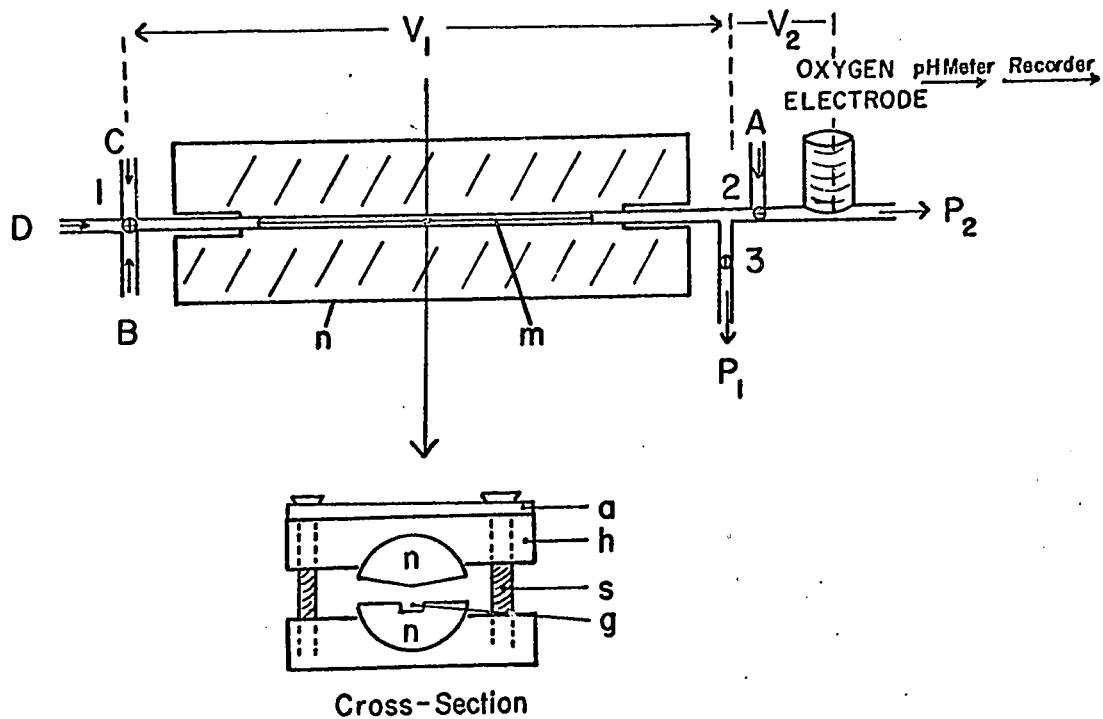


Fig.2. Diagram of apparatus for measuring oxygen consumption. The bath was constructed of Nylon (n), 2 toe muscles (m) were tied end to end in the groove (g). P_1 and P_2 are motor driven syringe pumps. Appropriate positioning of stopcock 2 permits Ringer's solution to flow from the muscle chamber or from reservoir A past the oxygen electrode. P_1 was used to rapidly change the Ringer's solution in the muscle chamber. Reservoirs B, C, and D contained Ringer's solution, 10 mM K^+ -Ringer's solution, and 17.5 mM K^+ -Ringer's solution, resp. In cross-section observe V-shaped cover also of Nylon (n), this shape permits a tight seal, stopcock grease was used only where the stopcocks were inserted. The Nylon blocks were fastened in acrylic plastic holders (h) and covered by an aluminium plate (a). The two halves were clamped together by 6 screws (s). Small tubes near stopcocks 1 and 2 which permitted continuous circulation of the Ringer's solutions in the reservoir are not shown.

syringe withdrawal pump (Model 901, Harvard Apparatus Co., Inc.), as indicated in Fig. 2. The composition of the Ringer's solution perfusing the bath could be changed quickly, within 2 - 4 sec, using pump 1 at a flow rate of 15.3 ml/min without altering the flow past the oxygen electrode. The flow past the latter was kept constant at a calibrated rate of 0.192 ml/min.

The volumes V_1 and V_2 (Fig. 2) should be considered as functional volumes, i.e. the absolute values are not as important as the time it takes to 'move' them through the system. Thus the volumes were estimated using a low pO_2 , 100% N_2 or 12.1 %, 16.3 %, or 19.7 % O_2 in N_2 , equilibrated Ringer's solution in either one of reservoir B, C or D (Fig. 2), with two muscles in the chamber and at the same flow rate used when muscle oxygen consumption was determined. In reservoir B Ringer's solution was equilibrated with a low pO_2 gas mixture then stopcock 1 was switched to pump 1 and the time taken to the beginning of the response by the oxygen electrode multiplied the flow rate (0.192 ml/min) gave the volume $V_1 + V_2$. To estimate V_2 , the chamber was perfused with pump 1 with room air equilibrated Ringer's in reservoir D then stopcock 2 was switched such that room air equilibrated Ringer's solution from reservoir A was withdrawn by pump 1 past the oxygen electrode while the chamber was switched to reservoir B and pump 2. Thus the volume V_1 became filled with low pO_2 Ringer's solution then

the chamber was switched to pump 1, the time taken to the beginning of the response of the oxygen electrode multiplied by the flow rate gave volume V_2 . V_1 was estimated by subtraction.

The oxygen electrode consists of a Pt electrode surrounded by a Ag/AgCl electrode, the electrodes are separated by a thin oxygen-permeable polyethylene membrane from the liquid whose partial pressure of oxygen has to be determined. The principle of the electrode is that the current between the Pt and Ag/AgCl electrodes is directly proportional to the partial pressure of oxygen (PO_2) of the liquid which passed by it. The oxygen electrode used was commercially available (Radiometer, Copenhagen). It was used in conjunction with a Radiometer, pH meter 27 with gas analyzer. The mV output from the pH meter was connected to an Esterline Angus S-601-S speed servo recorder. The linearity of the oxygen electrode was confirmed using calibrated gases, 12.1%, 16.3%, and 19.7% O_2 in N_2 , room air (20.9% O_2), and 100% N_2 .

The Ringer's solutions were bubbled with compressed air through fitted gas aerators. The oxygen content was determined using Henry's Law, values for Henry's Law Constant were obtained from tables in the Handbook of Chemistry and Physics (1962). The values for Henry's Law Constant are for the solubility of oxygen in distilled water, the solubility in Ringer's is slightly less by about 5% (Ritchie,

1967). No correction was made for the differences in solubility of oxygen in distilled water and Ringers.

Two toe muscles were mounted in the bath following dissection. The bath was closed and checked for leaks, the reservoirs containing the Ringer's solutions, and the oxygen electrode were connected and immersed in a constant temperature water bath which was kept at 24°C. Following 2 - 3 hours of equilibration during which Ringer's was withdrawn past the muscles at all times, the oxygen electrode was calibrated between 100% N₂ and room air equilibrated Ringer's in the reservoir connected to A (Fig. 2). The resting oxygen consumption was estimated at a flow rate of 0.192 ml/min, during this procedure the baseline pO₂, i.e. room air, was frequently checked by switching reservoir A to pump 2, and the flow past the muscle to pump 1 (Fig. 2). Ringer's solution containing 10 mM K⁺ or 17.5 mM K⁺ were introduced by switching the muscle chamber to pump 1 whose flow rate was increased to 15.3 ml/min for 2 - 4 sec following which the muscle chamber was switched back to pump 2, i.e. immediately the flow of elevated K⁺-Ringers went past the oxygen electrode. A change of elevated K⁺-Ringer's to Ringer's solution was accomplished in the same manner. Repeats were done when the resting oxygen consumption had stabilized although it never came back to the original resting value, i.e. determined prior to the introduction of elevated K⁺-Ringer's solutions. Upon conclusion of an experiment the

muscles were removed, lyophilized and weighed to constant weight on a microbalance.

III(e). Membrane Potential Measurements.

Toe-muscles were used for following the membrane potential changes with rapid changes of the potassium concentrations in the bathing medium. The half-times of repolarization of toe-muscle fibers located on the surface of the bundle were in the same range as those reported by Hodgkin and Horowicz (1960a) using single fibers of the semitendinosus of Rana temporaria.

The experimental bath was the same as that used in the mechanical experiments on semitendinosus preparations. The muscle was tied on to a small plastic tray with a small semicircular pedestal which supported the muscle in the middle. The bath was covered with a plastic coverslide in which a small hole was drilled and through which the microelectrode could be lowered. The flow rate of the choline-Ringers solutions was adjusted to between 3 - 5 ml/sec, usually it was 5 ml/sec. Using a dye it was found that at these flow rates the dye was flushed from the region of impalement within 0.5 sec. The volume of the bath was 0.6 ml and since 8 - 10 ml was allowed to flow through the bath a complete change had occurred when the flow was stopped.

Conventional Ling-Gerard type glass microelectrodes

filled with 3 M KCl and having a resistance of 10 - 15 M Ω were used. The potential was measured between the micro-electrode and a chloride silver electrode which was connected to the bath via a choline-Ringer agar bridge, through a negative capacitance electrometer (Argonaut, Model LRA 043) and thence to a Textronic Type 502 oscilloscope which was in parallel with an Esterline Angus S-601-S speed servo recorder.

Following dissection a toe muscle was mounted in the bath as described. The microelectrode to be used was lowered in the bath and the elevated K⁺-Ringer's solutions were flushed through the bath in order to check for junction potentials due to a change in the composition of the choline-Ringer's solution, at no time did the relatively small changes in K⁺, i.e. from 2.5 to 7.5, 10 or 17.5 mM, cause the appearance of junction potentials. The muscle was then left for 5 - 10 minutes in choline-Ringer's and impaled. If a stable membrane potential was recorded the chamber was flushed through with Ringer's solution to see whether the fluid flow had any influence on the membrane potential, if not the chamber was flushed with the appropriate elevated K⁺-choline. Ringer's solution and the flushing procedure was repeated once the new membrane potential was established. Whenever possible a two-step depolarization, i.e. 2.5 to 7.5 or 10 to 17.5 mM K⁺, and a one-step, i.e. 2.5 to 17.5 mM K⁺, each followed by repolarization was obtained from

the same fiber. The order in which one-step and two-step depolarizations were done on the same fiber was varied, no change in responsiveness was noted due to the order in which the depolarizations were carried out. In between tests on different fibers a period of 10 minutes was allowed to elapse.

CHAPTER IV

RESULTS

IV(a). Potassium-induced Contractures.

IV(i). Mechanical threshold.

The mechanical threshold of a potassium-induced contracture may be simply defined as the minimum potassium concentration in the solution bathing a muscle which will produce a discernible mechanical response of that muscle. Since this study was concerned with the events occurring in frog's skeletal muscle when exposed to potassium concentrations in the vicinity of the mechanical threshold, the determination of this value was of critical importance. Although it is usually assumed that the mechanical threshold for frog's skeletal muscle occurs at a K^+ concentration of about 20 mM, this is a gross over-simplification.

In the present study, over a period of about 3 years, it was observed that the mechanical threshold for individual muscles may be anywhere from 12 to 27 mM K^+ . In addition it was often observed that the threshold K^+ concentration might slowly decrease during the course of an experiment. Whenever such a decrease in the threshold was observed during the course of an experiment, the experiment was discontinued.

The variability in the mechanical threshold seemed to some extent to depend upon the time of the year and source of the frogs. In muscles from Rana pipiens from Canada or the northern part of the U.S.A., the mechanical threshold was about 18 - 20 mM K^+ , but during the period from January till early summer the threshold often decreased to about 12 mM during the course of an experiment. During the winter, frogs (Rana pipiens) were obtained from Mexico. Semitendinous preparations from these frogs had mechanical thresholds at about 25 - 27 mM K^+ and there was little if any decrease in the mechanical threshold concentration during the course of an experiment. Toe muscles from the same frogs had a mechanical threshold of about 20 mM K^+ . This variability was a problem requiring constant attention during the course of this study. It is also the reason why a variety of potassium concentrations had to be used in this study on threshold phenomena as will be seen below.

IV(ii). Time course of potassium-induced contractures.

The maximum tensions of potassium-induced contractures increase with increasing potassium concentrations from the mechanical threshold to approximately 80 mM at which a maximum is reached (Hodgkin & Horowicz, 1960b; Frank, 1960 a, b). Of equal or greater importance for the present study is that the rate at which tension is developed also is a function of the external potassium concentration (Fig. 3).

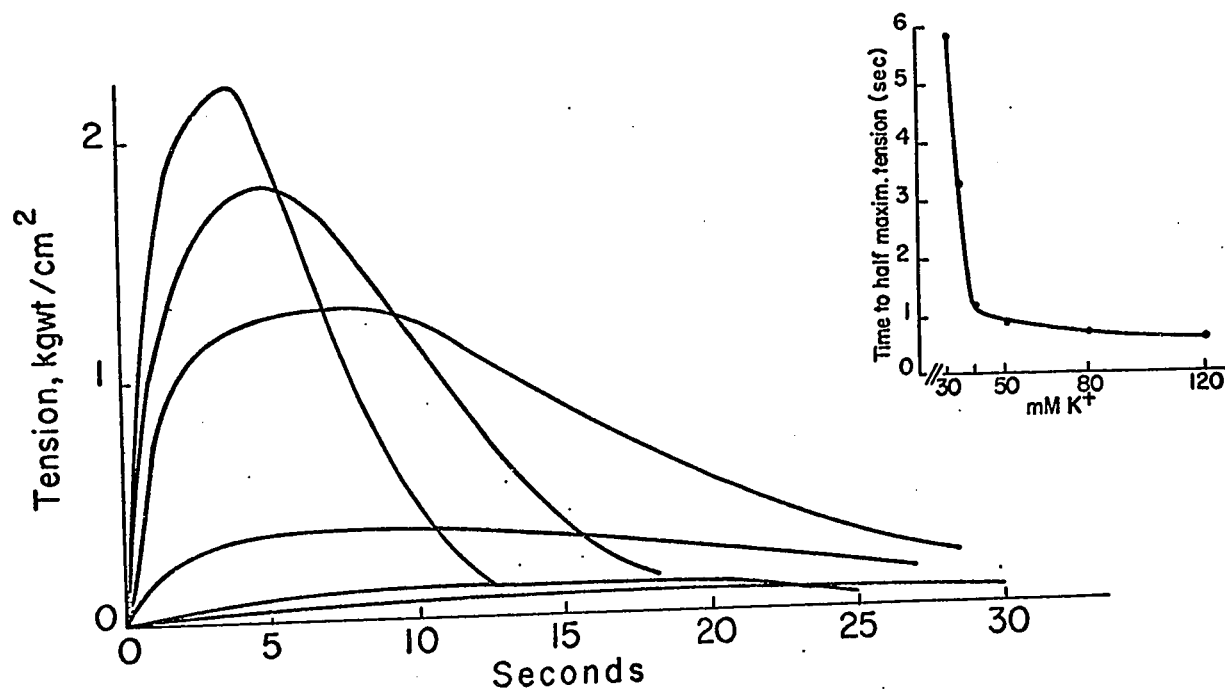


Fig.3. K⁺-contractures of the semitendinosus. In order of increasing maximum tensions contractures were elicited by 30, 35, 40, 50, 80 and 120 mM K⁺ at '0' length + 15%. Inset, relation between rate of tension development, expressed as the time taken to reach half maximum tension, and the K⁺ concentration. Semitendinosus, 140 X 320 μ , 10 fibers.

It was usually observed that the rate of tension development and the final tension achieved during potassium-induced contractures with low K^+ concentrations (20 - 35 mM) varied during a single experiment. In the early stages of an experiment both the rate of tension development and the maximum tension obtained increased slightly and later they diminished. The changes were of the order of about 10 - 15% and were corrected for as described below. They occurred more frequently and were of greater magnitude when small bundles of the semitendinosus were used.

When using small bundles of the semitendinosus the number of tests which could be obtained were limited. Usually up to 8 - 10 contractures were elicited from any one preparation before the muscle became nonresponsive; more than 10 tests were exceptional. Toe muscles, on the other hand, were far more durable but they had the disadvantage of being larger and thus increasing the time required for equilibrium of the potassium concentration between the bathing solution and the extracellular space surrounding the muscle fibers. In addition it was more difficult to estimate the resting length in toe muscles than in small bundles of the semitendinosus as described in Methods.

IV(iii). Time course of the resistance to stretch.

When a skeletal muscle begins to contract, the contractile elements become engaged and the muscle will resist

lengthening depending to a large extent upon the engagement of these contractile elements. Within the framework of the "sliding filament theory" (Huxley, 1957) engagement of the contractile elements is synonymous with the interaction of the actin and myosin filaments. Figures 4a and 4b show the time course of the resistance to stretch of respectively a low (35 mM) concentration and high (120 mM) concentration potassium-induced contracture.

All the experiments in which the resistance to stretch was estimated were done using small bundles (4 - 15 fibers) of the dorsalsemitendinosus of the frog, Rana pipiens. The time course of the resistance to stretch in the low potassium contracture (Fig. 4a) appears to be in step with tension development, i.e. the resistance to stretch increased slowly as did the tension. The resistance to stretch declined, however, more slowly during relaxation than the tension, so that, at any tension during relaxation the resistance to stretch was greater than at the same tension during development. This finding confirms those made by Frank (1965a,b).

The time course of the resistance to stretch of a maximum potassium contracture is somewhat different (Fig. 4b). The resistance to stretch did not reach a maximum till the tension was close to maximum. But in contrast to the time course during relaxation of a low potassium contracture, the resistance to stretch declined more rapidly during relaxation of a maximum contracture. The relatively slower

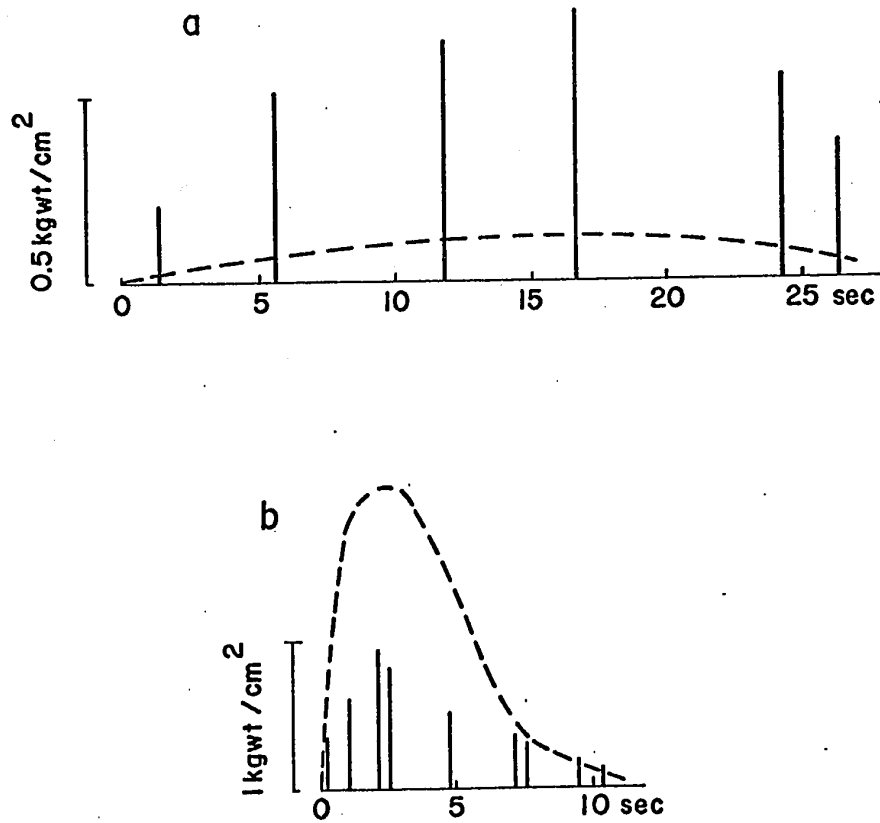


Fig. 4a, b. Time course of resistance to stretch in a 35 mM K^+ -contracture (a) and a 120 mM K^+ -contracture (b) of small bundles of the semitendinosus, a, 8 fibers measuring $100 \times 240 \mu$ and b, 6 fibers measuring $140 \times 140 \mu$. The stretch in both experiments was 15% of '0' length. Note difference in tension calibration of a and b.

onset of the resistance to stretch during tension development shown here is at variance with the results presented by Frank (1965a, b). The high values seen by Frank (1965a, b) are probably an artefact due to his use of a recorder with a slow response time, because immediately following the stretch there is a short rapid phase of tension development (Fig. 5) which would be obscured using a 'slow' recorder. A rapid phase of tension redevelopment following the stretch was seen by Frank (1965b) when the stretch was applied during relaxation, however, this rapid redevelopment of tension is much slower than the rapid tension development which occurs following a stretch during active tension development (Fig. 5). The discrepancy may also be due to a species difference; Frank (1965a, b) used Rana temporaria whereas in the present investigation Rana pipiens were used. It also may be noted that the maximum tension achieved using small bundles from the semitendinosus of Rana pipiens was usually between 2 and 2.5 kgwt. cm^{-2} while the maximum tetanic tension is in the order of 3.5 kgwt. cm^{-2} (e.g. Civan and Podolsky, 1966), Frank (1965a, b) using Rana temporaria was able to obtain maximum tensions using 123 mM K^{+} which were as great as the maximum tetanic tension (3 - 3.5 kgwt. cm^{-2}). The discrepancies noted above will be pursued separately; their existence do not affect the findings reported in subsequent sections.

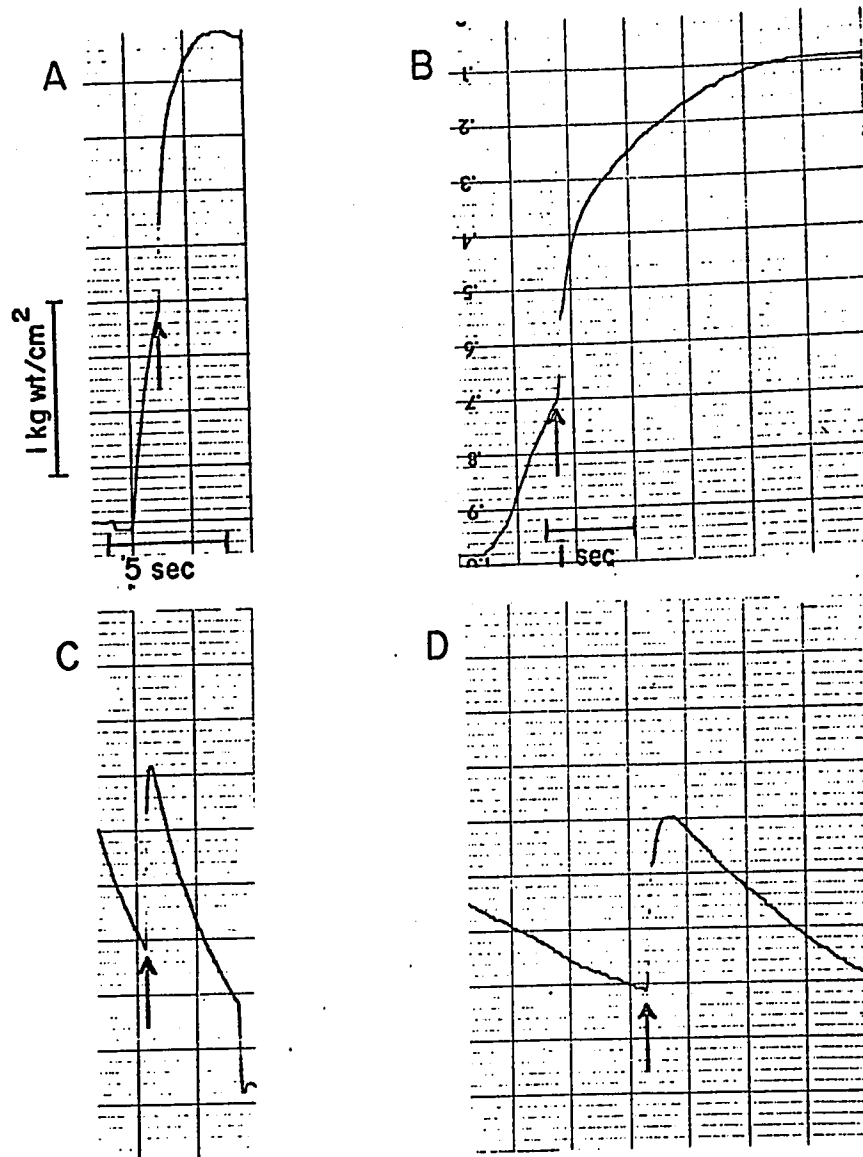


Fig.5. Transient increase in rate of tension development following a quick stretch (\uparrow) during a 120 mM K^+ -contracture. A and B stretch applied early in tension development, recorded at different recording speeds. C and D stretch applied during relaxation, recorded at different recording speeds. Semitendinosus, 4 fibers, 100 X 120 μ , stretch 15% of '0' length.

IV(iv). Pre-exposure to a potassium concentration just below the mechanical threshold followed by exposure to a concentration above the mechanical threshold.

Figure 6 illustrates the effect of a brief exposure to a sub-threshold potassium concentration upon a subsequent potassium-induced contracture. The dashed myograms (Fig. 6B and D) are 27 mM potassium-induced contractures obtained at '0' length plus 15%. Myograms C and E are also 27 mM potassium-induced contractures, however, contractures were immediately preceded by a 10 second exposure to 17.5 mM K^+ . Myogram A is an IsoKCl (122 mM)-induced contracture included for comparison. All the contractures shown in Fig. 6 were obtained during the course of a single experiment on the same preparation. It is apparent that contractures C and E develop more quickly, reach a greater maximum tension, and relax earlier and faster than contractures B and D without the pre-exposure to the low potassium concentration.

Since the above observation is basic to this study, it should be emphasized that all the contractures (except A) were elicited by exposing the same muscle preparation to the same external potassium concentration, yet, a short exposure to a potassium concentration close to the mechanical threshold resulted in a marked modification of the contracture. This potentiation which results from a short, below mechanical threshold potassium exposure has been observed without fail over the past two years, regardless

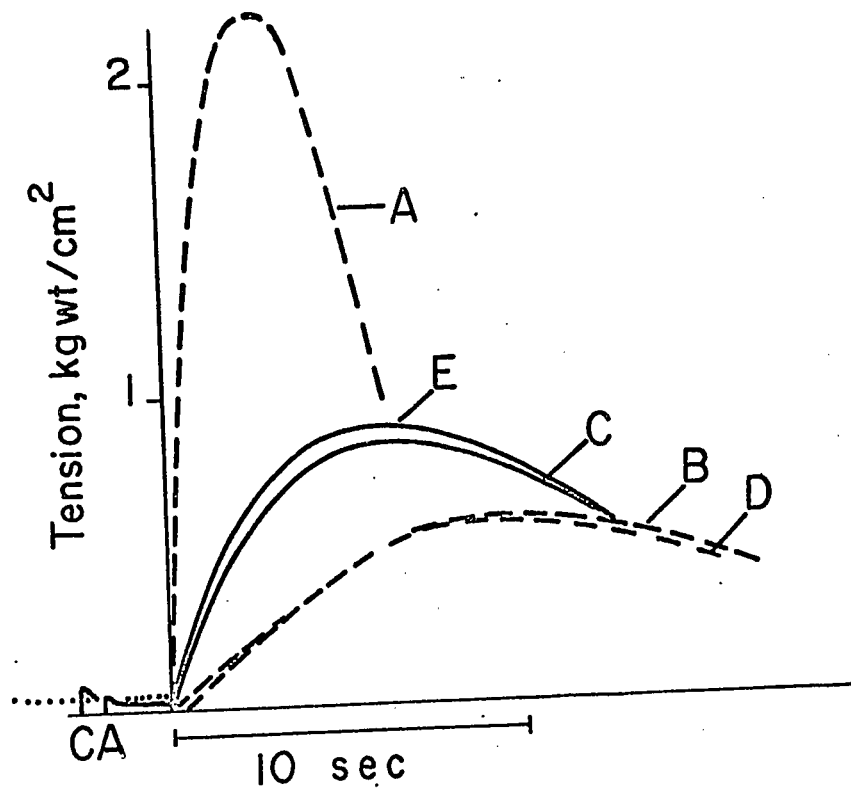


Fig.6. Isometric myogram of control, 27 mM K^+ (B and D) and 27 mM K^+ -contractures after pre-exposure to 17 mM K^+ for 10 seconds (E and C). B and C contracting at '0' + 15% with 15% stretch just prior to 27 mM K. D and E contracting from '0' + 15%. A, IsoKCl contracture. Semitendinosus 160 X 240 μ .

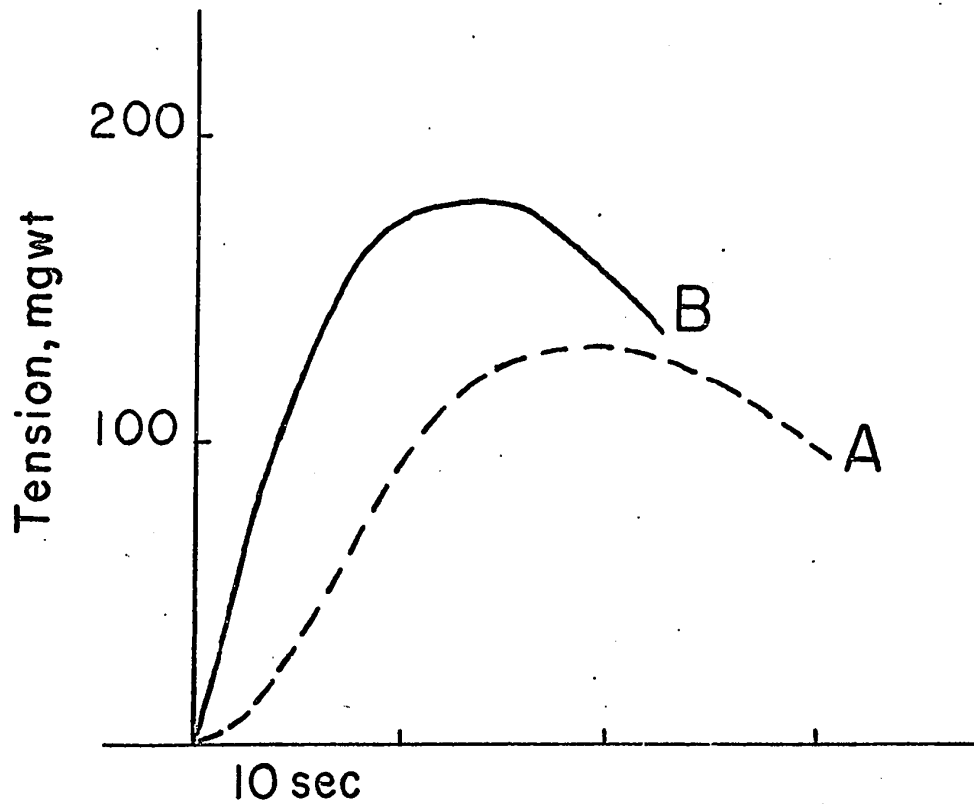


Fig.7. Potentiation of a submaximal contracture of the toe muscle of Rana pipiens. Dashed myogram is a control 25 mM K⁺-contracture, solid myogram 25 mM K⁺-contracture preceded by a 15 sec exposure to 17.5 mM K⁺.

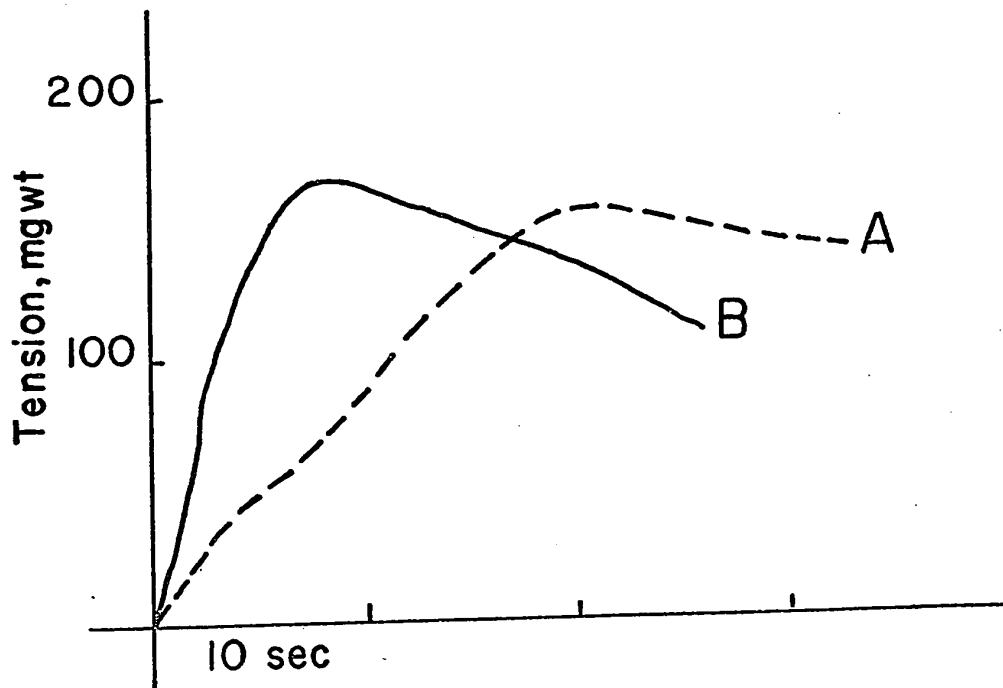


Fig.8. Potentiation of a submaximal contracture of the toe muscle of Rana temporaria. Dashed myogram is a control 25 mM K^+ -contracture, solid myogram 25 mM K^+ -contracture preceded by a 15 sec exposure to 17.5 mM K^+ .

of the season, in well over 40 experiments on both small bundles of the semitendinosus and the extensor digitorum longus IV (toe muscle) of Rana pipiens (Fig. 7). A few experiments were also done using the toe muscle of Rana temporaria and the same results were obtained (Fig. 8).

IV(v). Resistance to stretch during exposures to potassium concentrations below the mechanical threshold.

During the course of this work quick stretches were applied to resting and contracting muscles in order to obtain an estimate of the degree of activation. Since the sensitivity of the recorder was adjusted to record the tension developed during contractures the deflection due to the resistance of the resting muscle was very small. Thus if the compliance of the muscle had decreased only a small amount during exposures to potassium concentrations below the mechanical threshold it was possible that changes in the resistance to stretch would go unnoticed (Vos and Frank, 1968).

Upon reexamination there was a suggestion of an increase in the resistance to stretch on these 'low' gain records. This led to experiments to resolve at high gain (0.75 mg. wt per mm paper) whether the resistance to stretch did, in fact, increase when the potassium concentration was raised towards the mechanical threshold.

The resistance to stretch of the resting semitendinosus preparation varied from 5.08 to 29.0 mg. wt with

a 15% stretch from '0' length depending upon the size of the bundle used. Normalized, the resistance to stretch was 0.057 ± 0.006 kg. wt. cm^{-2} (mean \pm S.E. of mean, $N = 74$, 12 experiments).

With high gain recording it was found that the increase in the resistance to stretch at potassium concentrations just below the mechanical threshold can be quite striking (Fig. 9a). An example of a smaller response is shown in Fig. 9b. It is evident that the resistance to stretch does not markedly increase until the muscle has been exposed for 15 to 30 sec to the elevated K^+ . Also, the response was maximum at about a 30 sec exposure to the sub-threshold K^+ concentration following it declined; this will be more fully discussed below. An interesting feature of these records (Fig. 9a, b) is that there is no evidence whatever of active tension development; following the stretch in particular when the resistance is high, tension falls back in a manner quite similar to that seen when a preparation is stretched during the plateau of tension development, i.e. when no tension is being developed but maintained, or when the stretch is applied during relaxation (see Fig. 12, traces 7, 8, 9, and 10). In contrast, if the stretch is applied when tension is being developed in the beginning of a contracture the tension following the stretch increased (Fig. 12, traces 3 and 5; see also Frank, 1965, Figs. 5B, 6C, and 7C). Thus it seems likely that if tension was

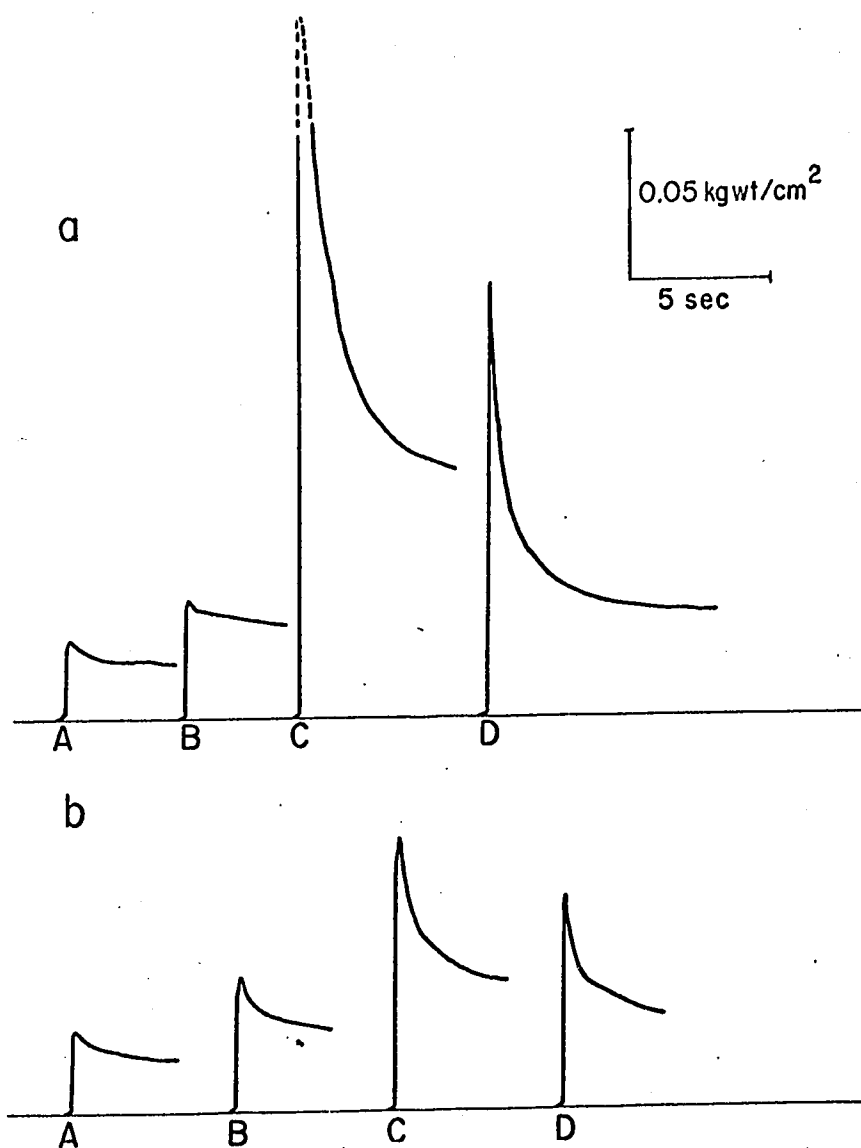


Fig.9. Resistance to stretch of the semitendinosus below the mechanical threshold. (a) 10 fibers, 180 X 280 μ , '0' length 18 mm, stretch 15%. (b) 9 fibers, 100 X 260 μ , '0' length 14 mm, stretch 15%. A, control (2.5 mM K⁺); B, C, and D fibers stretched after 15, 30, and 60 seconds of exposure to 25 mM K⁺. Dashed portion (a) C off scale.

actively developed (Fig 9a, b, traces C) stretching of the series elastic elements would have revealed it.

Fig. 10 illustrates the changes in the resistance to stretch observed when exposing the muscles to potassium concentrations below the mechanical threshold. It should be noted that in this series of tests the mechanical threshold of the muscles used was at about 27 mM K^+ . The increase in the resistance to stretch was dependent upon the concentration of potassium above 15 mM K^+ and there was also a tendency for the increase to vary with the period of exposure. Considering the resistance to stretch increases at potassium concentrations of 22.5 and 25 mM it may be seen that there is a strong tendency for it to peak when tested after a 30 sec exposure (also Fig. 9b).

When the resistance to stretch data of Fig. 10 were plotted independent of the period of exposure as a function of the potassium concentration a hyperbolic plot was obtained (Fig. 11, inset). A reciprocal plot therefore resulted in a straight line (Fig. 11). The least mean square plot so obtained only holds for the range of potassium concentrations below the mechanical threshold. At potassium concentrations which result in contracture development, the resistance to stretch becomes so great (up to 20X the resting value) as to cause a discontinuity in the curve at the point of the mechanical threshold. Thus the relationship between the resistance to stretch and the potassium concentration is not

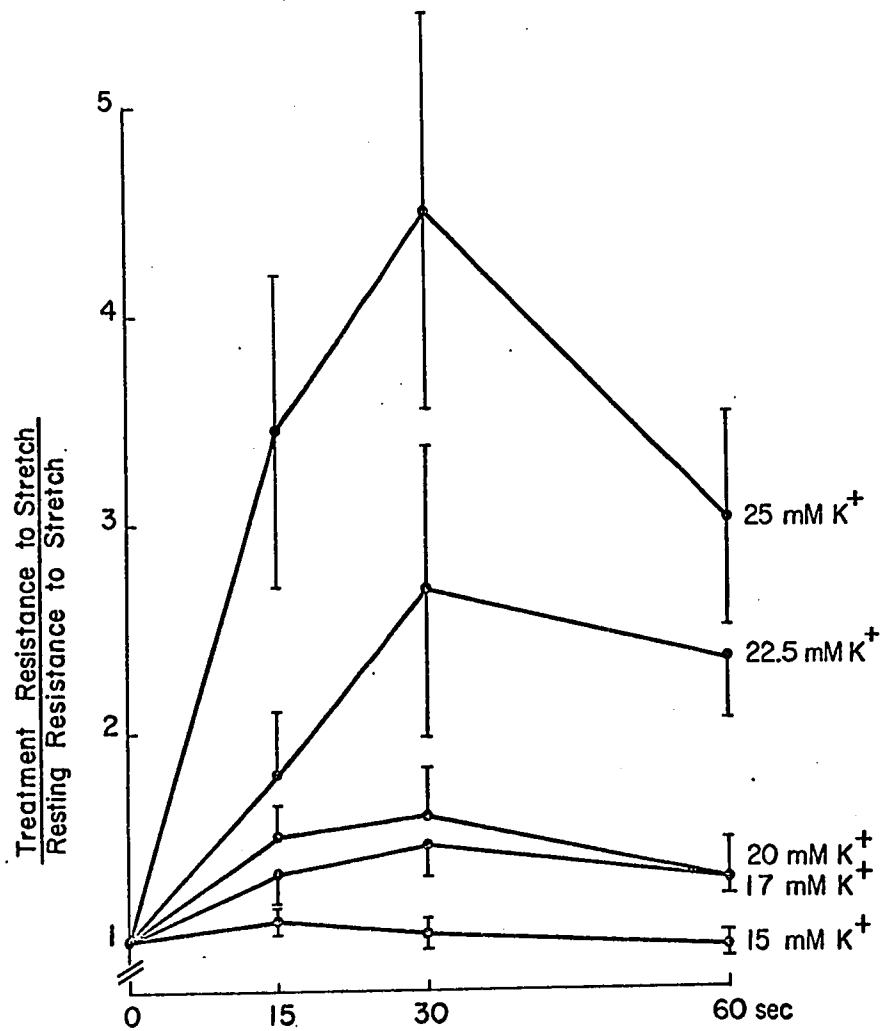


Fig.10. Relation between the resistance to stretch and duration of exposure to various concentrations of K^+ (7 experiments). The abscissa represents the ratio of the resistance to stretch at elevated K^+ to that of the control (2.5 mM K^+). The ordinate represents the duration of the elevated K^+ exposure. All K^+ concentrations were below the mechanical threshold. Preparations consisting of 5-11 fibers were used in each experiment. Each point is the mean (S.E. of mean) of 6-7 tests.

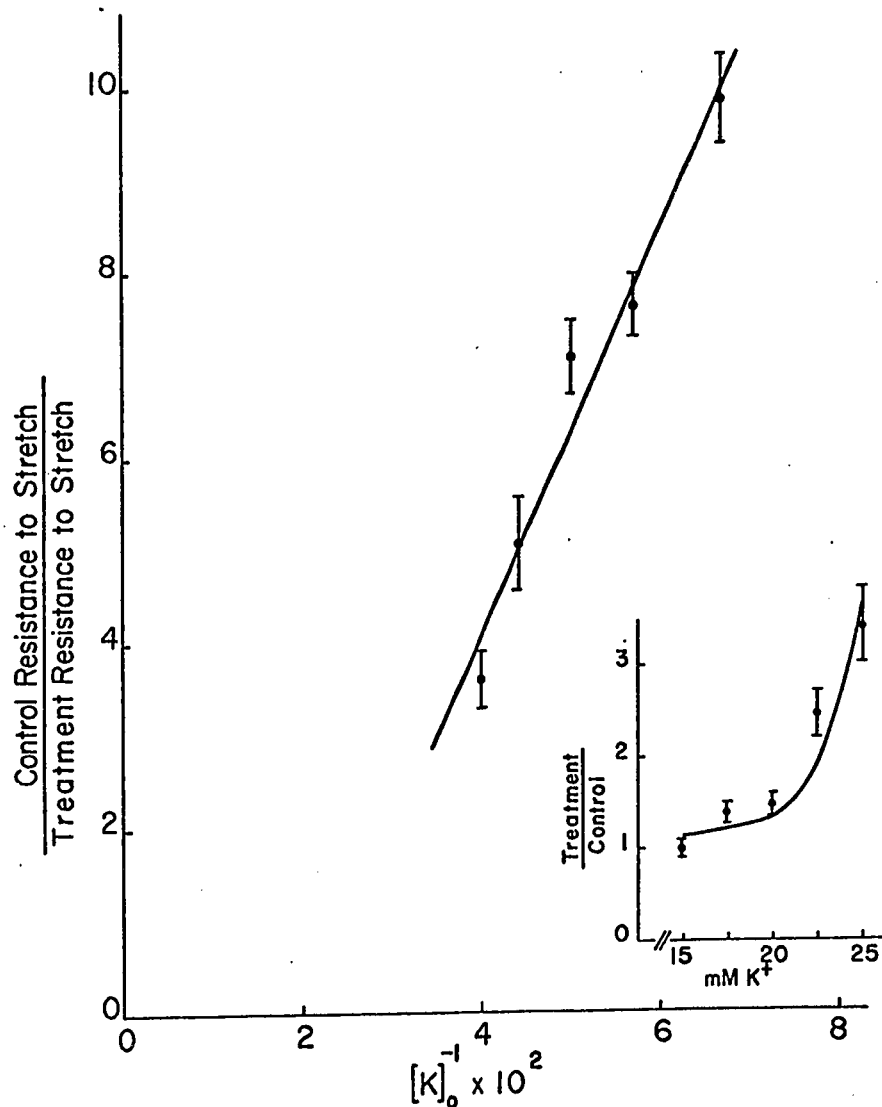


Fig. 11. Relation between resistance to stretch and various K^+ concentrations. The same data as in Fig. 9 have been used but the data from 15, 30, and 60 second exposures at each K^+ concentration have been pooled resulting in the graph shown in the inset. The main figure is a reciprocal plot of the data. Each point is the mean (\pm S.E. of mean) of 6-21 measurements.

the same above and below the mechanical threshold.

IV(vi). Pre-exposure to potassium below the mechanical threshold, time course of the resistance to stretch of subsequent contractures.

It was noted in Section IV(i) that the time-course of the resistance to stretch during a just above mechanical threshold potassium-induced contracture appeared to be in step with the development of tension. That is, the resistance to stretch is small at the beginning of the contracture and reaches a maximum value near or at the time of maximum tension. This is illustrated in Fig. 12A. In Fig. 12B the time course of the resistance to stretch of contractures due to 27 mM K^+ pretreated for 10 seconds with 17 mM K^+ . The responses shown in Fig. 12A and B were obtained using the same preparation. It can be seen that during the first 5 seconds of contractures obtained following pretreatment with 17 mM K^+ (Fig. 12B) the resistance to stretch is nearly twice that of the control 27 mM K^+ contractures (Fig. 12B). Furthermore, the resistance to stretch declines more rapidly than in the control responses. By looking at the tension development up to the time of stretching (Fig. 12) it can be seen that there is a small variability in the contractures, however, responses 10, 11, and 12 (dashed lines) are distinctly enhanced and therefore the resistance to stretch in these 3 responses should only be compared to each other.

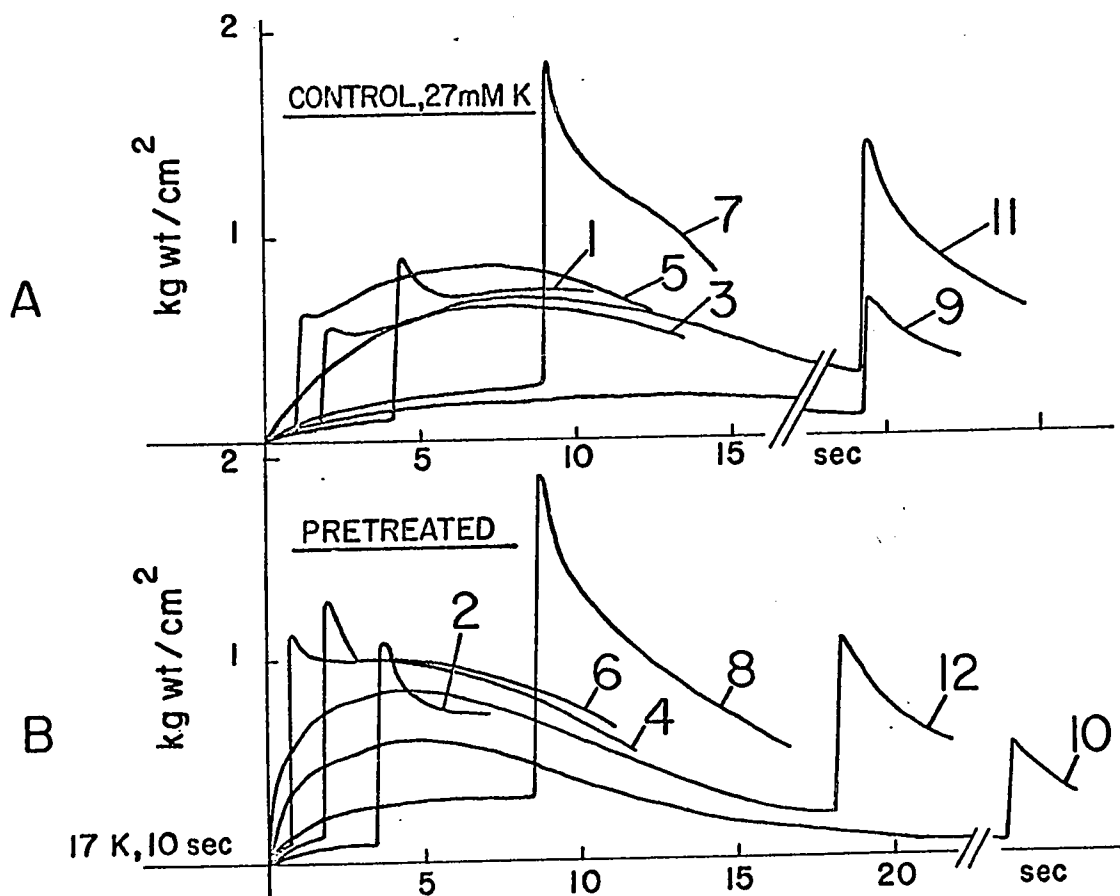


Fig.12. Comparison of time-course of resistance to stretch of a control, 27 mM K⁺ and a 17⁺ mM K 10 sec pretreated 27 mM K⁺ contracture. Numbers refer to test sequence, compare 1 with 2, 3 with 4 etc. Semitendinosus, 160 X 220 μ , stretch 15% of '0' length. Break in time base represents 10 sec.

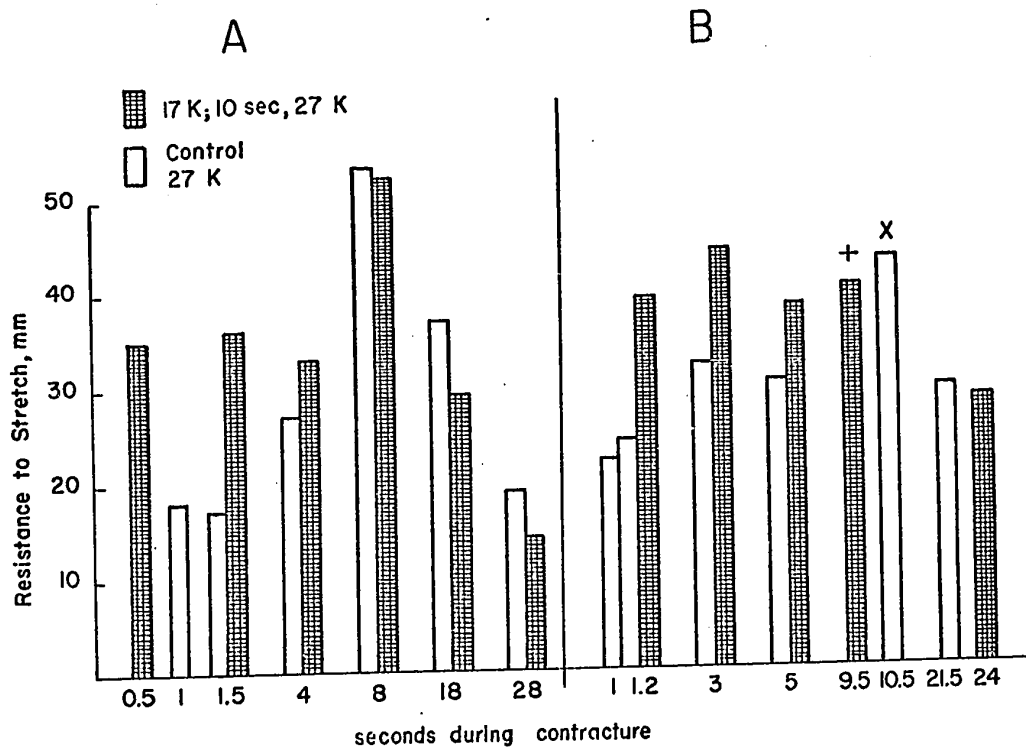


Fig. 13. Comparison of time-course of resistance to stretch of control 27 mM K^+ and 17 $^{+}$ mM K 10 second pre-treated 27 mM K^+ contracture (2 experiments). Ordinate resistance to stretch in mm of paper recorded, abscissa time during contracture at which stretch was applied. At (+) tension was still increasing and at (x) tension was at a maximum.

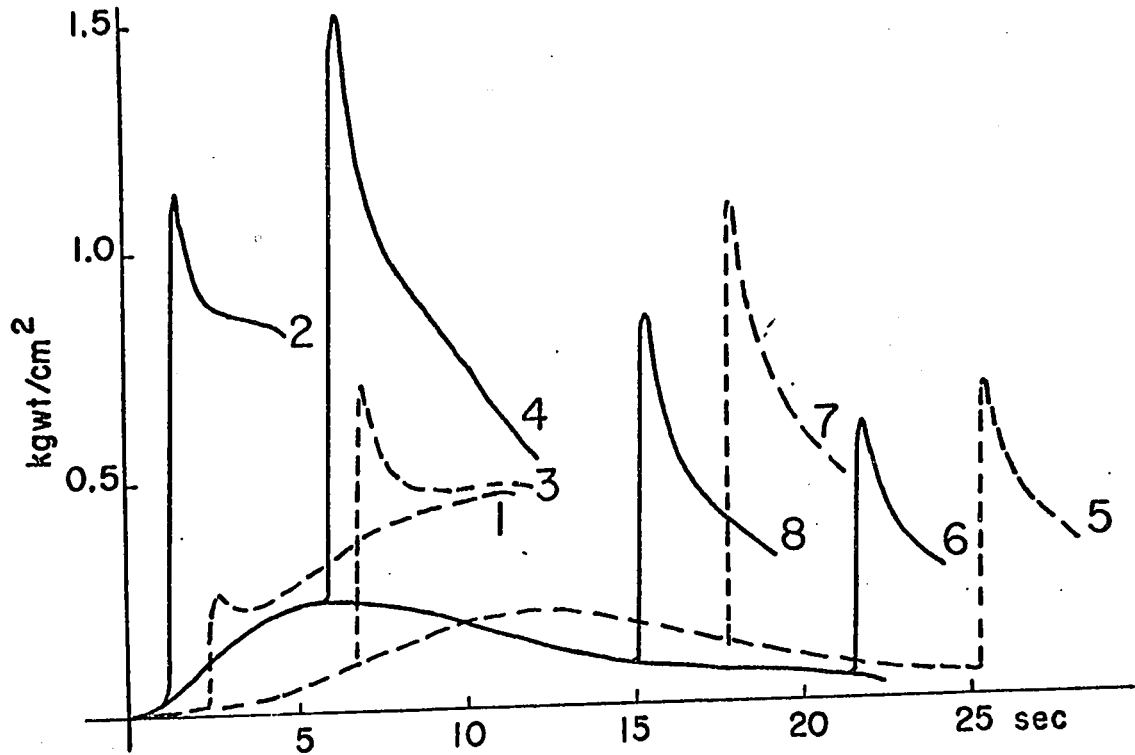


Fig.14. Comparison of time-course of resistance to stretch of a control, 35 mM K^+ and a 25 mM K^+ 20 sec pre-treated 35 mM K^+ -contracture. Numbers refer to test sequence, compare 1 with 2, 3 with 4, etc. Semitendinosus, 8 fibers, 160 X 240 μ , stretch 15% of '0' length. Dashed lines represent the control and solid lines represent the pre-treated contractures.

In order to simplify comparisons the time course of the resistance to stretch obtained in 2 experiments has been illustrated in the form of a bargraph in Fig. 13. Included in this figure are the results of another experiment which shows the same general trend (Fig. 13B). Figure 14 illustrates the same trend seen in Fig. 12, this experiment was performed about 18 months later. The mechanical threshold of the semitendinosus preparation was at about 27 mM K^+ which explains the pre-exposure to 25 mM K^+ and the contracture by 35 mM K^+ . Similar results were obtained in 3 more experiments carried out specifically to determine the time course of the resistance to stretch during the pre-treated and normal (control) contractures. Resistance to stretch measurements made during the course of other experiments designed to obtain different information, have always demonstrated that the resistance to stretch of a briefly pretreated contracture was larger by approximately 100% or more than the resistance to stretch of the control contractures when the measurements were made early in the contracture.

IV(vii). Effects of potassium concentrations up to mechanical threshold and duration of pre-exposure on subsequent contractures.

The toe muscle of the frog was used to determine the effects of varying the potassium concentration and the duration of the pre-exposure period on subsequent contractures.

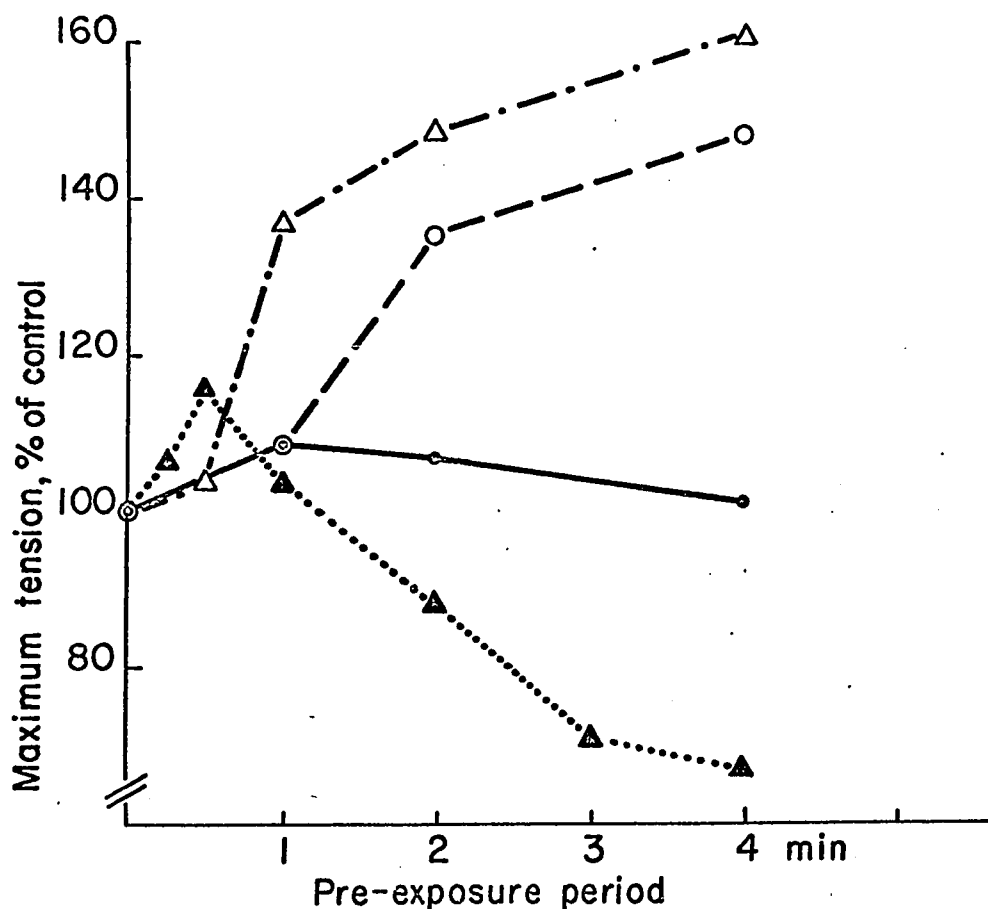


Fig.15. Relation of pre-exposure period of elevated potassium below the mechanical threshold to the maximum tension subsequently developed with 25 and 30 mM K⁺. Results are from a single experiment using the toe muscle. The abscissa indicates the time the preparation was exposed to 6.25 mM K⁺ (●), and 10 mM K⁺ (○) before a 30 mM K⁺-contracture; and to 10 mM K⁺ (Δ) and 17 mM K⁺ (▲) before a 25 mM K⁺-contracture. The ordinate represents the maximum tension developed as a percentage of the control contracture to 25 mM K⁺ or 30 mM K⁺ without pre-exposure.

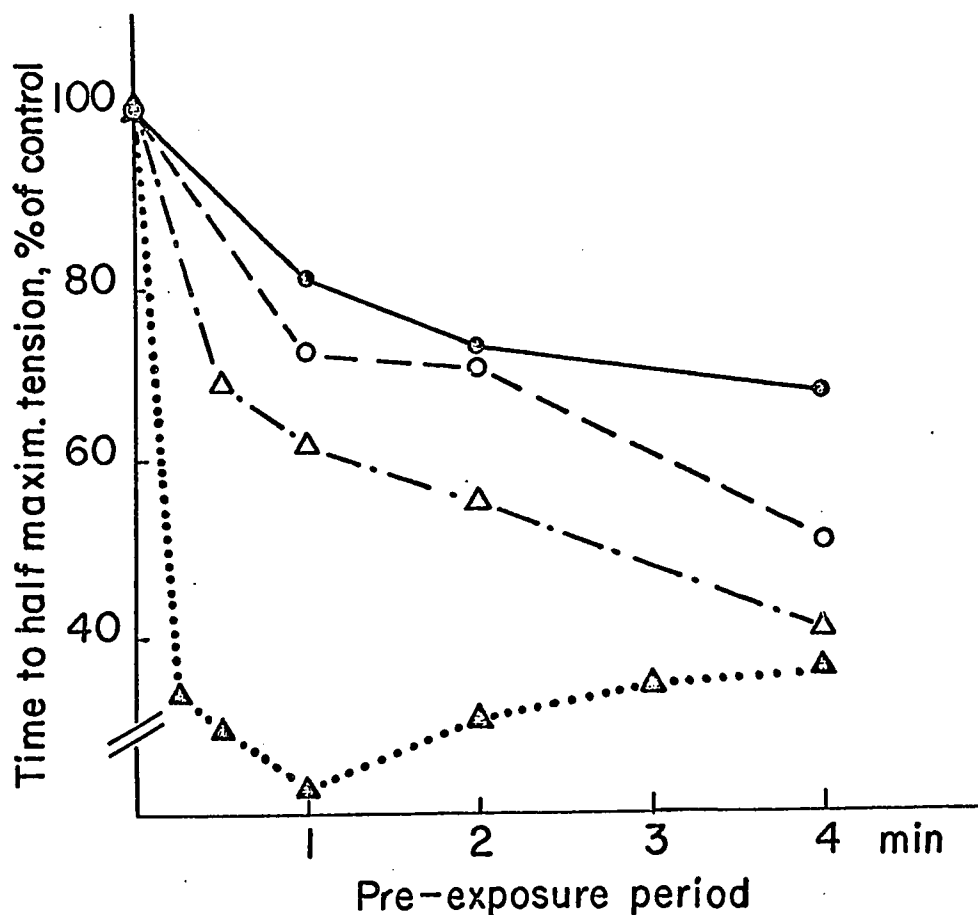


Fig. 16. Relation of pre-exposure period of elevated potassium below the mechanical threshold to the rate at which a subsequent 25 and 30 mM K^+ -contracture developed. Ordinate, the rate of tension development expressed as the time taken to half maximum tension as a percentage of the control. The abscissa represents the duration of the pre-exposure period. All symbols have the same meaning as in Fig. 15, from the same experiment shown in Fig. 15.

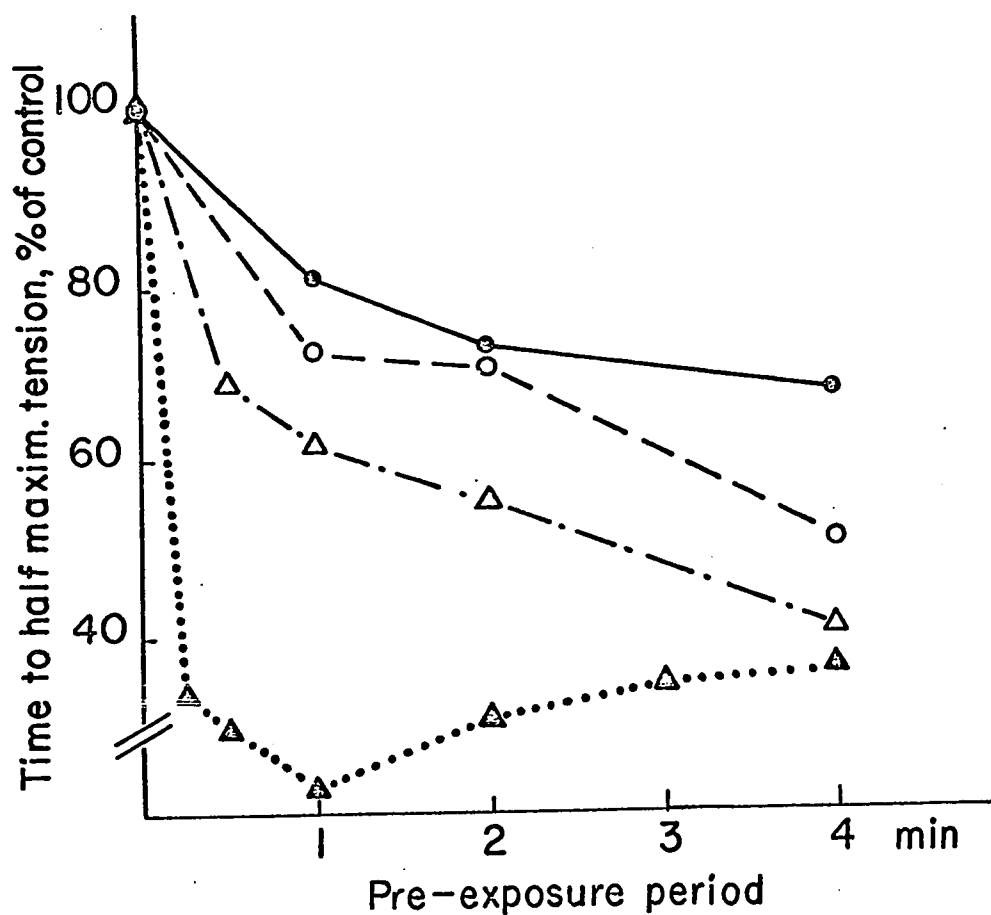


Fig. 16. Relation of pre-exposure period of elevated potassium below the mechanical threshold to the rate at which a subsequent 25 and 30 mM K^+ -contracture developed. Ordinate, the rate of tension development expressed as the time taken to half maximum tension as a percentage of the control. The abscissa represents the duration of the pre-exposure period. All symbols have the same meaning as in Fig. 15, from the same experiment shown in Fig. 15.

A few experiments using small bundles of the semitendinosus were done to confirm and extend the results.

Fig. 15 illustrates the effects of the period of pre-exposure and different concentrations of potassium on the maximum tension of subsequent contractures. It may be noted that at 17 mM K^+ the potentiation effect was very short lived, it reached a maximum by 30 seconds of pre-exposure after which the maximum tension of the subsequent contractures decreased below control levels as the period of pre-exposure was increased. At a potassium concentration of approximately 17 mM the Solandt effect is maximum (Hegnauer, Fenn and Cobb, 1935; Smith and Solandt, 1938; Hill and Howard, 1957) provided the mechanical threshold is at about 20 mM K^+ which it was in this muscle and four others which gave essentially the same results as shown in Fig. 16. At 10 mM K^+ a potentiation was observed after longer periods of pre-exposure and when the potassium concentration was 6.25 mM virtually no potentiation occurred. The threshold for the Solandt effect is at about 6 mM K^+ (Hegnauer, Fenn and Cobb, 1935; Smith and Solandt, 1938). Fig. 16 shows the effects of different potassium concentrations below mechanical threshold and the period of pre-exposure on the rate at which a subsequent contracture develops; the data are from the same experiment shown in Fig. 15. The rate of tension development is expressed as the time to half maximum tension and as a percentage of control contractures.

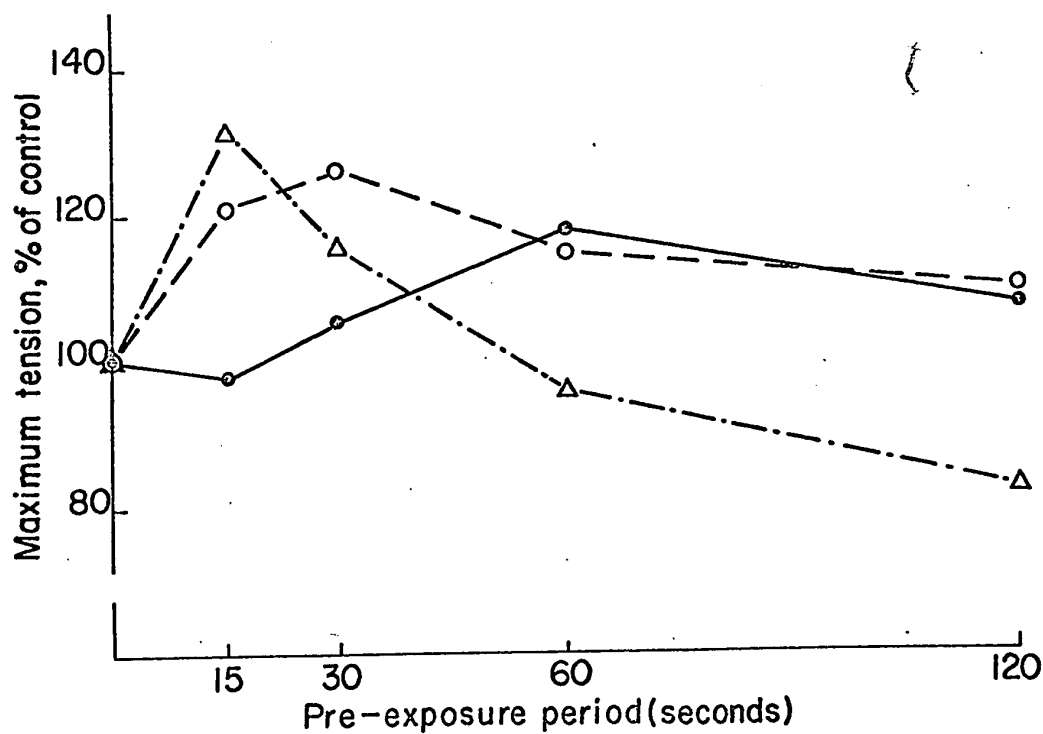


Fig. 17. Relation of pre-exposure period of elevated potassium below the mechanical threshold to the maximum tension subsequently developed with 25 mM K⁺. Results are plotted from a single experiment on the toe muscle done 18 months after the experiment shown in Figs. 15, 16. The preparation was pre-exposed to 12.5 mM K⁺ (•), 15 mM K⁺ (○), and 17.5 mM K⁺ (Δ) for periods indicated on the abscissa.

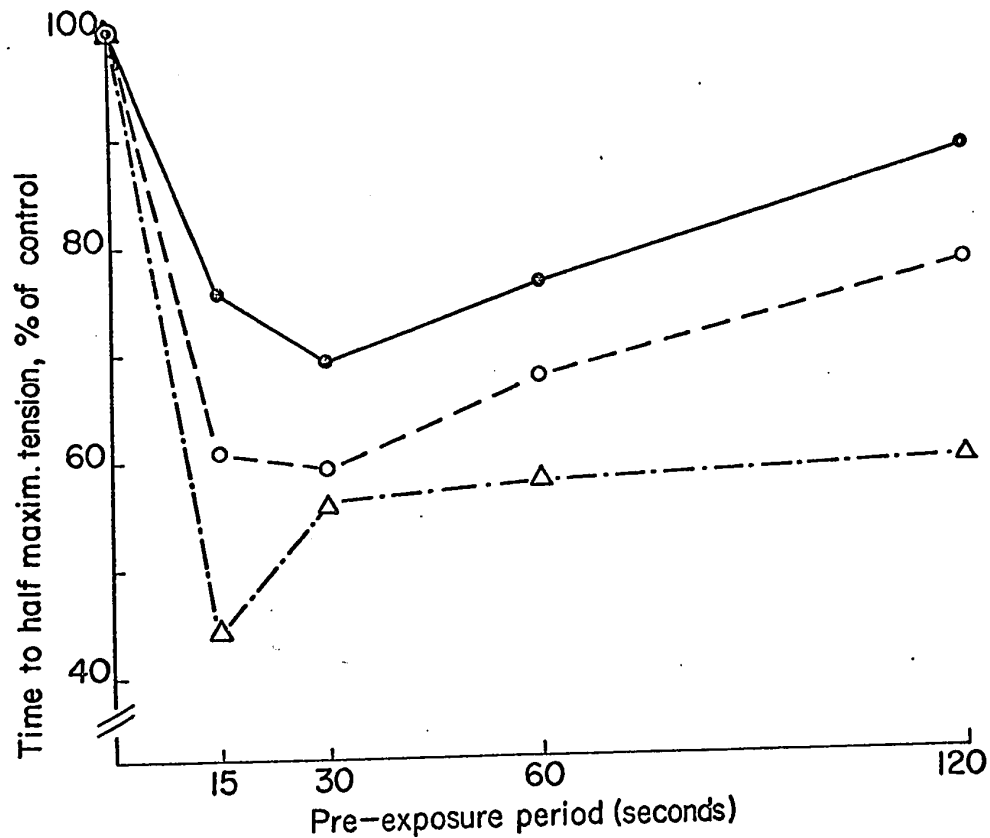


Fig.18. Relation of pre-exposure period of elevated potassium below the mechanical threshold to the rate at which a subsequent 25 mM K^+ -contracture developed. The rate is expressed as the time taken to reach half maximum tension as a percentage of the control. From the same experiment shown in Fig. 17. All symbols have the same meaning as in Fig. 17.

Thus when the percentage decreases the rate of tension development has increased. When the muscle was pre-exposed to 17 mM K^+ (i.e. close to the mechanical threshold) the rate of tension development increased rapidly. Prolonging the period of pre-exposure to 17 mM K^+ did not further alter the rate of tension development although the maximum tension did decline (Fig. 15). With lower concentrations of potassium the increase in rate of tension development was less marked and increased slightly with increasing the duration of the period of pre-exposure. Results on 8 experiments obtained some 18 months later when the mechanical threshold was at approximately 27 mM K^+ showed the same trends as reported above (Figs. 17 and 18).

The resistance to stretch early in contractures elicited after various periods of pre-exposure to low potassium close to the mechanical threshold showed an increase over controls with short pre-exposures (Section IIC) and then a decline below controls with longer pre-exposure periods (Fig. 19). In this particular experiment the peak of the potentiating effect occurred after a pre-exposure period of about 30 seconds. Following a 2 minute pre-exposure both the contracture and the resistance to stretch were distinctly depressed. It should be stressed, however, that in different preparations the peak of the potentiating effect may occur after a pre-exposure period of as little as 15 seconds or as long as 2 minutes; in all experiments prolonging the

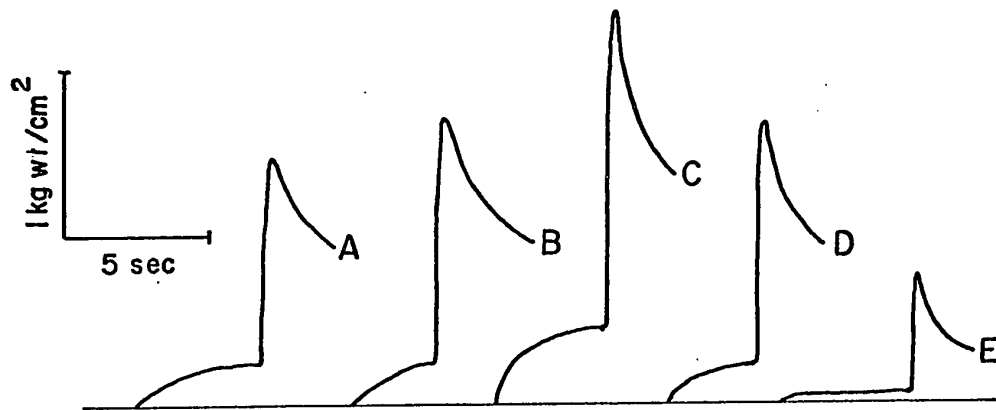


Fig.19. Resistance to stretch during a 35 mM K^+ -contracture and 35 mM K^+ -contractures following various periods of pre-exposure to 22.5 mM K^+ . The preparation was stretched between 2.5 and 5 seconds after introduction of 35 mM K^+ . A, 35 mM K^+ (control); B, C, D, and E, 35 mM K^+ preceded by 22.5 mM K^+ for 10, 30, 60, and 120 sec, respectively. Semiten-dinosus, 100 X 140 μ , 3 fibers, stretch 15% of '0' length.

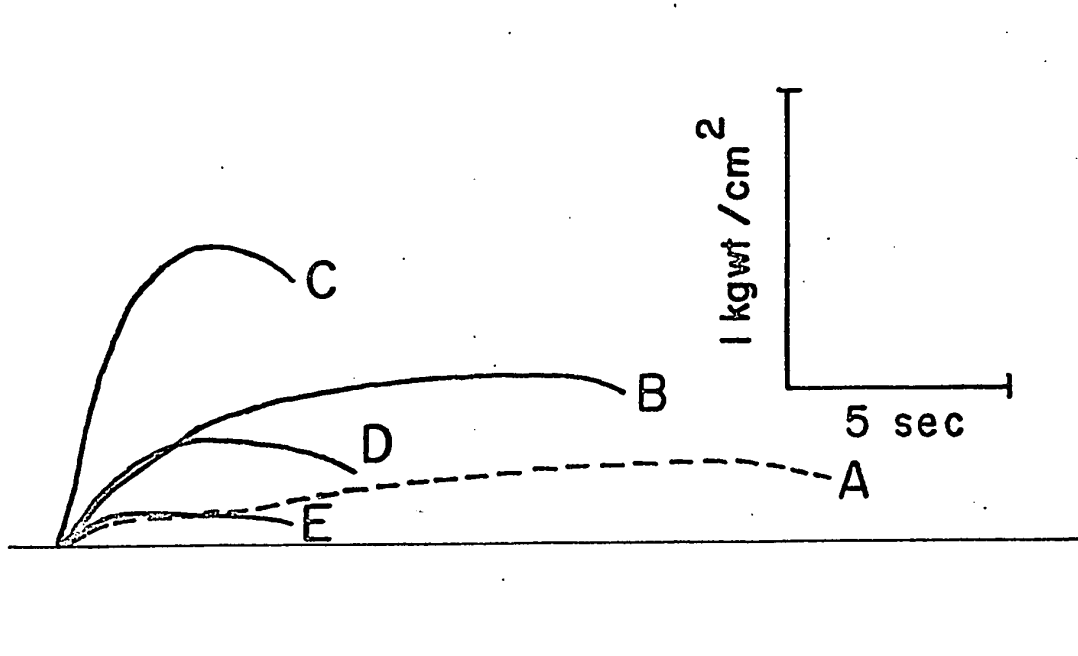


Fig.20. Isometric myograms of a control 35 mM K^+ -contracture (A) and 35 mM K^+ -contractures after various periods of a pre-exposure to 22.5 mM K^+ ; B, C, D and E, pre-exposures of 30 sec, 2, 4, and 8 min, respectively. Semitendinosus, 140 X 300 μ , 12 fibers at '0' length plus 15%.

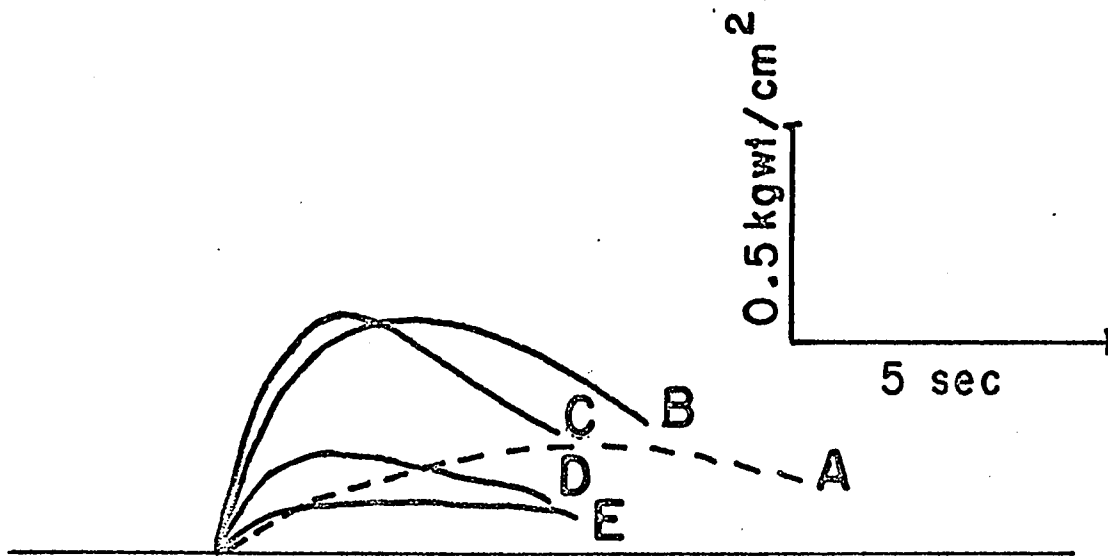


Fig.21. Isometric myograms of a control 25 mM K^+ -contracture (A) and 25 mM K^+ -contractures after various periods of pre-exposure to 17.5 mM K^+ . B,C,D, and E, pre-exposure periods of 10, 30, 60, and 120 sec, respectively. Semitendinosus, 100 X 300 μ , 10 fibers at '0' length plus 15%.

pre-exposure period beyond the period required for the peak potentiating effects resulted in a depression of tension and the resistance to stretch as seen in Fig. 19. An example of a preparation in which the potentiating effect reached a maximum and persisted after longer pre-exposures to potassium is given in Fig. 20. Inspection of the myograms shows that at 2 minutes of pre-exposure to 22.5 mM K^+ the contracture in this preparation was greatly potentiated and that depression set in at about 4 minutes. At 8 minutes of pre-exposure the contracture in response to 35 mM K^+ was distinctly depressed. Fig. 21 illustrates the results obtained in a similar experiment on another preparation. In this experiment it can be seen that after 1 minute of pre-exposure to 17 mM K^+ , the contracture to 25 mM K^+ was depressed as compared to the control and that after a 2 minute pre-exposure, a contracture was obtained which was similar to that seen after an 8 minute pre-exposure in the experiment shown in Fig. 19. In the experiment shown in Fig. 19 the mechanical threshold was at about 25 mM K^+ whereas in the Fig. 20 experiment the threshold was found to be at about 20 mM K^+ . However, there was no consistent relation between the mechanical threshold and the period of pre-exposure needed to obtain peak potentiating effects and depression measured either as the tension developed or the resistance to stretch.

IV(viii). Persistence of potentiation following removal of 17.5 mM K⁺ (Washout effect).

In addition to the potentiating effect of exposing the muscle to below mechanical threshold potassium concentrations, it was observed that the potentiation persisted for some time after placing the muscle back in ordinary Ringer's solution. For example, when a 30 sec exposure to 17.5 mM K⁺ was followed by 15 sec in Ringer's solution (i.e., with 2.5 mM K⁺) a potentiated contracture could still be elicited (Fig. 22). Thus even though the 17.5 mM K⁺ was removed and the membrane potential presumably had returned to normal (see IVb) potentiation still occurred. This has been termed the 'Washout effect.' To characterize this effect more fully further experiments were done using toe muscles. A factorial design with randomized blocks was used in these experiments. The test conditions are shown in tables 1 and 2. It can be seen that a complete experiment required that 13 separate tests be made on each muscle. Four complete experiments of this type were carried out. The treatment means (tables 1 and 2) were compared to the control mean using Dunnett's test (Edwards, 1968). This is a conservative test having the advantage that it gives a 95% confidence level for all the comparisons with the control mean. The treatment means are shown graphically in Fig. (23). Looking at the maximum tension means it will be observed that a significant potentiation occurred with pre-exposures to 17.5 mM K⁺ for 15

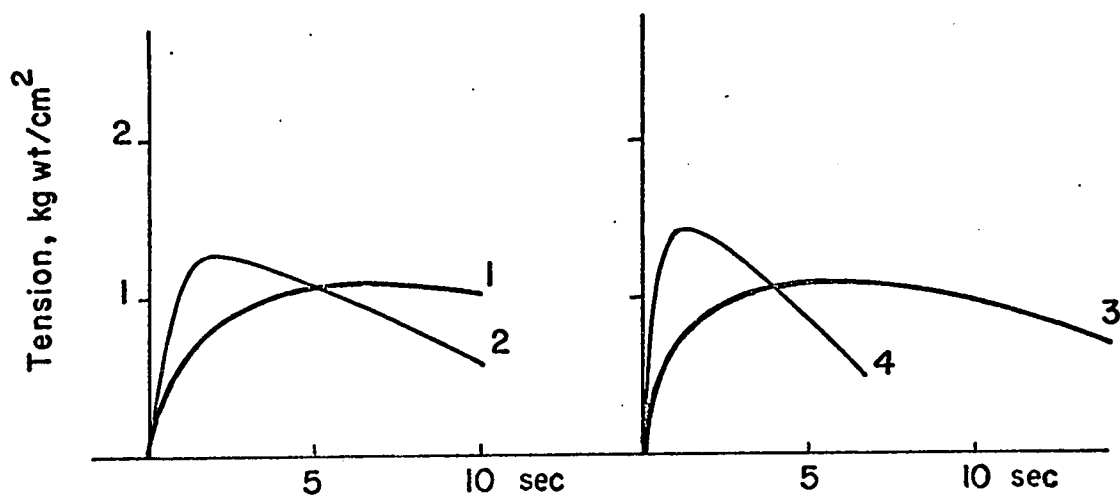


Fig.22. Persistence of potentiation following washout of 17.5 mM K^+ . Myograms 1 and 3 are control responses to 27 mM K^+ . Myogram 2 was obtained by 30 sec exposure to 17.5 mM K^+ followed by 27 mM K^+ . Myogram 4 was obtained by 30 sec exposure to 17.5 mM K^+ followed by a return to Ringer's solution (2.5 mM K^+) for 15 sec and then 27 mM K^+ . Semitendinosus, 4 fibers, 100 X 160 μ , at '0' length plus 10%.

Table 1. Comparison of maximum tensions of 25 mM K^+ -contractures in the Washout effect. (4 muscles)

Test Conditions	Control, 25 mM K ⁺	15 sec 17.5 mM K ⁺ followed by 2.5 mM K ⁺ for,				45 sec 17.5 mM K ⁺ followed by 2.5 mM K ⁺ for,				135 sec 17.5 mM K ⁺ followed by 2.5 mM K ⁺ for,			
		0 sec	15 sec	45 sec	135 sec	0 sec	15 sec	45 sec	135 sec	0 sec	15 sec	45 sec	135 sec
Mean Tensions, mg.	84	167*	139*	140*	142*	156*	121	134*	134*	129	119	132*	123

* significantly greater than the control mean, p<0.05 (k=12 with 36 df)

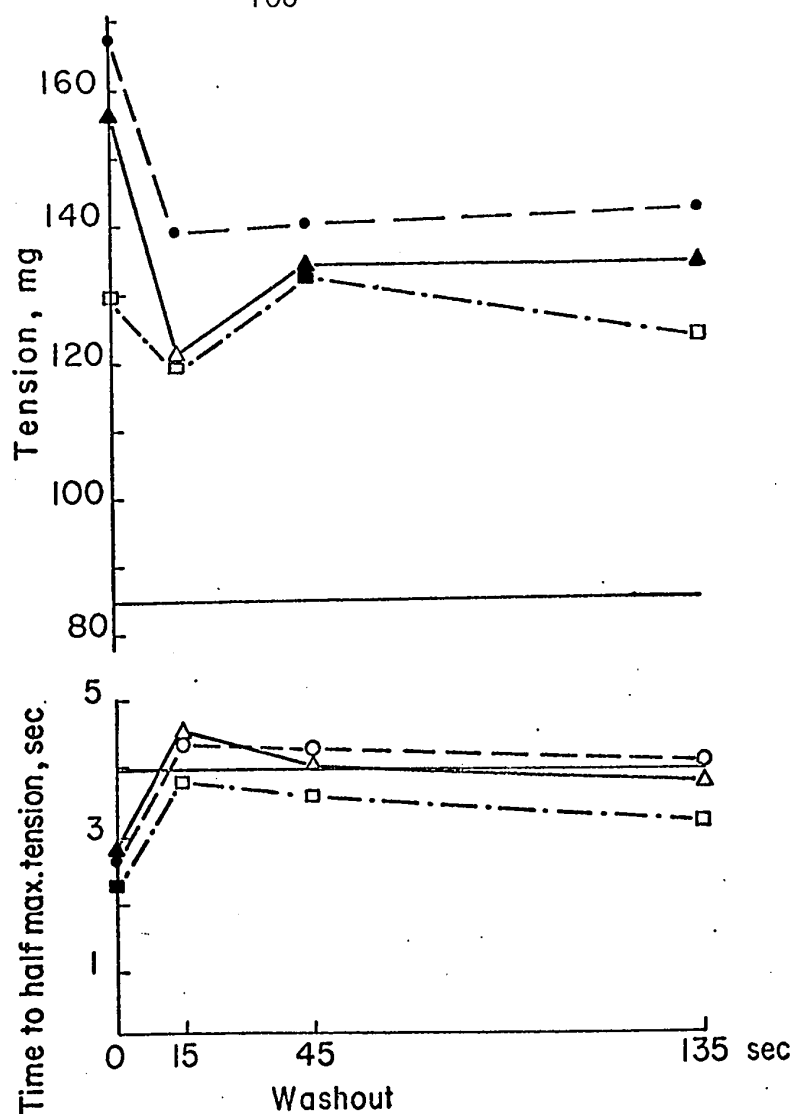


Fig.23. Persistence of potentiation following washout of 17.5 mM K^+ . Means of 4 complete experiments on toe muscles are shown. Contractures were elicited by 25 mM K^+ following exposure to 17.5 mM K^+ for 15 sec (●,○), 45 sec (▲,△), and 135 sec (■,□) plus the washout period in Ringer's solution as indicated on the abscissa. The ordinates represent maximum tension achieved and the rate of tension development as the time taken to half maximum tension. Solid horizontal lines represent control means. Filled symbols indicate that the experimental means are significantly different from the control means ($p < 0.05$). Data from tables 1 and 2.

and 45 sec but not for a 135 sec pre-exposure period. There was a small drop in the maximum tension after a washout period of 15 sec, with longer washout periods the maximum tensions increased and levelled off. The rate of tension development was significantly increased only if no washout period was interposed. With washout of the 17.5 mM K^+ the rate of tension development decreased and the means were clustered about the control mean (Fig. 23). Thus, potentiation of tension development will occur after a washout period of up to 135 sec if the pre-exposure to 17.5 mM K^+ was kept to 15 to 45 sec. Six additional but incomplete experiments were performed. Although the lack of a complete series of tests prevented their inclusion in the statistical analysis, the results obtained in these tests in so far as they could be compared, were the same as the results obtained in the 4 complete tests. Similar results also were obtained in another 4 experiments in which a variety of pre-exposure potassium concentrations, pre-exposure periods, and washout periods were used.

IV(ix). Miscellaneous, effects of isotonic sucrose and subthreshold caffeine on subsequent contractures.

Since it appeared that the potentiating effects observed with potassium were related to the Solandt effect, it was of interest to know whether other agents which cause an increase in resting metabolism will also cause potentiation

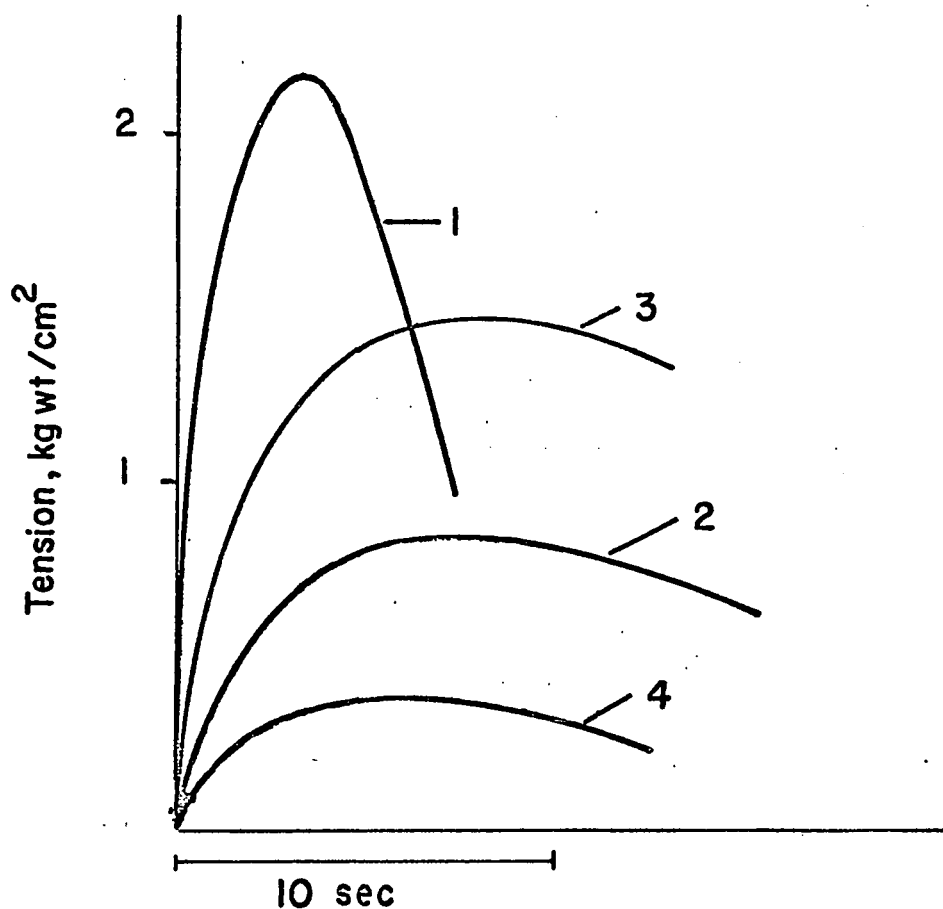


Fig.24. Effect of 0.65 mM caffeine on a subsequent 25 mM K^+ -contracture. Numbers beside the myograms indicate the order in which the experiments were done. Myogram 1, response to isotonic KCl; 2, response to 25 mM K^+ plus 0.65 mM caffeine in Ringer's solution; 3, response to 25 mM K^+ following 10 sec of 0.65 mM caffeine; 4, response to 25 mM K^+ (control). Semitendinosus, 140 X 340 μ at '0' length plus 15%.

of a submaximal potassium contracture.

Caffeine in concentrations insufficient to initiate tension development cause an increase in resting metabolism and Ca^{++} -exchangeability (Novotny and Vyskocil, 1966). Caffeine in a concentration of 0.65 mM was used to explore this possibility (Fig. 24). It was observed that a short, 10 sec, preexposure to 0.65 mM caffeine greatly augmented a 25 mM K^{+} -contracture (Fig. 24, trace 3), this potentiation was greater than when the caffeine was added together with the 25 mM K^{+} -Ringer's solution (Fig. 24, trace 2). Although small preparations of the semitendinosus were used in these caffeine potentiating experiments one cannot discount the fact even using single fibers that diffusion of caffeine to its site(s) of action may be slower than the rate at which a fiber (or fibers) is depolarized by quickly raising the external potassium concentration. Thus Fig. 24, trace 2 may not be as large as trace 3 (Fig. 24) because caffeine was not given sufficient time to diffuse to its site of action. This uncertainty as well as the observation that following a few tests the muscle preparation began to go into a contracture to 0.65 mM caffeine, led to abandoning further experiments using caffeine.

Fenn (1931) had shown that isotonic sucrose causes an increase in resting metabolism which does not occur if the ionic strength of the isotonic sucrose is increased. Fig. (25) illustrates the effect of a 10 sec preexposure

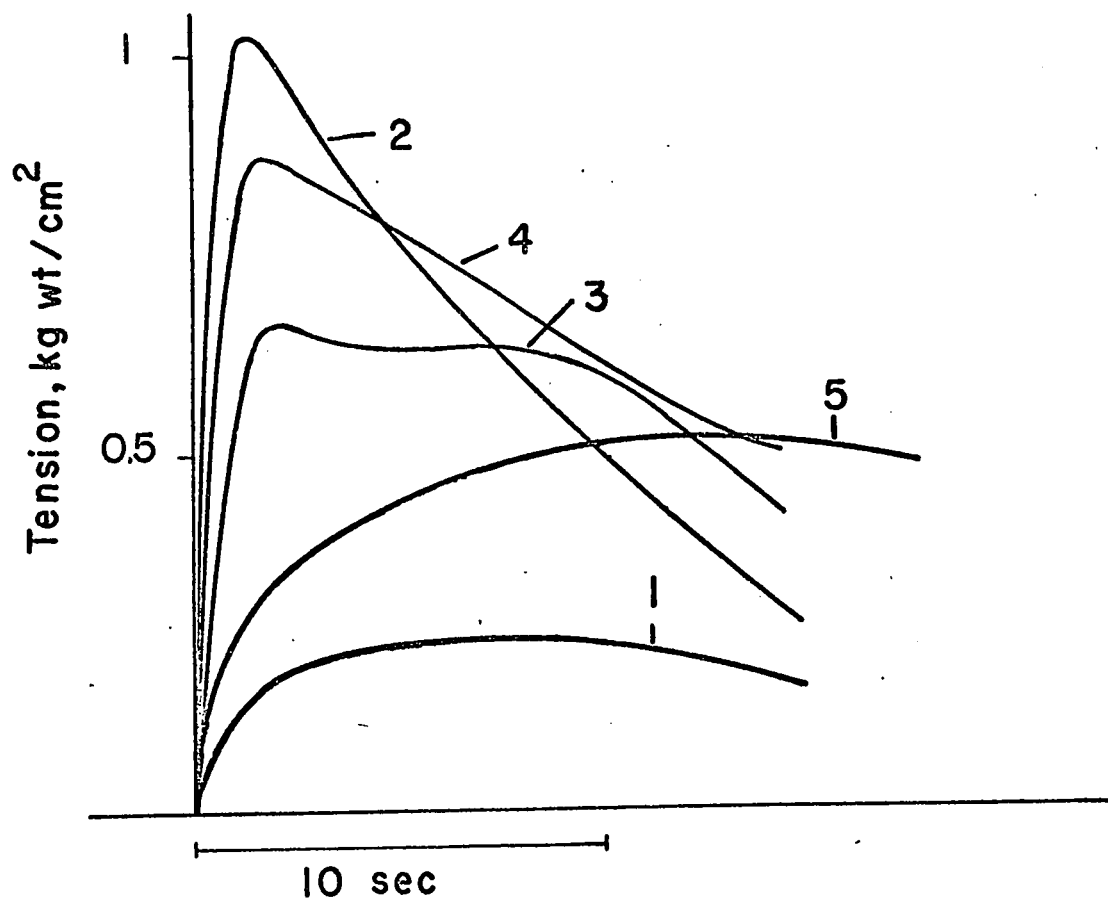


Fig.25. Effect of 10 sec pre-exposures to isotonic sucrose on a subsequent 27 mM K^+ -contracture (2,3, and 4); controls 27 mM K^+ only (1 and 5). Numbers refer to order in which tests were done. Semitendinosus, 180 X 360 μ at '0' length plus 10%.

to isotonic sucrose on a subsequent 27 mM K^+ -contracture (traces 2, 3, and 4). It is immediately evident that the 27 mM K^+ -contracture was greatly potentiated but also that the contour of the myogram changes markedly with each test. Also, it may be noted that the control contracture changed, this change is more than the 5-10% normally encountered during an experiment in which below the mechanical threshold concentrations of potassium were used to induce potentiation. It should also be pointed out that isotonic sucrose induced contractures, probably due to Cl^- withdrawal, occurred very frequently. The results obtained in a small series of experiments on the toe muscle using isotonic sucrose and isotonic sucrose with added electrolytes are presented in table 3. The effect on maximum tensions developed in the toe muscle with 10 sec pre-exposures to isotonic sucrose, isotonic sucrose with 3 mM PO_4 -buffer, or isotonic sucrose with 1.08 mM $CaCl_2$ was not marked, however, the effect on the rate of tension development was large. Isotonic sucrose with both PO_4 -buffer and $CaCl_2$ did not cause any increase in the maximum tension developed compared with controls, whereas the rate of tension development was decreased compared to the control and to isotonic sucrose with either PO_4 -buffer or $CaCl_2$. This latter result is probably related to Fenn's (1931) finding that the increase in oxygen consumption of frog's sartorius in isotonic sucrose is prevented by the progressive addition of electrolytes.

Table 3. Effect of pre-exposure with sucrose solutions on 30 mM K⁺-contractions.

Treatment	Max. tension, pretreated Max. tension, control	Time to $\frac{1}{2}$ max. tension, pretr. Time to $\frac{1}{2}$ max. tension, control	
Isotonic sucrose, 10 sec, 30 mM K ⁺ .	1.16 \pm 0.02 (n=7)	2.40 \pm 0.31 (n=7)	
Isotonic Sucrose + PO ₄ (3mM), 10 sec, 30 mM K ⁺ .	1.21 \pm 0.04 (n=11)	1.86 \pm 0.22 (n=11)	
Isotonic Sucrose + PO ₄ (3mM) + 1.08 mM CaCl ₂ , 10 sec, 30 mM K ⁺ .	1.02 \pm 0.06 (n=6)	0.68 \pm 0.08 (n=6)	
Isotonic Sucrose + 1.08 mM CaCl ₂ , 10 sec, 30 mM K ⁺ .	1.17 \pm 0.02 (n=9)	1.56 \pm 0.11 (n=9)	

Mean \pm S.E. of mean

Thus, it was found that either caffeine or isotonic sucrose which can cause an increase in resting metabolism, produced potentiation of submaximal K^+ -contractures.

Unfortunately the development of contractures to either low concentrations of caffeine or to isotonic sucrose by themselves during the course of a single experiment would make extensive investigation of the potentiating effects of these agents extremely difficult. For this reason their effects were not pursued further in this study.

IV(b). Membrane Depolarization.

Experiments in which the membrane was depolarised in one or two steps by increasing the potassium concentration in the bath were done using the extensor digitorum longus IV. As described in Methods the solution was changed within 0.5 seconds at the site of penetration while care was taken to penetrate a fiber located on the surface of the muscle. Potassium concentrations below the mechanical threshold were used because this is the region of interest in this work. In addition because in a rapidly flowing system it was possible to keep the microelectrode in the fiber only if no contracture took place. Thus, the stepwise changes were 2.5 to 7.5 to 17.5 to 2.5 mM K^+ , 2.5 to 10 to 17.5 to 2.5 mM K^+ or directly from 2.5 to 17.5 to 2.5 mM K^+ . Figures 26 and 27 illustrate the time courses of the membrane potential when the potassium concentrations were altered. Of interest in these records is that when the membrane was depolarised in one step from 2.5 to 17.5 mM K^+ there was an initial quick drop, followed by a slower fall in the membrane potential; this was also described by Hodgkin and Horowicz (1960a). However, when the depolarization occurred in two small steps, i.e. 2.5 to 7.5 or 10 to 17.5 mM K^+ , the initial quick drop was not seen, instead there were two slow depolarization steps, each with about the same time course. Also, the final membrane potential change was the same regardless whether it was arrived at in one or two steps. In depolarizations

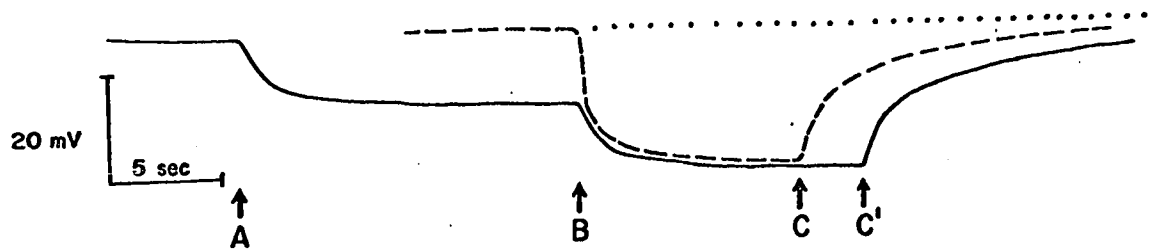


Fig.26. Changes of the membrane potential in a peripheral fiber of the extensor digitorum longus IV of the frog. At A, 7.5 mM K^+ -choline Ringer's solution was introduced; at B, 17 mM K^+ ; at C and C' return to 2.5 mM K^+ . Calibration as indicated.

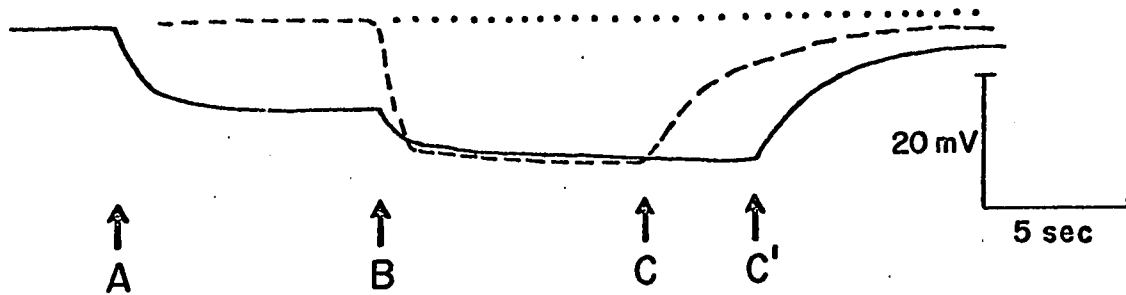


Fig.27. Changes of the membrane potential in a peripheral fiber of the extensor digitorum longus IV of the frog. At A, 10 mM K^+ -choline Ringer's solution was introduced; at B, 17.5 mM K^+ ; at C and C', return to 2.5 mM K^+ . Calibration as indicated.

produced in one or two steps repolarization occurred with the same time course; an initial fast phase followed by a slow rise. In 6 experiments it was possible to obtain both a one and a two step depolarization and repolarization from the same fiber in 7 instances. As mentioned above the rate of repolarization was not affected by the procedure used to obtain depolarization; paired t-test showed that there was no significant difference between the rate of repolarization following a one step or two step depolarization. The half time for repolarization was found to be 2.92 seconds \pm 0.24 (S.E. of mean), 23 measurements in 7 experiments. This figure is similar to the half times of 2.6 to 3.2 seconds found by Hodgkin and Horowicz (1960a) using single fibers of the semitendinosus. The time taken for repolarization to within 5 mV of the initial membrane potential was 12.4 seconds \pm 0.56 (S.E. of mean), 23 measurements in 7 experiments.

It might be pointed out here that the rate of depolarization and the time to reach a stable level of depolarization are both much more rapid than required to achieve the maximum potentiating effect of subthreshold K^+ concentrations (e.g. Figs. 15, 16, 17, 18, 19). Also, repolarization to levels which are below the values required to produce potentiation is much more rapid than the elimination of the potentiation upon re-exposure to 2.5 mM K^+ (Figs. 26, 27).

IV(c). Oxygen Consumption.

Oxygen consumption measurements were made in an attempt to answer the question whether the potentiation of a low K^+ -contracture resulting from a short pre-exposure to potassium concentrations just below the mechanical threshold was a result of increased metabolism, i.e. when the Solandt is established. And if so, when potentiation became inhibited by prolonging the pre-exposure period, what relationship did this inhibition have to the increase in metabolism.

The resting oxygen consumption of frog's sartorius at 20°C may be taken as 0.47 l/g (wet wt)/min (Hill, 1965), recent determinations on the sartori of Rana pipiens which are in agreement with this value was 0.18×10^{-4} cal/mg (dry wt)/min at 22°C (Gore and Whalen, 1968). Assuming a wet/dry weight ratio of 5 and a Q_{10} of 2.5 these values convert to 1.54×10^{-4} moles O_2 /mg (dry wt)/min at 24°C. The mean resting oxygen consumption at 24°C for the 5 pairs of toe muscles reported on in this section was $4.78 \pm 0.73 \times 10^{-4}$ moles O_2 /mg (dry wt)/min (\pm SE of mean) or about 3 times the computed value for frog's sartorius.

Since it was of importance to know how soon the increase in metabolism set in after the elevated K^+ -Ringer's solution was introduced, measurements were made from a continuously flowing system. As outlined in Methods the flow past the oxygen electrode was constant at all times

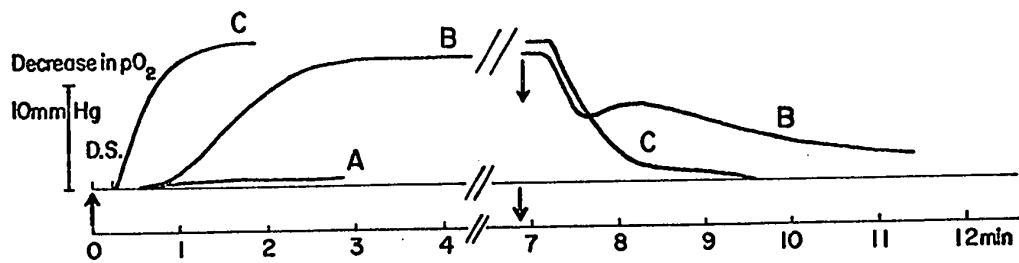


Fig. 28. Change of pO_2 in the solution flowing past a pair of toe muscles following rapid introduction (\uparrow) and removal (\downarrow) of a 17.5 mM K^+ -Ringer's solution in the flow system (B). A, resting oxygen consumption of the same muscles following a 2-3 sec flush of the chamber (by use of pump 1, Fig. 2) at 15.3 ml/min. C, control response recorded using the same flushing sequence but with Ringer's solution equilibrated with 19.7% O_2 in N_2 . Change in pO_2 was followed at a flowrate of 0.192 ml/min. Upward deflection indicates a decrease in the pO_2 . D.S., delay due to dead space corresponding to V_2 , Fig. 2.

while the flow past the muscle was increased for 2 to 3 sec to permit a rapid change of solutions following which the muscle chamber was switched back to the oxygen electrode so that the pO_2 of the elevated K^+ -Ringer's solution is continuously monitored (Fig. 2). When the pO_2 of the fluid flowing through the muscle chamber is rapidly changed by this procedure there was a constant delay of 13-14 sec between the end of the rapid flush period and the start of the response of the oxygen electrode (Fig. 28, trace C). This delay was presumably due to the dead space between stopcock 2 (Fig. 2) and the oxygen electrode. This delay was the same in 6 different control experiments in which the pO_2 of the solution was changed from room air pO_2 (144 mm Hg) to solutions with lower pO_2 values between 135 mm Hg and 0 mm Hg. Following this delay there was a rapid phase in the decrease in the pO_2 recorded followed by a slow phase of decreasing pO_2 to the final level. Since mixing in this system was unlikely to be a large factor, the shape of trace C (Fig. 28) presumably shows the response of the oxygen electrode to a step decrease in O_2 tension.

Following the delay due to the dead space, there was a further delay of about 30 sec before the oxygen consumption of the K^+ -stimulated muscle started to increase above the resting oxygen consumption. Thus there was about a 30 sec delay between the time that the muscle was exposed to the elevated K^+ -Ringer's solution and the start of the

increase in oxygen consumption. Following this second delay the pO_2 fell rapidly (Fig. 28, trace B). Nevertheless, even in this rapid phase the rate of pO_2 decline was slower than the response time of the oxygen electrode (Fig. 28, trace C) and would indicate that during this rapid phase which lasted about 1.5 min the rate of oxygen consumption was increasing.

When the elevated K^+ -Ringer's solution was flushed out of the muscle chamber, the Ringer's solution used had a pO_2 of room air. Thus following the removal of the elevated K^+ -Ringer's solution there was an initial rise in the pO_2 (Fig. 28, trace B) which paralleled the rise with a stepped reduction in the pO_2 to the room air level (Fig. 28, trace C). However, the metabolic rate of the muscles was still above the resting rate and this appeared as a secondary, slower increase in the pO_2 . Following removal of the elevated K^+ -Ringer's solution it took about 5 min or more before the metabolic rate returned to the resting level.

In order to estimate the rate of oxygen consumption increase successive 30 sec areas were measured from the time the initial delay was over. Thus the increased amount of oxygen consumed in elevated K^+ -Ringer's solution was equal to the area under the curve above the resting oxygen consumption multiplied by the flowrate. The results for 10 mM K^+ and 17.5 mM K^+ are shown in Figs. 29 and 30, it is evident that the plateau of oxygen consumption increase

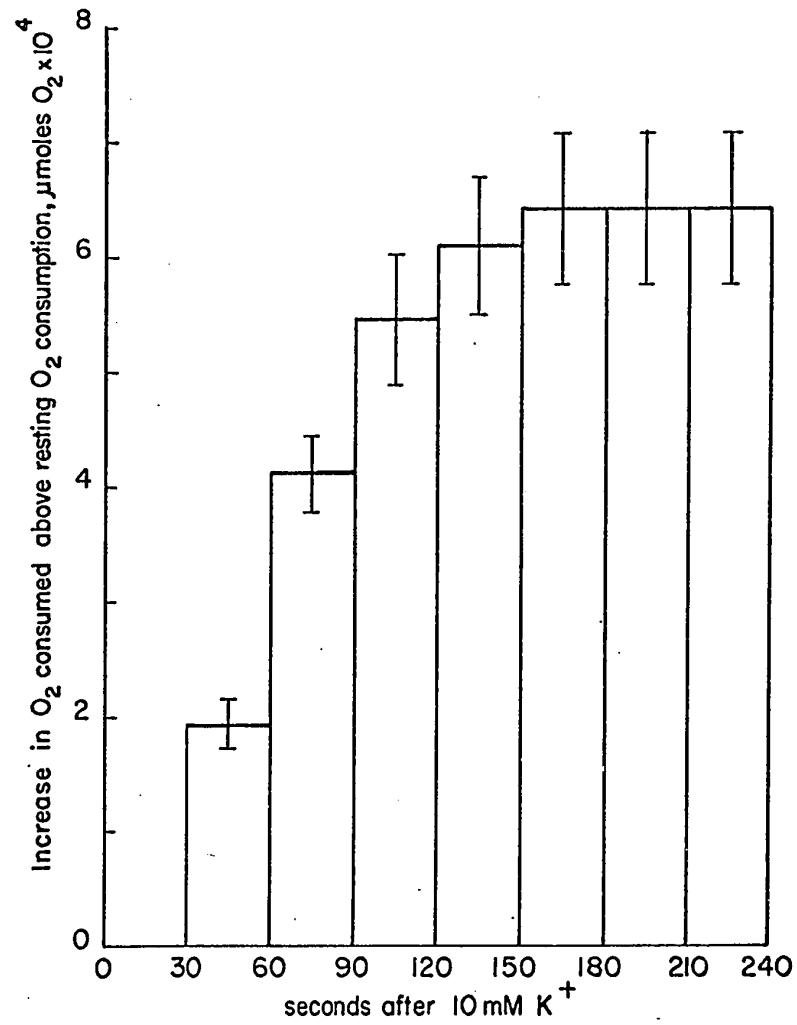


Fig.29. The amount of oxygen consumed by two toe muscles above the resting oxygen consumption for 30 sec intervals following introduction of 10 mM K⁺-Ringer's solution. Each value given is the mean ± S.E. of mean, n=8.

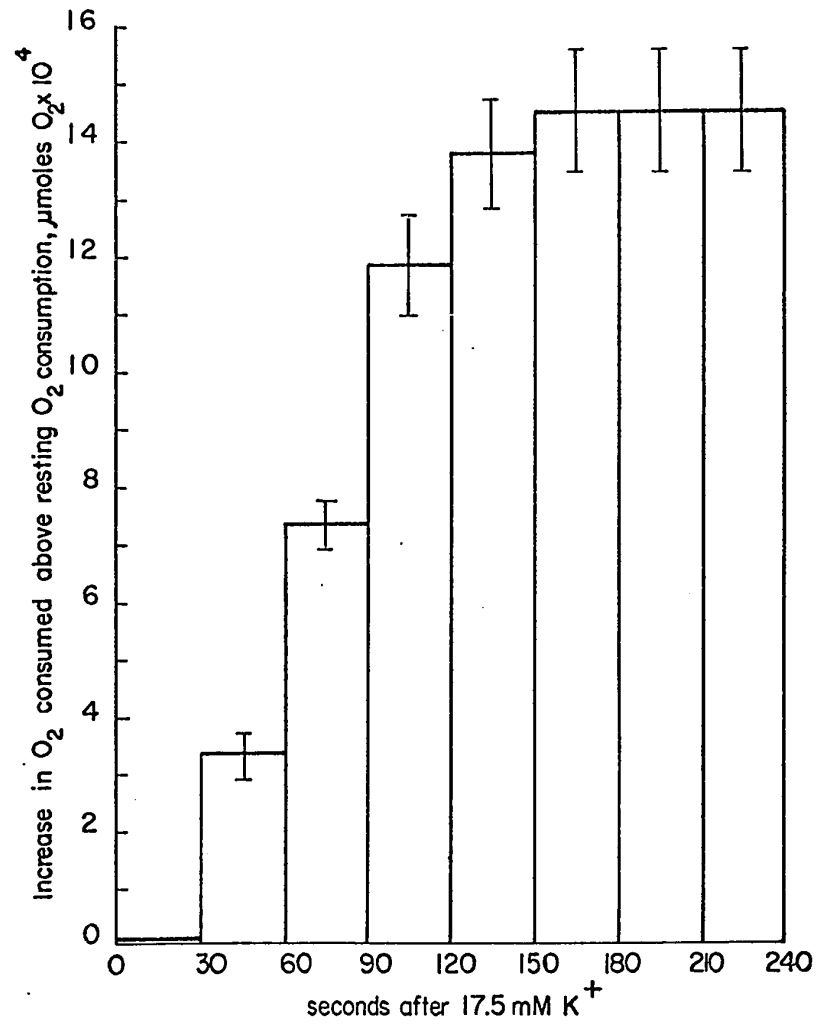


Fig. 30. The amount of oxygen consumed for two toe muscles above the resting oxygen consumption for 30 sec intervals following the introduction of 17.5 mM K⁺-Ringer's solution. Each value given is the mean \pm S.E. of mean, n=11.

was reached in about 150 sec with both concentrations of potassium. This plateau could be maintained for up to 24 minutes which was the maximum period it was followed. The half times for the oxygen consumption increase to reach the plateau phase were about 60 sec for both concentrations of potassium (Figs. 29 and 30), whereas the half times for the return of oxygen consumption to resting levels was found to be 147 ± 9 sec (\pm SE of mean, $n=9$).

CHAPTER V

DISCUSSION

The mechanical threshold has been defined as that membrane potential or potassium concentration at which striated muscle switches from rest to activity; activity being either tension development or shortening. This viewpoint was undoubtedly encouraged by the views of Hill (1949) and the relationship between tension developed and membrane potential described by Hodgkin and Horowicz (1960b). Although there can be little doubt that the change from zero tension to tension development is rather abrupt, there is ample evidence to suggest that this abrupt change in state is actually preceded by a transitional phase during which mechanical changes occur in the muscle (p. 4). This investigation has been limited to the changes which occur at or about the mechanical threshold because if excitation-contraction coupling is to be explained fully a greater understanding of the events occurring at the mechanical threshold is required.

When a skeletal muscle is stimulated to contract, several changes are known to occur in the muscle. From among these many changes, changes in membrane potential, elasticity ('resistance to stretch'), tension, and oxygen

consumption were investigated. Potassium-induced contractures were employed both because the events occurring during activation proceed more slowly and therefore are more amenable to measurement and experimental manipulation than comparable events following an electrical stimulus and because the intensity of these changes could be varied easily by alterations in the potassium concentration of the solution bathing the muscle. Since there is a considerable body of evidence showing that membrane potential changes are responsible for initiating and controlling the mechanical events during a contraction (Frank, 1958, 1960; Sten-Knudsen, 1954, 1960; Hodgkin and Horowicz, 1960b), it seems reasonable to use K^+ -induced contractures, in which the membrane potential is varied by changes in K^+ -concentrations, as a model for the events occurring during a twitch or tetanus. However, it must be kept clearly in mind that this is only a model and whenever possible comparisons must be made with the results obtained by other workers in studies on contraction.

The picture of the events occurring during the early stages of activation which emerges from the results of the present investigation and from the related results of other workers may be briefly summarized as follows. When the muscle is depolarized to a level below the mechanical threshold there is an increased level of free Ca^{++} in the sarcoplasm. This increase in the level of free Ca^{++} results in the start of activation even though no tension development

occurs. The evidence for the start of activation during this outwardly quiescent period is that the muscle elasticity ('resistance to stretch') is increased and that a contracture produced during this time is increased above control both in rate of tension development and in the maximum tension developed. However, if the exposure to subthreshold potassium concentrations is prolonged, further activation does not proceed to potentiation but rather activation becomes reversed as indicated by a loss in the increase in elasticity and an actual inhibition of subsequent contractures elicited by exposure to higher potassium concentrations. Concurrent in time with this reversal in the initial stages of activation there is the full development of the increase in oxygen consumption (or the Solandt effect). Presumably the increased level of free Ca^{++} stimulates the active Ca^{++} -uptake by the sarcoplasmic reticulum resulting eventually in a lowering of the free Ca^{++} towards the resting level, i.e. as during relaxation. Thus it is suggested here that the Solandt effect mainly is a reflection of the metabolic events involved in the active uptake of Ca^{++} by the sarcoplasmic reticulum and not the reflection of some form of chemical interaction between the actin and myosin filaments.

The rest of this Discussion Section will be concerned with a consideration of the evidence leading to the above picture of activation and a more detailed description of events occurring during activation.

V(i). Resistance to stretch.

One of the first events found to occur following stimulation of a striated muscle is an increase in elasticity or resistance to stretch. This change is observed during the mechanical latent period (Hill, 1949). In the present study it was observed that the muscle fibers show an increase in the resistance to stretch without observable tension development when the fibers are briefly exposed to sub-mechanical threshold potassium concentrations (Figs. 9, 10, and 11). Considering that during the mechanical latent period preceding the twitch the resistance to stretch and the torsional rigidity of the muscle increase (Hill, 1949; Sten-Knudsen, 1953) the finding of an increased resistance to stretch below or at the mechanical threshold is not surprising although the increase in the resistance to stretch and torsional rigidity follows the action potential, i.e. a depolarization well above the mechanical threshold. The relationship which was observed between the resistance to stretch below the mechanical threshold and the time of exposure in the elevated K^+ -Ringer's solution (Figs. 9, 10) appears to correlate with the observation that contracture potentiation was greatest with brief pre-exposures of about 30 sec at which time the resistance to stretch also appeared to be at a maximum; with more prolonged exposures to elevated potassium the resistance to stretch declined. Thus, at potassium concentrations close to the mechanical threshold

the activation process starts but does not continue indefinitely.

Within the limits of the experimental techniques employed only an increase in the resistance to stretch was seen when using potassium concentrations below the mechanical threshold. It is, of course, possible that microscopical observation would uncover sliding of the filaments. However, Costantin (1968) microscopically observed the mechanical threshold during voltage clamp experiments on Rana temporaria sartori and found that in general mechanical activity started at a membrane potential corresponding to about 20 mM K^+ , i.e. the 'average' potassium concentration at which the mechanical threshold was found to occur in experiments reported here and by Hodgkin and Horowicz (1960b). Also, the fact that there appears to be a relationship between the potassium concentration and the resistance to stretch which does not extend above the mechanical threshold (Fig. 11) would indicate that the initial activation process is probably limited to a locking of the actin and myosin filaments only. There is no direct biochemical evidence for locking of the actin and myosin filaments; the curves relating superprecipitation and the ATP or Ca^{++} concentrations are sigmoid in shape (e.g. Levy and Ryan, 1966; Edwards, Lorković and Weber, 1966). It could be argued that since the flattening of the sigmoid curve at high ATP or high Ca^{++} concentrations is the result of no further sliding between

the filaments, the bottom flat portion (low ATP or Ca^{++}) represents the same; however, this is far from conclusive evidence. Nevertheless, it may be envisaged that at very low levels of activation the number of sites participating would be so small that any force generated by sliding of the filaments would be balanced by the viscous resistance, the net result being a slight increase in the resistance to stretch.

The time course of the resistance to stretch of submaximal K^+ -contractures was similar to that described by Frank (1965a, b). The resistance to stretch increases slowly during the contracture reaching a maximum near maximum tension and it declines more slowly during the relaxation phase than it increased during tension development (Fig. 4a). It was found that the resistance to stretch early in the submaximal contracture could be increased with short pre-exposures to submechanical threshold concentrations of potassium (Figs. 12, 13, and 14). This increase over the control could be observed only in the first few seconds of the potentiated contracture and it was considerably more than the sum of the resistance to stretch below the mechanical threshold and the control resistance to stretch. Also, this increase in the resistance to stretch over the control became less with longer periods of pre-exposure and eventually the resistance to stretch became less than control (Fig. 19). Thus the

modifications of the resistance to stretch early in a potentiated submaximal contracture followed a pattern similar to that seen in the resistance to stretch changes below the mechanical threshold and that seen in tension changes of subsequent contractures. The time-course of the resistance to stretch of a maximal K^+ -contracture was quite similar to that of the submaximal contracture (Fig. 3b), i.e., it does not reach maximum until the tension developed in near maximum, however, the resistance to stretch during relaxation decreases more rapidly than it does during relaxation of a submaximal K^+ -contracture. The finding that the maximum resistance to stretch of a maximal K^+ -contracture is not reached till near maximum tension is at variance with the observations of Frank (1965b). The possible reasons for this discrepancy have already been given (p. 75). If the resistance to stretch is a measure of the active state (Gasser and Hill, 1924; Hill, 1949) then the observations on the maximal K^+ -contracture suggest that the active state is not maximally developed at the beginning of maximal contracture which is quite similar to that observed by Jewell and Wilkie (1958) for the tetanus. These workers found that redevelopment of tension following a quick release during a tetanus was faster than the initial development of tension. This implies that the active state in a twitch is not maximal at the start of tension development since the initial rate of tension development was the same for

both twitch and tetanus. Thus, a finite time is required for the active state to develop fully contrary to Hill's interpretation (Hill, 1949); who it appears, ignored his own evidence (Hill, 1949, Figs, 3, 4, and 6). Another observation although peripheral to this thesis was the increased rate of tension development following the stretch whether applied during tension development or relaxation (Fig. 5); the transient increase in tension development following a quick stretch during relaxation was also reported by Frank (1965b). It is plausible that a sudden length change affects the state of activation of the muscle, the observation shown here is similar to that reported by Armstrong, Huxley, and Julian (1966) who showed that a sudden small increase in length during an isometric tetanus gave rise to a small delayed rise of tension and conversely a small decrease in length led to a small delayed fall in tension (also, Civan and Podolsky, 1966); the delay between the conclusion of the length change and the beginning of the transient was about 10 to 15 msec. In Fig. 5, traces C and D a small delay is also discernable (see also Frank, 1965b, Fig. 8D).

V(ii). Effects of exposing frog striated muscle to elevated potassium concentrations on subsequent K^+ -induced contractures.

It was found in this study that submaximal K^+ -contractures could be potentiated by pretreating frog striated

muscle for 10 to 30 sec with Ringer's solution in which the potassium concentration is elevated to just below the mechanical threshold prior to the addition of, say, 25 mM K^+ -Ringer's solution (Figs. 6, 7, and 8). The potentiation was characterized by an increase in the rate of tension development, a small increase in the maximum tension developed, an earlier and faster decline in tension, and an increase of the early resistance to stretch as compared to control (Figs, 6, 7, 8, 12, 13, 14).

When the period of exposure to below mechanical threshold concentrations of potassium was prolonged beyond 30 to 60 sec the potentiation of a subsequent contracture diminished and was replaced by inhibition (Figs. 15 - 21). It should be pointed out, however, that since the $[K^+] \times [Cl^-]$ product was not held constant in these experiments, the membrane potential would be expected to show a slow constant drift (Hodgkin and Horowicz, 1959); this drift would not amount to more than a 5 to 10 mV further depolarization over 60 - 120 sec. Since this drift would be in the direction of the mechanical threshold it unlikely to account for the inhibition. Moreover, inhibition or refractoriness also was observed following high K^+ exposures by Hodgkin and Horowicz (1960b) when the $[K^+] \times [Cl^-]$ product was held constant and hence no drift of the membrane potential occurred. Therefore, the inhibition observed following prolonged exposures to potassium below the mechanical

threshold must be ascribed to process(es) other than a change or continuing change of the membrane potential per se. On the other hand, if the muscle was returned to Ringer's solution after a short exposure to, say, 17.5 mM K^+ contracture potentiation persisted for a considerable time (Figs. 22, 23, table 1). Foulks, Pacey, and Perry (1965) observed a similar effect. They found that exposing a frog's toe muscle to 10 to 15 mM K^+ for 2 to 3 min could enhance a chloride withdrawal contracture, and this enhancement persisted when the muscles were returned to Ringer's solution for 1 to 2 min before a chloride withdrawal contracture was elicited. In the present investigation additional details have been observed, namely, potentiation apparantly diminished in the first 15 sec or so after washout, i.e. after return to Ringer's solution, following which potentiation increased (Fig. 23). Also, potentiation is more likely to persist during washout following a short, 15 to 45 sec, exposure to 17.5 mM K^+ than following a long, 135 sec, exposure (table 1). It is tempting to suggest that following the removal of 17.5 mM K^+ two opposing processes operate within the muscle, (1) the refractoriness which is dissipated within 1 min (Hodgkin and Horowicz, 1960b), and (2) the potentiating process which declines at a slower rate than (1). Then the 'dip' in the potentiation of tension at 15 sec washout (Fig. 23) would be due to the refractoriness predominating whereas

by 45 sec washout refractoriness would be gone or nearly gone leaving the potentiating process. The effect is independent of the membrane potential as was the refractoriness effect observed by Hodgkin and Horowicz (1960b) who found that although the membrane potential had returned to resting values the action potential could not be generated. Holloszy and Narahara (1967a, b) reported that the increase in permeability to sugar of frog's sartorius which accompanies depolarization, electrically induced or by increasing the external potassium concentration, does not cease when the muscle is no longer stimulated or depolarized but the increased permeability may persist for several hours. Also, the influx of sugar can be increased by NO_3^- and by increasing the external Ca^{++} concentration. Whether the persistent increase in sugar permeability is related to the mechanism by which potentiation persists is, of course, a matter of speculation, however, it underscores the findings that return to the resting membrane potential does by no means imply a return to a resting membrane.

V(iii). Temporal sequence of the changes occurring during and following exposure to submechanical threshold concentrations of potassium.

For the sake of convenience in making comparisons, several of the measurements made in this study have been scaled to common time bases and drawn diagrammatically in

Figs. 31, 32, and 33.

Some of the changes observed under conditions for obtaining contracture potentiation are shown in Fig. 31. From the oxygen consumption measurements it is evident that the potentiating effect is not the result of increased oxidative metabolism since potentiation is obtained before there is any increase in the oxygen consumption. It also can be seen that the increased rate of tension development is not due to an increased rate of depolarization. In fact, the rate of depolarization in the tension inducing step, i.e. increasing K^+ from e.g. 17.5 to 25 mM is slower than the initial rate of depolarization to just below the mechanical threshold, i.e. the step 2.5 to 17.5 mM K^+ , and it is also slower than in the control where the step change is from 2.5 to 25 mM K^+ . It can therefore be concluded that the manner in which the membrane potential is changed as such bears no relationship to the subsequent time course of tension development but rather the change in the membrane potential from fully polarized to just below the mechanical threshold causes a change to be transmitted into the muscle which in turn leads to a facilitation of the response to a subsequent further depolarization above the mechanical threshold. It also should be noted that the pretreatment did not increase the subsequent amount of depolarization (Figs. 26, 27) over the control depolarization by the same final potassium concentration.

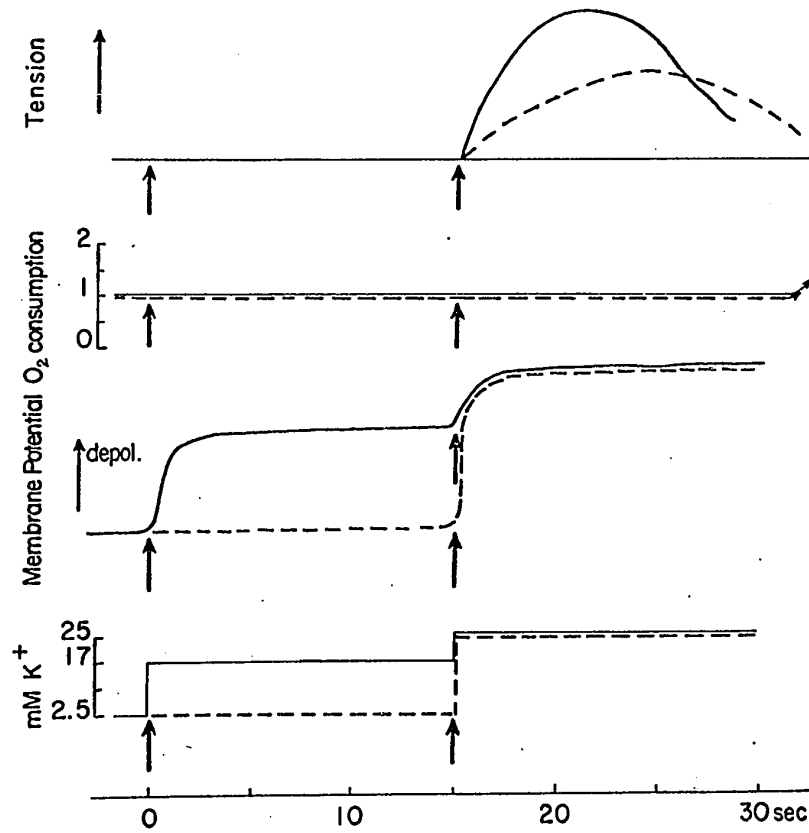


Fig.31. Comparison of tension, oxygen consumption, membrane potential, and potassium concentration changes in producing potentiation of a submaximal contracture. Solid traces represent treatments, and dashed traces the controls.

Another comparison of some changes observed during and following somewhat longer exposures to a sub-mechanical threshold potassium concentration is presented in Fig. 32. One of the most interesting findings in this study was that both the resistance to stretch below the mechanical threshold and the contracture potentiation increase before the oxygen consumption starts to increase, both of these effects reach a maximum at about the time that the oxygen consumption starts to increase, thereafter both decline as the oxygen consumption increases, and finally that the contracture potentiation changes to contracture inhibition at about the time that the increase in oxygen consumption reaches a maximum. Thus it would appear that the increase in resting metabolism produced by exposing a frog's skeletal muscle to elevated submechanical threshold potassium concentrations cannot be responsible for the contracture potentiating effects observed under similar experimental conditions. Indeed the increase in oxygen consumption rather seems to be associated with a secondary inhibitory effect on contractile activity.

It is in the work of Bianchi and Shanes (1959), Weiss and Bianchi (1965), and Novotny and Vyskocil (1966), on Ca^{45} movements during K^+ -induced depolarization that at least a partial answer may be found to the mechanism by which potentiation was produced. Of particular interest to this discussion was the observation that above a potassium

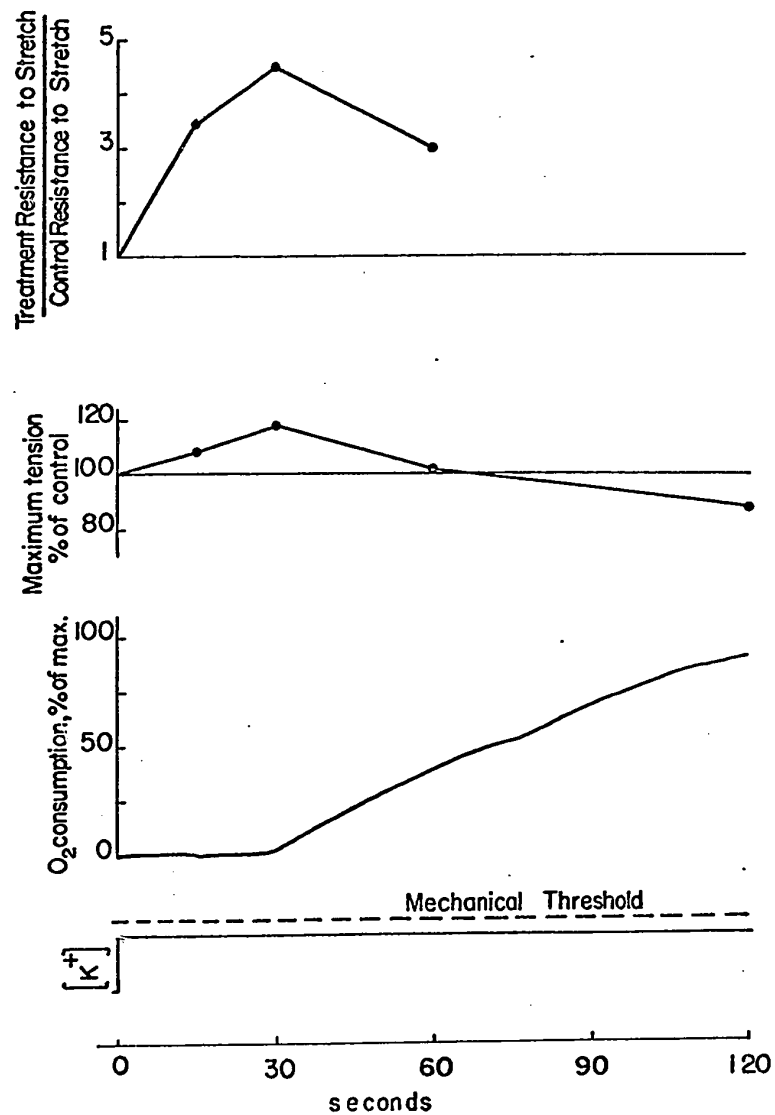


Fig. 32. Comparison of some of the effects of exposing frog's skeletal muscle to just submechanical threshold potassium concentrations. Resistance to stretch data taken from Fig. 10; contracture potentiation from Fig. 15; oxygen consumption from Fig. 30. Curves modified to fit a common time base.

concentration of 1.6 mM the Ca^{45} influx increases as a function of the degree of depolarization, and also that the depolarization induced influx is very brief with K^+ -depolarization above 35 to 40 mM but is prolonged at lower potassium concentrations while at submechanical threshold concentrations of potassium it may last for an hour or more. Although as is generally accepted (Sandow, 1965), the amount of Ca^{++} which moves into the muscle is insufficient to initiate a mechanical response, the influx may be part of a process which causes a further release of Ca^{++} internally. It also has been shown that the Ca^{++} influx, a subsequent increase in Ca^{++} -exchangeability (i.e. both increased influx and efflux), and the increase in metabolism (the Solandt effect) are all susceptible in a similar manner to the various agents and procedures by which events above the mechanical threshold may be affected. So that, if the mechanical threshold is lowered by, for example NO_3^- , the threshold for the increase in metabolism is likewise lowered (Hill and Howarth, 1957), as well, the Ca^{45} influx is increased (Weiss and Bianchi, 1965). Similarly, if the mechanical threshold is raised by increasing the external Ca^{++} concentration, the threshold for the Solandt effect is increased (e.g. Solandt, 1936) and also the increase in Ca^{45} influx or Ca^{++} -exchangeability occurs only with greater depolarization. In general therefore, the events which lead to the Solandt effect are closely tied

to the mechanical threshold and Ca^{++} movements. It is possible that as a result of depolarization below the mechanical threshold there occurs an influx of Ca^{++} and probably a release of Ca^{++} from the lateral cisternae (Winegrad, 1967) which results in an increase in the sarcoplasmic free Ca^{++} concentration to a level which is insufficient to initiate tension development. As a result of a further depolarization to above the mechanical threshold an additional amount of Ca^{++} is released. Thus, in its simplest form, when the muscle is depolarized by increasing the external potassium concentration from 2.5 to 25 mM a contracture results because an amount A of Ca^{++} is released. When the muscle is depolarized by increasing the potassium concentration from 2.5 to 17 mM an amount B of Ca^{++} is released, a further depolarization to 25 mM K^+ causes again a release of an amount A of Ca^{++} , in total therefore, A + B. In this scheme it is assumed that the amount of Ca^{++} released is a function of the membrane potential and independent of how the membrane potential is reached. The rate of tension development and the final tension achieved appear to be critically dependent on the rate of release and the amount of Ca^{++} released, as was shown by Ridgway and Ashley (1967) and Ashley and Ridgway (1968) on barnacle muscle.

When the exposure period to below mechanical concentrations of potassium is prolonged beyond 30 to 60 sec the resistance to stretch of the preparation kept in this

solution declined and also the potentiation of subsequent contractures diminished, disappeared and was replaced by inhibition as the duration of the exposure period increased (Fig. 32). As mentioned above, the decline of potentiation and its subsequent inhibition occurs at about the time when the oxygen consumption reaches its maximum increase (Fig. 32). In considering muscle oxygen consumption increases and mechanical activity it should be pointed out that one generally measures a change after the fact, i.e. after at least the initial force development. Cytochrome b does not start to become oxidized at an increased rate until tension has reached maximum in a twitch (Jöbsis, 1959, 1963), and following a series of twitches oxygen consumption rises fairly slowly (e.g. Baskin and Galuzzi, 1966). Thus the increase in metabolism observed here might be considered a result of the process(es) attempting to return the muscle to resting conditions. It is possible that the sarcoplasmic reticulum presented by an increased and continuous Ca^{++} load responds by increasing its Ca^{++} -pumping activity. The initial increase in free internal Ca^{++} is small and the sarcoplasmic reticulum initially responds slowly (Weber, Herz, and Reiss, 1966). The steady state level of Ca^{++} -uptake, i.e. when the Ca^{++} -load is continuously elevated, however, is much higher; that is, the steady state curve relating Ca^{++} -uptake to the log concentration of Ca^{++} is situated to the left of the initial rate of Ca^{++} -uptake

curve (Weber, Herz, and Reiss, 1966). On the basis of the above evidence it may be reasonable to suggest that when the resistance to stretch below the mechanical threshold declines and when potentiation ceases, these changes occur as a result of an adjustment by the sarcoplasmic reticulum to the increased Ca^{++} presentation. A new steady state condition is set up which operates with a higher metabolic rate. In a sense this adjustment may be considered analogous to what happens during relaxation with the qualification that during relaxation the muscle returns again to its original resting level.

Thus, it is proposed here that prolonged partial depolarization is accompanied by a continued release of Ca^{++} into the sarcoplasm. This in turn stimulates active Ca^{++} -uptake resulting in the splitting of ATP, increased ADP and thus an increase in metabolism, the Ca^{++} level would be reduced from the initial, up to 30 sec, increase in free Ca^{++} resulting in a diminished potentiation of a subsequent contracture. With very long pre-exposures the sarcoplasmic reticulum has reached its steady level of Ca^{++} -uptake and a further depolarization leads to an inhibited contracture because the Ca^{++} -uptake mechanism is already operating at a higher rate rather than responding to an increase in free Ca^{++} as is the case during a normal or potentiated contracture. Such a process might also account for the refractoriness which follows a K^{+} -induced contracture.

However, it must be pointed out that other possibilities exist and further experimentation is required to test the mechanism suggested above.

V(iv). Washout effect.

The contracture potentiation which persists for several seconds following the return of the muscle to Ringer's solution after a brief exposure to submechanical threshold potassium concentrations (or the Washout effect, Figs. 22, 23), was an interesting finding. As can be seen in Fig. 33 membrane potential changes cannot be responsible for this effect.

The relation of oxygen consumption changes to the Washout effect is not so clear cut. Thus, it appeared that the Washout effect was larger the briefer the duration of the initial exposure to 17.5 mM K^+ (Fig. 23) during which it would be expected that there was either no increase in oxygen consumption or only a small increase (Fig. 33). Interestingly, there was a large drop in potentiation during the first 15 sec of washout (Fig. 23). Considering the slow decline in the oxygen consumption following reexposure of the muscle to Ringer's solution (Fig. 28) it is possible that the active uptake of Ca^{++} by the sarcoplasmic reticulum and recovery processes continue during this period resulting in a continued reduction of free Ca^{++} and thus a decrease in contracture potentiation. Then as the exposure to Ringer's continues and the oxygen consumption

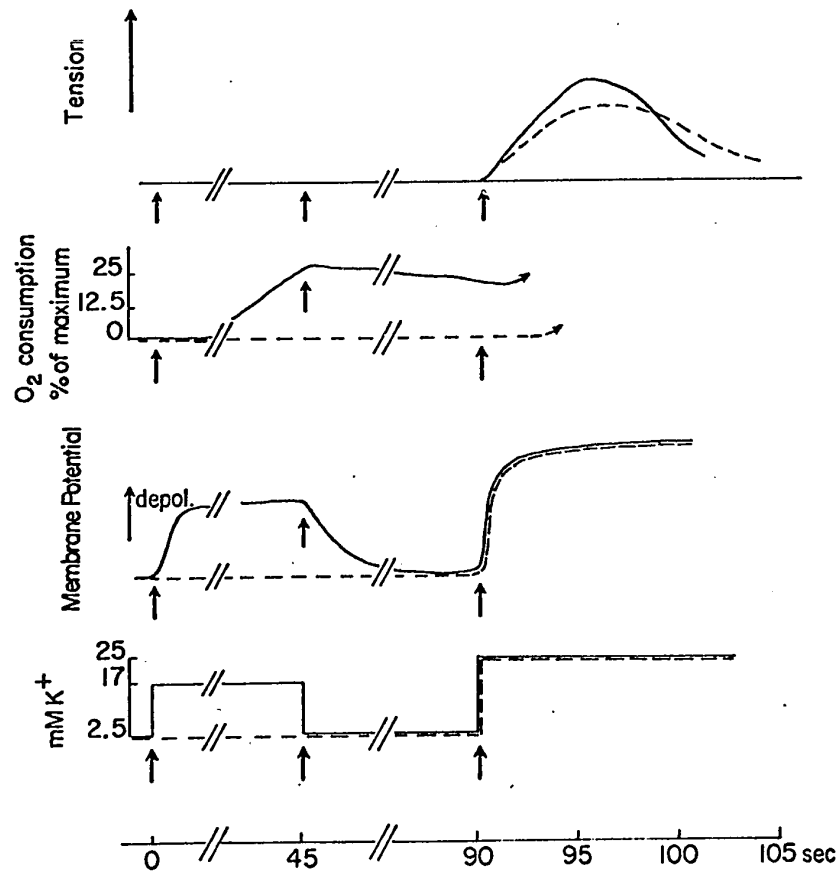


Fig.33. Comparison of tension, membrane potential, oxygen consumption, and potassium concentration changes in the Washout effect. Treatments are presented by solid curves and controls by dashed curves.

further declines this would result in an free Ca^{++} level still above the resting level and potentiation increases once more. Whereas this hypothetical mechanism would fit in with the interpretation proposed above, evidence supporting this mechanism is lacking and it can be considered little more than an interesting speculation until further work is done on this problem. For the purpose of this study, the one thing clearly demonstrated by the Washout effect is that the contracture potentiating effect of brief exposures of frog's striated muscle to submechanical threshold concentrations of potassium is completely independent of membrane potential changes during or immediately prior to exposure to suprathreshold potassium concentrations. And it would appear that the potentiation is due to alterations occurring at a later stage in the excitation-contraction coupling process.

V(v). Effects of caffeine and isotonic sucrose.

The few experiments done using caffeine in sub-threshold concentrations and isotonic sucrose as pre-exposure solutions confirmed the general nature of the potentiating effect (Figs. 24, 25), namely, a stimulus which affects Ca^{++} movements and consequently metabolism may be utilized to potentiate a subsequent submaximal K^{+} -contracture. The effects of caffeine on metabolism and its action in potentiating the twitch are well known (e.g. Hartree and Hill, 1924; Saslow, 1936a, b, 1937; for reviews

see Gasser, 1930; Sandow, 1965). Of direct interest to this study is the caffeine-induced increase in Ca^{++} -exchangeability (Bianchi, 1961; Novotny and Vyskocil, 1966). The increase in Ca^{++} -exchangeability and consequent increase in metabolism produced by caffeine can be inhibited by local anesthetics (Feinstein, 1963; Novotny and Vyskocil, 1966). These observations also suggest that there is a causal relation between Ca^{++} movements and metabolism. The effects of isotonic sucrose on Ca^{++} movements are not known, however, since isotonic sucrose can cause contracture development through depolarization due to chloride withdrawal and since the isotonic sucrose-induced increase in metabolism can be inhibited under certain conditions by local anesthetics, it is reasonable to assume that even in the fortuitous circumstance that isotonic sucrose did not cause a chloride withdrawal contracture the potentiation observed (Fig. 25, table 3) was by the same general mechanism as that observed with subthreshold caffeine and a pre-exposure to, say 17 mM K^+ .

V(vi). Recommendations.

A number of observations made in the course of this study are sufficiently interesting to deserve further investigation, the main ones may be summarized as follows. (1) The resistance to stretch below the mechanical threshold, further investigation should include microscopical, possibly

cinematographical, observation of the fibers when the potassium concentration is raised, this would settle the question whether sliding occurs. Also, quick release experiments and stretches at various speeds should be done in conjunction with simultaneous length measurements. (2) The transient increase in tension development following a quick stretch (Fig. 5) and the redevelopment of tension following a quick stretch during relaxation should be pursued with stretches applied at various speeds and simultaneous length measurements, as well the converse, i.e. does the rate of tension development decrease following release, should be looked into. (3) Ideally internal Ca^{++} concentration changes following initial elevation of the potassium concentration and during the Solandt effect should be followed. Perhaps the luminescent Ca^{++} -sensitive protein aequorin and the barnacle muscle fiber could be used. (4) The Washout effect should be pursued further in relation to the properties of the membrane and Ca^{++} movements, this would necessarily extend to an investigation of the refractoriness observed by Hodgkin and Horowicz (1960b). It may also be of interest to see whether the resistance to stretch of the muscle changes during the washout period.

Summary and Conclusions

1. The nature of the Solandt effect was further investigated with the object of determining its relation to events known to occur at the mechanical threshold, i.e. when excitation-contraction coupling takes place.
2. Whereas by definition below the mechanical threshold no tension development occurs, it was found by applying quick stretches to the muscle that the resistance to stretch can increase up to about 4-5 fold over that of the resting muscle when the muscle was exposed to potassium concentrations which approached the mechanical threshold.
3. A hyperbolic relationship exists between the resistance to stretch and the potassium concentrations below the mechanical threshold. It appeared that this relationship is restricted to below the mechanical threshold.
4. The findings of an increased resistance to stretch when the muscle was exposed to concentrations of potassium below the mechanical threshold, and that following the stretch the tension fell back to a steady level indicated that a slow sliding of the filaments which could be taken up by the series elastic elements without the expression of tension, probably does not take place but that the filaments interact or lock without sliding. The locking of the filaments,

it is suggested, is part of the process of activation normally taking place during the mechanical latent period between action potential and tension development or shortening.

5. A submaximal K^+ -contracture could be potentiated by a brief pre-exposure to potassium in concentrations below but approaching the mechanical threshold, i.e. concentrations of potassium which will induce the Solandt effect.
6. The period of pre-exposure to 'Solandt' concentrations of potassium which will result in maximum potentiation of a subsequent submaximal K^+ -contracture was variable. In general peak potentiation was achieved following a 30 sec pre-exposure period but it could also occur after 2 min of pre-exposure. When the pre-exposure period was prolonged the potentiating effects were abolished and inhibition of a subsequent contracture resulted.
7. The greatest potentiation with the shortest pre-exposure period was achieved when the potassium concentration was just below the mechanical threshold, i.e. at concentrations at which the Solandt effect is also maximal. Lower potassium concentrations used during pre-exposure yielded a smaller potentiating effects and there was less tendency for depression of the subsequent contracture to occur with prolonged pre-exposures.

8. The resistance to stretch within the first 5 sec of the potentiated contracture was about twice as large than that observed for the control contracture elicited by the same final concentration of potassium.
9. Potentiation was still observed when following the pre-exposure period to elevated but below mechanical threshold potassium the muscle was returned to normal Ringer's solution and then exposed to a contracture concentration of potassium (Washout effect). During the washout period the potentiation initially decreased and then increased to remain for about 2 minutes if the initial exposure period to elevated potassium was held to 15-45 sec.
10. Potentiation was also observed when the muscle was pre-exposed to a subthreshold concentration of caffeine, to isotonic sucrose or isotonic sucrose with a small amount of electrolytes. Higher concentrations of electrolytes in isotonic sucrose resulted, if anything, in an inhibition of a subsequent contracture. The latter solutions have been reported to prevent or reverse the increase in metabolism due to exposure to isotonic sucrose. Caffeine in concentrations insufficient to cause tension development can also induce an increase in metabolism.
11. Potentiation did not appear to be a result of an increased

rate of depolarization. In the Washout effect potentiation was independent of the membrane potential.

12. Potentiating effects do not appear to be a consequence of the increase in metabolism (the Solandt effect) resulting from the exposure to elevated potassium in the bathing medium. Following exposure to elevated potassium concentrations, toe muscles of the frog showed a rapid increase in oxygen consumption after a delay of about 30 sec. Thus, peak potentiation occurred when the oxygen consumption has not or hardly increased, inhibition is definitely associated with an increased oxygen consumption. It is suggested, that the potentiating effects can be obtained during that period when the internal free Ca^{++} rises and the sarcoplasmic reticulum has not or hardly reacted to the increased Ca^{++} load.
13. The overall findings suggest that the Solandt effect, i.e. the increase in metabolism, is the second step of a process initiated by exposure of the muscle to potassium just below the mechanical threshold. The first step, namely an increase in internal free Ca^{++} , is the trigger for the increase in metabolism. This first step can be manifested mechanically as potentiation of a subsequent contracture if the muscle is further depolarized during that first step. The duration of the first step may be variable but it can be

determined in a muscle preparation. Once the duration is known the experimenter could manipulate the muscle during that period which is probably equivalent to the mechanical latent period between electrical stimulus and twitch. Hence, there is now a period of 30 seconds to 2 minutes available to study excitation-contraction coupling proper.

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