*J. Physiol.* (1963), **169**, *pp.* 353–372
*With* 11 *text-figures*
*Printed in Great Britain*



# STUDIES ON THE MICRO-INJECTION OF VARIOUS SUBSTANCES INTO CRAB MUSCLE FIBRES

BY P. C. CALDWELL AND G. WALSTER

*From the Department of Zoology, University of Bristol, the Laboratory of the Marine Biological Association of the U.K., Plymouth and Plymouth College of Technology*

(*Received* 13 *February* 1963)

The squid giant axon has, on account of its size, made possible a large number of experiments which would have been extremely difficult or impossible to carry out with the smaller axons from other species (see, for example, Hodgkin, 1958). The leg muscles of crabs have been shown to consist of large fibres (Fatt & Katz, 1953), the diameters of which fall in the same range as those of squid giant axons. Hitherto it has been found difficult to work with these fibres in the same way as with squid axons because they are relatively short, and when bathed in crab saline deteriorate in regions in which they have been damaged. The largest fibres obtained so far from a British species of crab are those from the Spider Crab (*Maia squinado*), and work on internal pH in which single-fibre preparations from *Maia* were used has been described previously (Caldwell, 1958).

In the work on internal pH it was necessary to damage the fibre at the point of entry of the electrodes, and once this had been done retraction and clotting started at the point of entry and spread gradually down the fibre. This meant that it was not possible to carry out experiments, involving the longitudinal insertion of micro-electrodes, which lasted more than about 15 min. In this paper a way of overcoming this difficulty is described. This involves cannulation of a single *Maia* fibre, and experiments lasting 2–3 hr and involving the longitudinal insertion of micro-electrodes and micro-injectors have been carried out.

A number of substances have been micro-injected into *Maia* muscle fibres and their effects on the contractile mechanism studied. A brief account of some of this work has already appeared in the published proceedings of a Ciba Foundation Study Group (Caldwell, 1960) and of a meeting of the Deutsche Physiologische Gesellschaft (Caldwell, 1961). The cannulated preparation has been demonstrated to the Society (Caldwell & Walster, 1961).

## METHODS

*Dissection and cannulation of the fibres.* Single fibres, normally 0·9–1·8 mm wide in the direction of maximum width and 0·5–1·2 mm thick, were dissected from the leg muscles of *Maia squinado*. Usually a fibre from the flexor muscle of the carpopodite was used. The fibres were exposed by removal of the extensor muscle, the nerve and as much of the shell as possible. The fibres on one side of the flexor muscle were then cut and a further quantity of the shell removed until the preparation shown diagrammatically in Fig. 1*a* was obtained.

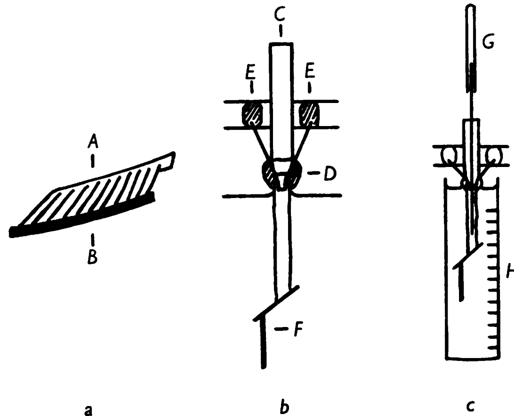


Fig. 1. Stages in the dissection and setting up of the *Maia* single muscle fibre preparation for micro-injection studies. *a*, Muscle, with most of the shell and fibres from one side removed, from which single fibres can be dissected: *A*, apodeme; *B*, shell. *b*, Diagram of the cannulated fibre set up for the insertion of an electrode or micro-injector: *C*, cannula attached to the Perspex stand with plasticine; *D*, paraffin/petroleum jelly mixture; *E*, plasticine with which the threads from the fibre are attached to the Perspex stand; *F*, platinum weight attached to the piece of apodeme at the end of the fibre. *c*, Diagram of a cannulated fibre, with a micro-injector, *G*, inserted, surrounded by a small graduated vessel, *H*, containing bathing solution. The injection is carried out and the injector withdrawn from the fibre within 2 sec. The progress of any resultant isotonic contraction is followed by observation of the position of the bottom of the platinum weight in relation to the graduations on the vessel.

The fibre was freed from its surroundings by carefully cutting the connexions between it and adjacent fibres. The section of the apodeme to which the fibre was attached was cut out and when as much of the fibre as possible had been freed from its surroundings it was cut from the shell at the point at which it joined the shell. After further removal of adhering material a looped bit of thread was passed round the cut end of the fibre and tightened so that the loop just gripped the fibre at the cut end which was by now slightly swollen. The fibre was next lifted by means of the thread and the cut end eased on to the tip of a cannula, supported vertically with plasticine on the edge of a Perspex stand, so that the tip of the cannula went about 1 mm into the fibre. The fibre was suspended in position by pressing the ends of the thread into the plasticine used for supporting the cannula. The fibre, suspended in air, was then gently pulled up with the thread until the cannula tip was about 0·3 cm from the cut end. When the fibre was finally in position the ends of the thread were pressed firmly into the plasticine. The region of the cut end was dabbed liberally with a

mixture of liquid paraffin and petroleum jelly and then a small platinum weight (weight in the region of 90 mg) was tied to the apodeme. The fibre was then immersed in a bathing solution (usually crab saline) up to about the position of the tip of the cannula, the final arrangement being that shown in Fig. 1*b*. Usually there was 1–2 cm of fibre between the cannula tip and the point of attachment of the fibre to the apodeme. It seems that entry of crab saline through the point at which the membrane has been injured is the cause of the deterioration which normally takes place when injured crab muscle fibres are immersed in crab saline. The coating of the cut end of the fibre with the paraffin/petroleum jelly mixture appears to delay this deterioration for long periods by preventing contact of the injured part with the crab saline.

*Insertion of micro-electrodes and micro-injectors.* Glass capillary micro-electrodes of about 100 $\mu$ diameter and filled with 0·6 M-KCl were used for the measurement of resting potentials. In the micro-injections, micro-injectors similar to those described by Hodgkin & Keynes (1956) were used. A slight modification was introduced during the course of the work in that the diameter of the glass tube to which the inner capillary of the injector was attached was reduced to about 2 mm and filled with a silicone (Silicone Fluid M.S. 200/1000 cs.) instead of with mercury. The narrowness of the glass tube relative to the inner capillary resulted in a certain amount of mechanical support for the latter and enabled the outer capillary to be moved over a much greater length of it (about 5–6 cm) without it bending and breaking, thus making micro-injections over lengths up to about 5 cm possible. It was found that the silicone provided a more satisfactory seal at the point at which the open end of the outer capillary moves over the inner capillary and the fact that the silicone is transparent makes it possible to check that the parts of the micro-injector are functioning properly.

The micro-electrodes and micro-injectors were inserted longitudinally down the fibres through the cannula, the technique used being based on that worked out by Hodgkin & Huxley (1945) for work on squid giant axons. The experimental arrangement used was very similar to that used for the insertion of micro-glass pH electrodes into cannulated squid giant axons (Caldwell, 1958, Fig. 1). The micro-electrode or micro-injector was kept fixed while the whole platform to which the cannula, fibre, beaker of bathing fluid, reference electrode, etc., were attached was moved upward. As in previous work on crab muscle fibres (Caldwell, 1954) it was found relatively easy to push the electrode or injector down the fibre, since it was, to a large extent, guided by the internal structure of the fibre.

*Technique for the study of the effect of injected substances on the contractile mechanism.* In order to study the effects of the micro-injection of various substances on the contractile mechanism, the fibre was bathed in a solution contained in a small graduated vessel, as shown in Fig. 1*c*. The contents of the micro-injector were injected over 0·5–1·0 cm of the fibre as rapidly as possible and the injector was then withdrawn by rapidly lowering the platform. These operations were usually completed in about 1–2 sec. The changes in the length of the fibre were then followed by observation of the position of the bottom of the platinum weight, attached to the fibre, relative to the graduations on the side of the small vessel containing the bathing solution. The contractions studied were therefore all isotonic. No attempt has been made so far to study isometric contractions and to measure tension development, on account of the ease with which the fibres pull themselves off the cannula when they develop tension.

The volume of fluid injected was usually in the region of $6–30 \times 10^{-3}$ of that of the part of the fibre into which it was injected, so that if the injected material diffused throughout the injected part of the fibre its concentration would fall to roughly $6–30 \times 10^{-3}$ of that in the injected fluid.

*Substances injected.* Most of the substances injected were obtained from British Drug Houses and were made up in distilled water. The adenosine triphosphoric acid (ATP) and adenosine monophosphate (AMP) were made up from samples supplied by the Sigma Chemical Co. and were injected as the potassium salts. The caffeine was Caffeine B.P.

anhydrous. The pH of the solutions used was in the range 6·4–8·0, most of the solutions being between 6·7 and 7·6. The pH of solutions of non-buffering substances was usually stabilized with a small amount of $KHCO_3$.

*Other technical details.* The crab saline used had the following composition (mM): NaCl, 510·4; KCl, 12·9; $MgCl_2$, 23·6; $CaCl_2$, 11·8; $NaHCO_3$, 2·6. In the KCl crab saline used to depolarize the fibres the NaCl was replaced by KCl and the $NaHCO_3$ by $KHCO_3$. The experiments were done at 15–22° C. The resting potentials were recorded with a Vibron Model 33*b* electrometer. In some of the work with caffeine the output of the electrometer was connected to a recording ammeter so that continuous records of the resting potential over a period could be obtained.

## RESULTS

### *Effects of the micro-injection of various substances on the contractile mechanism of Maia muscle fibres*

Calcium and barium chlorides have been reported previously to cause contraction when introduced into frog muscle fibres by micro-injection or micro-electrophoresis, whereas magnesium, sodium and potassium chlorides have been reported to be without effect (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955). The effects of the micro-injection of solutions of these substances were therefore among the first experiments tried.

Figure 2 shows the results of an experiment in which distilled water and solutions of $CaCl_2$ (10 mM), $MgCl_2$ (10 mM), KCl (10 mM) and NaCl (10 mM) were injected into the same fibre. It will be seen that the $CaCl_2$ caused quite a marked contraction which was followed by relaxation. A very slight contraction was obtained after the first injection of distilled water, but the injections of $MgCl_2$, KCl and NaCl had virtually no effect. A further test with 50 mM-$MgCl_2$, 50 mM-KCl and 50 mM-NaCl showed that at these concentrations these substances did not cause contraction. Slight contractions after the injection of distilled water, like that shown in Fig. 2, were sometimes obtained in other experiments in the course of this work. It is not certain whether this effect was due to the presence of traces of calcium in the distilled water or to some other cause such as the transient hypotonicity produced.

An interesting illustration of the ability of calcium-containing solutions to cause contraction when injected and of the inability of calcium-free potassium chloride solutions to do so is given by the experiment illustrated in Fig. 3 which shows that the internal effects of crab saline and of 0·6 and 3 M-KCl on a *Maia* muscle fibre are the opposite of their external effects. Normally fibres bathed in crab saline remain relaxed unless stimulated electrically or treated with caffeine. The effect of crab saline after micro-injection into the fibre is in sharp contrast to this, since a marked contraction was produced, this contraction being due to the 11·8 mM-$CaCl_2$ in the saline. On the other hand, external application of 0·6 or 3 M-KCl to *Maia* muscle fibres causes a sharp contraction which

arises from the depolarization of the membrane, whereas injection of 0·6 or 3 M-KCl into the fibre failed to cause any contraction. However, when KCl crab saline, which contains 523 mM-KCl and 11·8 mM-$CaCl_2$, was injected a contraction was obtained on account of the calcium present.

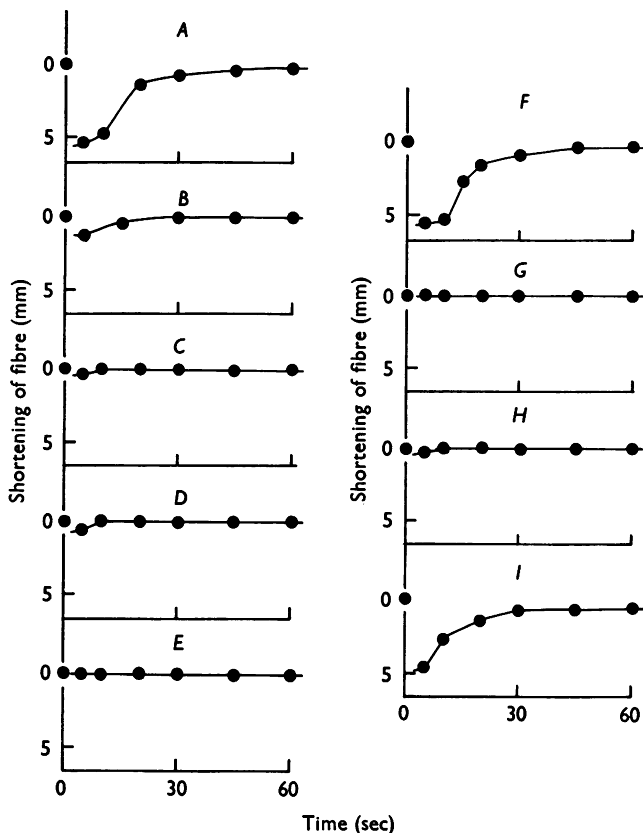


Fig. 2. The effects of the injection of solutions of various substances on a *Maia* muscle fibre bathed in crab saline. The shortening produced at various times after the injection of each solution over a 5 mm length of the fibre is shown. Injections: *A*, *F* and *I*, 10 mM-$CaCl_2$; *B* and *C*, distilled water; *D* and *E*, 10 mM-$MgCl_2$; *G*, 10 mM-KCl; H, 10 mM-NaCl. Average width of relaxed fibre, 1·1 mm. Resting potential throughout −50 (cannula tip) to −53 mV.

The injection of crab saline still produced a contraction after the membrane potential of the fibre had been made positive by immersion in KCl crab saline and the fibre had been allowed to relax from the initial contraction caused by the rise in the external potassium concentration.

In Fig. 4 the results are shown of two further experiments in which the membrane potential of the fibres was removed or made positive by increasing the potassium concentration of the bathing solution, the effects of the

micro-injection of 10 mM-$CaCl_2$ after the change of bathing solution being studied after the fibre had relaxed from the contraction caused by the rise in the external potassium. In one experiment (Fibre 1) the membrane potential was reduced to about zero by immersion of the fibre in 50 % crab saline + 50 % KCl crab saline while in the other (Fibre 2) the membrane potential was reversed to a positive value of 20–22 mV by immersion in

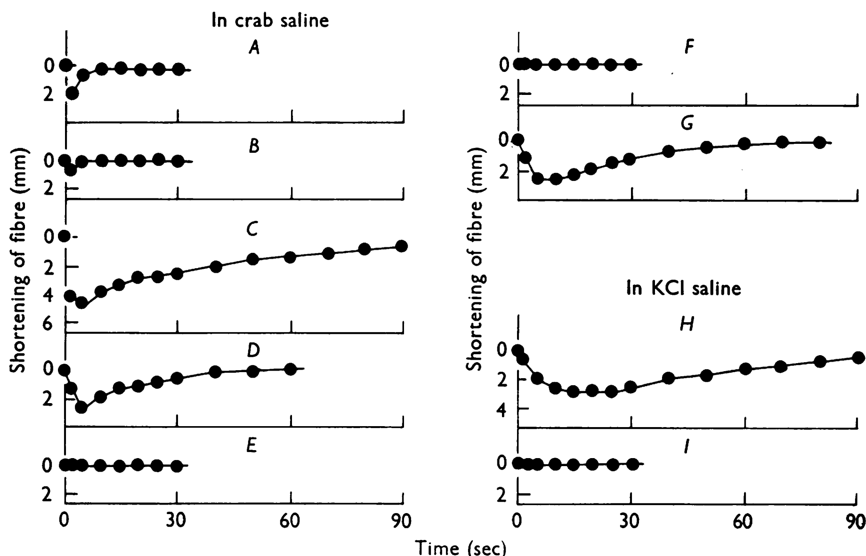


Fig. 3. The effects of the injection of various solutions on a *Maia* muscle fibre. The shortening produced at various times after the injection of each solution over a 5 mm length of the fibre is shown. Injections: *A*, distilled water; *B*, *E* and *I*, 0·6 M-KCl; *C*, *G* and *H*, crab saline (Ca content, 11·8 mM); *D*, KCl crab saline (Ca content, 11·8 mM); *F*, 3 M-KCl. The fibre was bathed in crab saline during injections *A*–*G*; resting potential −45 (cannula tip) to −55 mV. The fibre was then transferred to KCl crab saline, and when it had relaxed from the KCl-induced contraction injections *H* and *I* were carried out; resting potential +17 (cannula tip) to +19 mV. Dimensions of relaxed fibre, 1·5 mm wide × 0·78 mm thick. Temperature, 19° C.

0·6 M-KCl containing 2·5 mM-$NaHCO_3$. It will be seen from Fig. 4 that in spite of the removal or reversal of the resting potential the injection of $CaCl_2$ still caused a contraction, indicating that the calcium-induced contraction is independent of any change in membrane potential.

Figure 5 shows the effects of the injection of gradually increasing amounts of calcium into a fibre. In general the duration of each contraction appears to be related to the amount of calcium injected.

Similar experiments, in which increasing amounts of $SrCl_2$ and $BaCl_2$ were micro-injected, are shown in Figs. 6 and 7. It will be seen that in both cases the duration of each contraction appears to be related to the

amount of strontium or barium injected. Furthermore, the results in Figs. 5, 6, and 7 suggest that at lower concentrations equivalent amounts of calcium, strontium and barium bring about contractions of roughly the

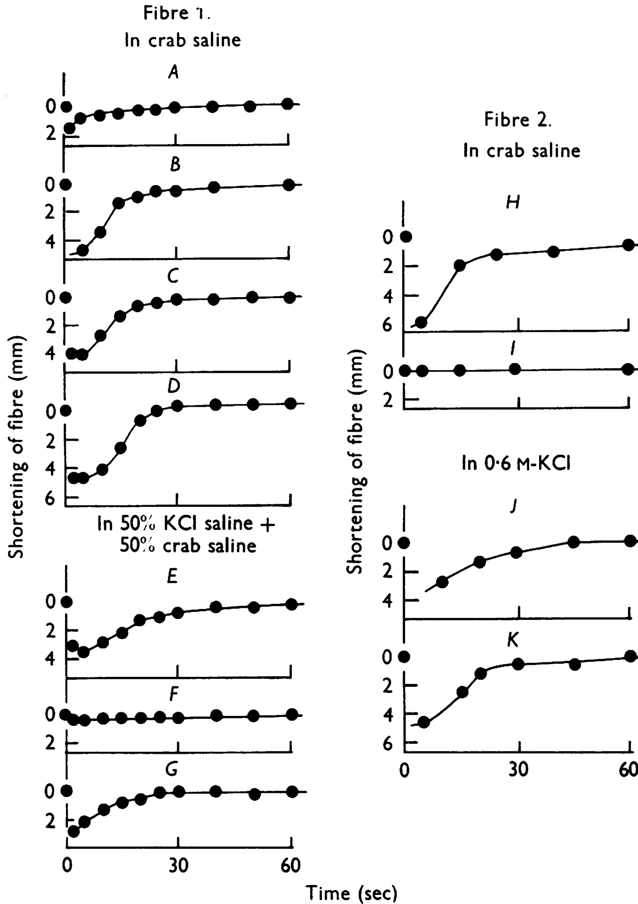


Fig. 4. The effects of the injection of 10 mM-$CaCl_2$ and distilled water on polarized and depolarized *Maia* muscle fibres. The shortening produced at various times after each injection is shown. The solutions were injected over a 5 mm length, with the exception of injection *D* which was over 10 mm. Injections: *A*, *F* and *I*, distilled water; *B–E*, *G*, *H*, *J* and *K*, 10 mM-$CaCl_2$. Injections *A–D*, *H* and *I* were carried out with the fibres in crab saline: resting potentials, Fibre 1, −56 (cannula tip) to −64 mV; Fibre 2, −52 (cannula tip) to −57 mV. The fibres were then immersed in a high-potassium solution and allowed to contract and relax and then injections *E*, *F*, *G*, *J* and *K* were carried out. Fibre 1 was immersed in a mixture of 50 % KCl crab saline and 50 % crab saline (K content of mixture, 269 mM); resting potential −1 to +2 mV (cannula tip); Fibre 2 was immersed in 0·6 M-KCl containing 2·5 mM-$NaHCO_3$; resting potential, +20 to +22 mV (cannula tip). Average width of relaxed fibre: Fibre 1, 1·0 mm, Fibre 2 not measured. Temperature: Fibre 1, 22° C; Fibre 2 not measured.

same duration while at higher concentrations strontium appears to cause shorter contractions than calcium and barium.

The effects of the injection of a number of other substances were tried. No significant contraction was obtained after the injection of solutions of the potassium salts of ATP (0·28 M and 0·38 M ATP containing 0·22 M

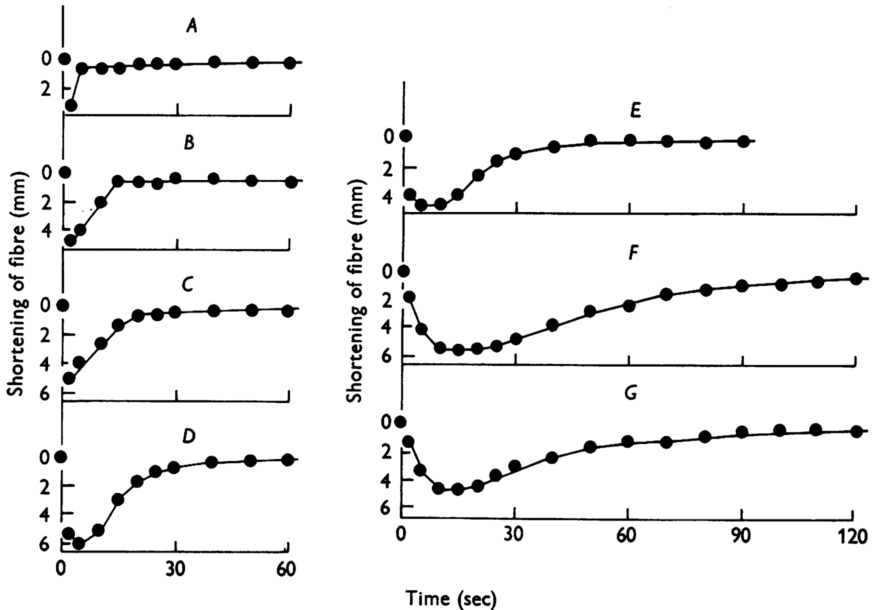


Fig. 5. The effects of the injection of solutions containing various concentrations of $CaCl_2$ and of distilled water on a *Maia* muscle fibre bathed in crab saline. The shortening produced at various times after the injection of each solution over an 8 mm length of the fibre is shown. Injections: *A*, distilled water; *B*, 1 mM-$CaCl_2$; *C*, 5 mM-$CaCl_2$; *D*, 10 mM-$CaCl_2$; *E*, 20 mM-$CaCl_2$; *F*, 50 mM-$CaCl_2$; *G*, 100 mM-$CaCl_2$. Average width of relaxed fibre, 1·0 mm. Initial resting potential, −48 (cannula tip) to −55 mV; final resting potential, −34 to −48 mV (injected region −34 to −48 mV). Temperature, 20–22° C.

and 0·18 M ADP, respectively), the potassium salt of AMP (1·3 M), potassium orthophosphate (1·0 M), the sodium salt of ethylenediamine-tetra-acetic acid (EDTA) (0·2 M) and arginine hydrochloride (2·0 and 2·2 M, the pH being adjusted with KOH). The results of an experiment in which ATP was injected are shown in Fig. 8. It will be noted that the ATP injections weaken and eventually bring about the virtual disappearance of $CaCl_2$-induced contractions. A similar effect was found when AMP was injected. It is difficult to assess the significance of this effect, however, since the amounts of ATP or AMP injected were large and the injected region tended to become more transparent than the rest of the fibre. It is

possible that the effect was due to the higher ionic strength in the injected region brought about by the ATP or AMP.

*The effects of the external application and micro-injection of caffeine on Maia muscle fibres*

Taylor (1953), Axelsson & Thesleff (1958), and Conway & Sakai (1960) showed that the external application of 1·3–5 mM caffeine in Ringer's solution to frog muscle fibres could cause a contraction with little or no

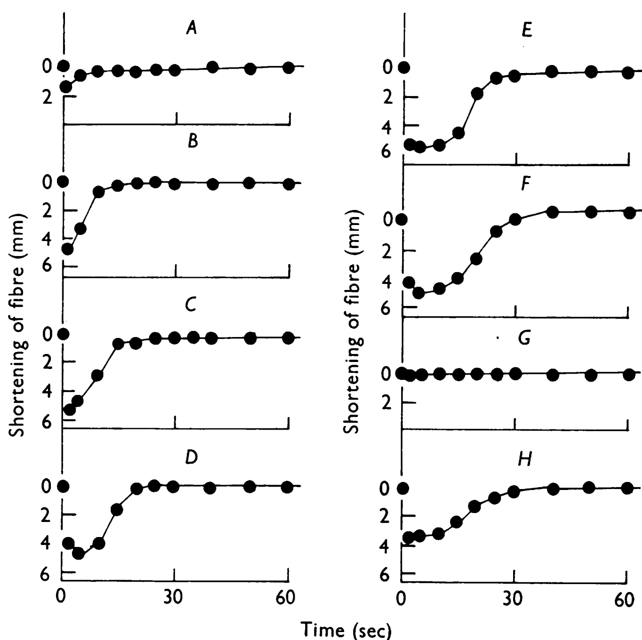


Fig. 6. The effects of the injection of solutions containing various concentrations of $SrCl_2$, 10 mM-$CaCl_2$ and distilled water on a *Maia* muscle fibre bathed in crab saline. The shortening produced at various times after the injection of each solution over an 8 mm length of the fibre is shown. Injections: *A* and *G*, distilled water; *B*, 1 mM-$SrCl_2$; *C*, 5 mM-$SrCl_2$; *D*, 10 mM-$SrCl_2$; *E*, 20 mM-$SrCl_2$; *F*, 50 mM-$SrCl_2$; *H*, 10 mM-$CaCl_2$. Average width of relaxed fibre, 1·2 mm. Initial resting potential, −55 to −57 mV; resting potential after the $SrCl_2$ injections, −29 to −57 mV (injected region −29 to −57 mV); resting potential during injections *G* and *H* (carried out about 1 hr after injections *A–F*), −36 (cannula tip) to −58 mV (injected region −47 to −58 mV). Temperature, 20° C.

accompanying depolarization of the fibre membranes. Caffeine contractions were also obtained with fibres which had been previously depolarized in high-potassium solutions, when such fibres were treated with high-potassium solutions containing caffeine (Axelsson & Thesleff, 1958; Conway & Sakai, 1960). On the other hand, when Axelsson & Thesleff

(1958) attempted to apply caffeine internally to the fibres they came to the conclusion that the internal application did not cause contraction.

The effects of 5 mM caffeine in the bathing solution on single *Maia* muscle fibres were tried, to see if they behaved in the same way as frog

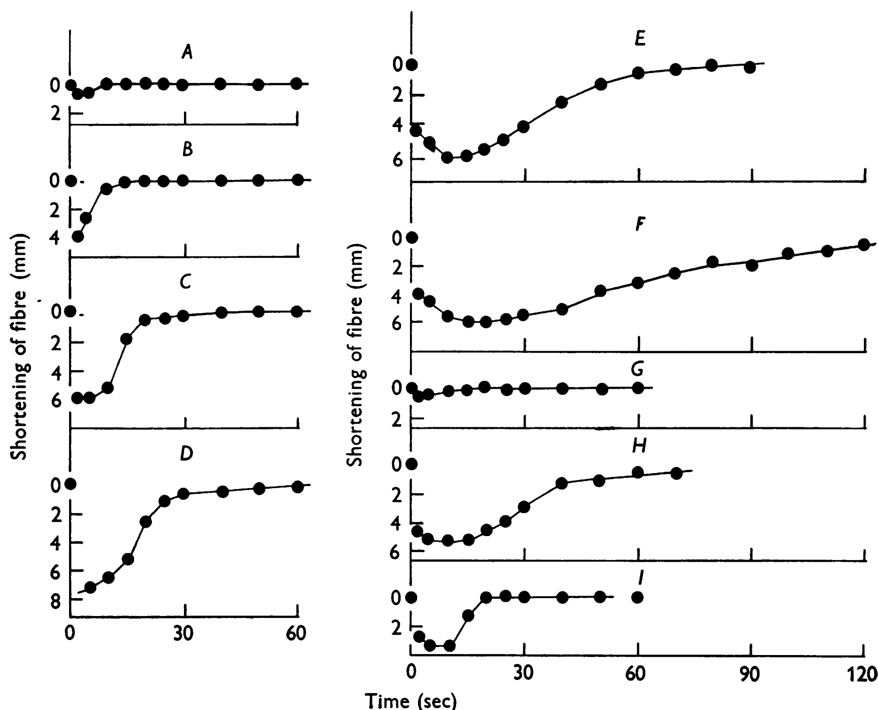


Fig. 7. The effect of the injection of solutions containing various concentrations of $BaCl_2$, 10 mM-$CaCl_2$, 10 mM-$SrCl_2$ and distilled water on a *Maia* muscle fibre bathed in crab saline. The shortening produced at various times after the injection of each solution over an 8 mm length of fibre is shown. Injections: *A* and *G*, distilled water; *B*, 1 mM-$BaCl_2$; *C*, 5 mM-$BaCl_2$; *D*, 10 mM-$BaCl_2$; *E*, 20 mM-$BaCl_2$; *F*, 50 mM-$BaCl_2$; *H*, 10 mM-$CaCl_2$; *I*, 10 mM–$SrCl_2$. Average width of relaxed fibre, 0·87 mm. Initial resting potential, −51 (cannula) to −57 mV; resting potential after $BaCl_2$ injections, −41 (near cannula) to −57 mV; resting potential during injections *G*, *H* and *I* (carried out further down the fibre after recannulation), −50 (cannula) to −55 mV. Temperature, 19° C.

muscle. The membrane potential was recorded continuously, before and during the application of caffeine, by means of a 50–100 $\mu$ capillary electrode inserted longitudinally down the fibre through the cannula, the output of the electrometer being connected to a recording ammeter.

Before immersion in the caffeine-containing solution, the circuit between the electrode in the caffeine-containing solution, the electrode inside the fibre and the electrometer was completed by immersion of the tip of the

apodeme at the bottom of the fibre, but not the fibre itself, in the caffeine-containing solution. A similar initial procedure was adopted when membrane potential changes following the immersion of fibres in KCl crab saline were followed. In all cases the initial membrane potential recorded with fibres in this position was similar to that found in the preceding bathing solution. It was found, in confirmation of the previous work,

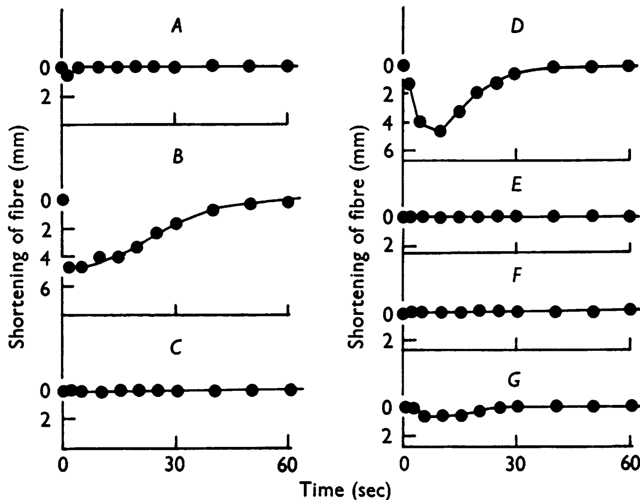


Fig. 8. The effects of the injection of solutions containing the potassium salt of ATP (0·38 M ATP together with 0·18 M ADP as an impurity), 10 mM-$CaCl_2$ and distilled water on a *Maia* muscle fibre bathed in crab saline. The shortening produced at various times after the injection of the ATP solution over a 10 mm length of the fibre and the injection of 10 mM-$CaCl_2$ and distilled water over a 5 mm length is shown. Injections: *A*, distilled water; *B*, *D* and *G*, 10 mM-$CaCl_2$; *C*, *E* and *F*, 0·38 M ATP. Initial resting potential, −51 (cannula) to −57 mV; final resting potential, −20 (cannula) to −58 mV (resting potential in the region into which ATP was injected, −20 to −58 mV; resting potential in the region into which $CaCl_2$ was injected −47 to −58 mV). Average width of relaxed fibre, 1·1 mm. Temperature, 18° C.

that the external application of 5 mM caffeine in crab saline to the fibres caused a sustained contraction without altering the membrane potential by more than a few millivolts. Similarly, application of 5 mM caffeine in KCl crab saline to fibres, which had previously been immersed and allowed to contract and relax in KCl crab saline, caused a sustained contraction, even though the fibres had a small positive membrane potential (+13 to +19 mV), which changed by no more than a few millivolts. In order to investigate further the extent of the dissociation of caffeine-induced contractions from decreases in a negative membrane potential the following type of experiment was carried out. A 50–100 $\mu$ capillary electrode, with which the membrane potential was recorded continuously, was inserted longitudinally down a single *Maia* fibre. The fibre was first

depolarized with KCl crab saline and allowed to relax. The fibre was then transferred from KCl crab saline to ordinary crab saline containing 5 mM caffeine when a caffeine-induced contraction was observed even though the transfer from the high-potassium KCl crab saline to the low-potassium crab saline caused a simultaneous negative repolarization of the fibre

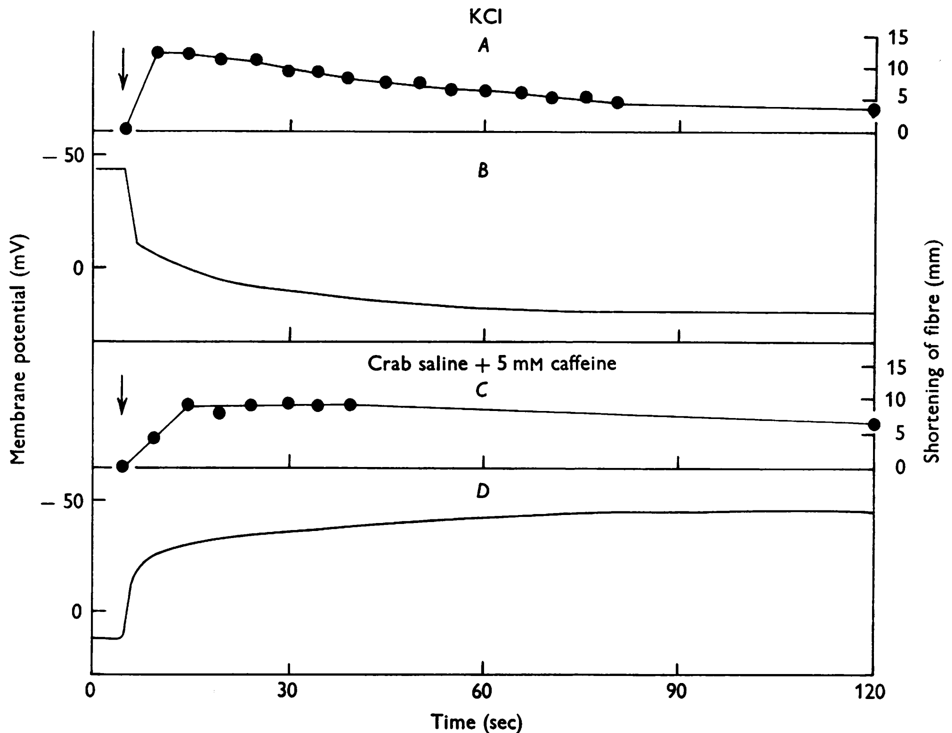


Fig. 9. The effects of the external application of crab saline containing 5 mM caffeine on a *Maia* muscle fibre previously treated with KCl crab saline. *A* shows the shortening of the fibre and *B* the resting potential at various times after the fibre had been immersed in the KCl crab saline (KCl content, 523 mM) at the point indicated by the arrow (time = 5 sec). The fibre had previously been in crab saline (KCl content, 12·9 mM). When the fibre had relaxed in the KCl crab saline it was immersed in crab saline containing 5 mM caffeine at the point indicated by the arrow on *C* (time = 5 sec). *C* shows the shortening of the fibre caused by the 5 mM caffeine, and *D* shows the simultaneous repolarization of the fibre brought about by the low potassium content of the crab saline. Temperature, 18° C.

membrane. The results of an experiment of this type are shown in Fig. 9. The fact that caffeine can still induce a contraction when the membrane potential is changing in a direction normally regarded as the opposite of that required for the initiation of contraction provides further evidence that caffeine is acting on a stage in the initiation of contraction which is subsequent to the membrane depolarization.

Experiments in which caffeine was injected into *Maia* muscle fibres led to conclusions about the intracellular effects of caffeine, which differ from those of Axelsson & Thesleff (1958), since contractions were obtained. The first injection of a solution containing a given concentration of caffeine

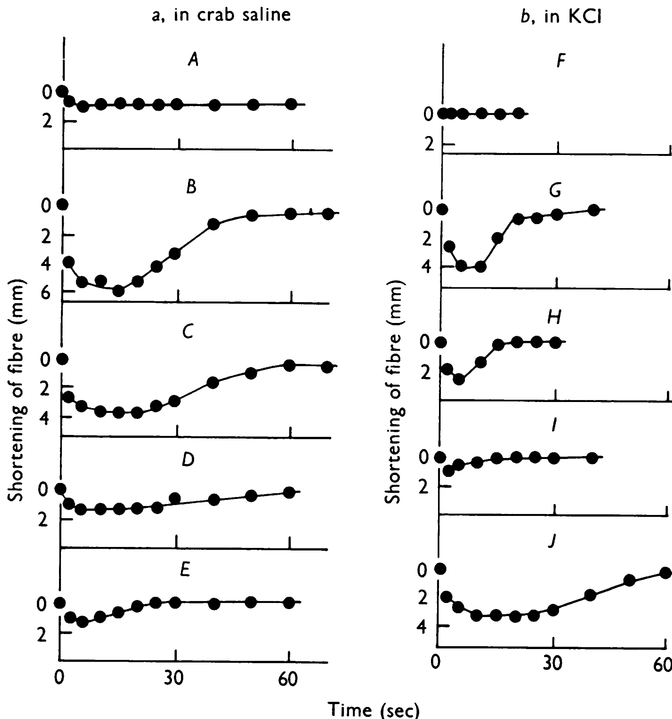


Fig. 10. *a*, The effects of the injection of solutions containing 20 mM caffeine and of distilled water on a *Maia* fibre bathed in crab saline. Injections: *A*, distilled water; *B–E*, 20 mM caffeine. Fibre dimensions, 1·78 mm wide × 0·78 mm thick. Initial resting potential, −42 (cannula) to −55 mV; final resting potential, −12 (cannula) to −58 mV; the resting potential in the injected region was −49 to −58 mV throughout. Temperature, 16° C. *b*, The effects of the injection of solutions containing 10 mM caffeine, 10 mM-$CaCl_2$ and of distilled water on a *Maia* fibre bathed in KCl crab saline. This fibre had a positive resting potential as a result of the high K content of the saline; resting potential throughout, +10 to +18 mV. Injections: *F*, distilled water; *G*, *H* and *I*, 10 mM caffeine; *J*, 10 mM-$CaCl_2$. Dimensions of relaxed fibre, 1·1 mm wide × 0·75 mm thick. Temperature, 17° C. In both *a* and *b* the solutions were injected over 5 mm of the fibres.

caused a fairly marked contraction followed by relaxation (Fig. 10*a*, *B*). Further injections of the same solution however tended to cause weaker contractions and in some cases very weak contractions were obtained (Fig. 10*a*, *C–E*). In two cases fibres were left soaking for a while at this stage and there was some indication of a recovery of the response to the

injection of the caffeine solution. Successive injections of a caffeine solution into a fibre which had first been depolarized by immersion in KCl crab saline and allowed to relax caused a weakening series of contractions (Fig. 10*b*). In three cases 10 mM-$CaCl_2$ was injected into polarized or depolarized fibres with a weakened response to caffeine resulting from successive caffeine injections, when a contraction was obtained (Fig. 10*b*, *J*), which if anything was slightly longer lasting than that usually obtained with this concentration of $CaCl_2$.

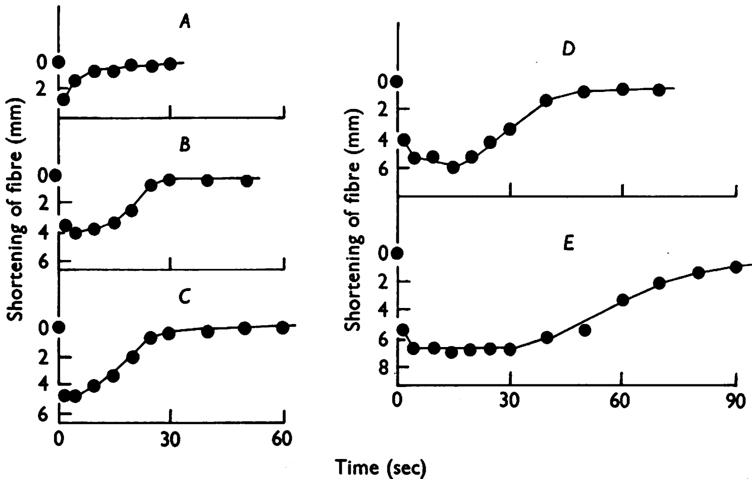


Fig. 11. The effects of the injection of a series of caffeine solutions on a series of fibres bathed in crab saline. In each case the injection was the first injection of caffeine into an individual fibre. The caffeine solutions were injected over 5 mm of the fibres. *A*, 1 mM caffeine; *B*, 5 mM caffeine; *C*, 10 mM caffeine; *D*, 20 mM caffeine; *E*, 50 mM caffeine. Widths of relaxed fibres, 1·5–1·8 mm; resting potentials in the injected region, −46 to −58 mV. Temperature, 15–17° C. The contraction produced by 1 mM caffeine (*A*) was only slightly greater than that produced by a preceding control injection of distilled water.

In Fig. 11 the time courses are given of the contractions observed after the injection of caffeine solutions of various concentrations into a series of *Maia* fibres, the injection in each case being the first injection of caffeine into a particular fibre, following an initial injection of distilled water. It will be seen that the increase in the time course of these contractions with increasing caffeine concentration is very similar to that obtained with increasing concentrations of $CaCl_2$, $SrCl_2$ and $BaCl_2$. Furthermore, the actual time course of the contraction caused by the injection of a solution containing a given concentration of caffeine is similar to that produced by the injection of a solution containing the same concentration of $CaCl_2$ or $BaCl_2$ and, at low concentrations, of $SrCl_2$.

## DISCUSSION

The results described in this paper show that the muscle fibre preparation from *Maia squinado* facilitates the investigation of problems which are more difficult to study with smaller fibres from other animals. The effects of the injection of substances such as calcium chloride, barium chloride, magnesium chloride, potassium chloride, sodium chloride, distilled water, and ATP on the contractile mechanism are in agreement with previous work by others (Heilbrunn & Wiercinski, 1947; Falk & Gerard, 1954; Niedergerke, 1955) in which frog muscle fibres were used. The experiments in which caffeine was injected, however, do not support the conclusion of Axelsson & Thesleff (1958) that the intracellular application of caffeine to muscle does not cause contraction.

In the present work the first injection of caffeine into a *Maia* muscle fibre produced a contraction which appeared to be similar, at lower concentrations, to that produced by equivalent amounts of calcium, strontium or barium, but subsequent injections following immediately tended to produce contractions which became weaker. Axelsson & Thesleff state that with frog muscle fibres they failed to get any contraction as the result of the intracellular application of caffeine. This difference in the results may be due to a species difference. Alternatively, it is possible that caffeine acts on the inner surface of the sarcoplasmic reticulum but not on the side in contact with the sarcoplasm. If this was so and if the somewhat coarser method of injection used in the present work damaged the reticulum sufficiently to allow penetration of caffeine to the inner surface whereas the method used by Axelsson & Thesleff did not, the difference in the results could be accounted for. Another, more likely, explanation, is as follows. In Axelsson & Thesleff's experiments 80 mM caffeine was introduced into the fibres by allowing it to diffuse from the 2–3 $\mu$ tip of a micropipette which had been inserted into the fibre. They state, however, that the fibres were already contracted in the region of the insertion, before the pipette was inserted, as a result of the leakage of caffeine, from the pipette into the external solution in this region. Since they observed a slow relaxation of the fibre in this region when they inserted the pipette they assumed that the caffeine leaking into the inside of the fibre was unable to cause a contraction. It is, however, possible that any initial contraction which the caffeine applied intracellularly might have caused was masked by the contraction already caused by the extracellular caffeine, and that during the slow relaxation the gradual weakening of the contraction, observed in the present work during successive injections of caffeine, was taking place as further caffeine diffused into the fibre.

The results obtained in the present work must now be related to theories

about the role of calcium in muscular contraction (Bailey, 1942; Heilbrunn, 1943; Sandow, 1952; Lüttgau & Niedergerke, 1958; Huxley & Taylor, 1958; Caldwell, 1960; Porter, 1961; Ebashi, 1961). It is thought that the effect of the membrane depolarization spreads down the sarcoplasmic reticulum (Huxley & Straub, 1958; Huxley & Taylor, 1958) causing the release into the sarcoplasm from the reticulum of ionized calcium which then activates the contraction and the ATPase of the actomyosin system. Relaxation may first involve the release of a soluble relaxing factor from the sarcoplasmic reticulum of the type studied by Briggs & Fuchs (1960), Parker & Gergely (1960) and Nagai, Uchida & Yasuda (1962), this factor possibly acting by removing the ionized calcium as a complex. The cycle of events is, however, only completed by the re-accumulation of the calcium by the reticulum, microsomes from which have been shown to have relaxing-factor activity which is associated with an ability to accumulate calcium (Portzehl, 1957; Nagai, Makinose & Hasselbach, 1960; Hasselbach & Makinose, 1961; Ebashi, 1961; Muscatello, Andersson-Cedergren & Felice Azzone, 1962). This sequence of events implies that activation of contraction via the membrane would require a membrane depolarization to bring about the release of calcium from the reticulum. On the other hand, contractions independent of membrane depolarization would be obtained if a substance was injected or allowed to diffuse into the fibre which either activated the contractile mechanism directly or else displaced ionized calcium from the reticulum or from some form of bound calcium.

Injected calcium would, with this scheme, be expected to be equivalent in its effects to calcium released from the sarcoplasmic reticulum and would therefore be expected to cause the contraction which is observed experimentally. Since in this case a release of calcium from the sarcoplasmic reticulum is not involved, the effects of the injected calcium would be expected to be independent of the state of the membrane polarization, and this is in fact the case. The increasing times required for relaxation as the amount of calcium injected is increased would represent the time required to remove the injected calcium by accumulation into the sarcoplasmic reticulum and for enough of a soluble relaxing factor to be formed to interact with any calcium not accumulated.

An interesting feature of the effects, at low concentration, of injected barium and strontium and of the first injection of caffeine, is that equivalent amounts of these substances produce contractions of roughly the same duration. The duration of the contractions is also similar to those produced by an equivalent amount of calcium. It seems unlikely that barium, strontium and caffeine all interact with the contraction–relaxation system in precisely the same way as calcium, even though this is suggested by

these observations. They would, however, be expected to produce effects similar to injected calcium if they each caused the displacement, from the sarcoplasmic reticulum or from some form of bound calcium, of an equivalent amount of ionized calcium which acted on the contractile system and was then reaccumulated by the reticulum or rebound in some other way. Frank (1962) has suggested a mechanism of this type, involving displacement of calcium by caffeine from a binding site, to account for some of the effects of externally applied caffeine on frog muscle in calcium-free bathing solution. If internally or externally applied caffeine in fact causes such a direct displacement of calcium, then the observed independence of caffeine contractions from membrane depolarizations is to be expected. The extent of this independence is shown in the experiments described in this paper, in which contractions were obtained with externally applied caffeine while the fibre membrane was being simultaneously repolarized. A puzzling feature of the effects of injected caffeine, for which an explanation cannot as yet be given, is the tendency of the contractions produced to weaken with successive injections of the same amount. It is possible, however, that this effect has the same origin as the decline in the effect of externally applied caffeine observed by Frank (1962) during successive applications of caffeine to frog muscle in calcium-free bathing solution.

Of the other substances injected into *Maia* muscle fibres, potassium chloride, sodium chloride, potassium phosphate and arginine hydrochloride were injected as controls and, as expected, failed to cause contraction. With regard to the lack of effect of magnesium and ATP, it must be remembered that, in the light of the foregoing discussion, the ionized calcium level is probably being kept at an extremely low level in the sarcoplasm as a result of the accumulation of calcium into the sarcoplasmic reticulum. In the absence of calcium, magnesium at higher concentrations tends to inhibit the shortening and ATPase activity of glycerol-extracted muscle-fibre models containing relaxing factor (Bendall, 1953) and the ATPase activity of isolated myofibrils (Perry, 1951), even though it causes contraction and activation of the ATPase at lower concentrations. The magnesium level in relaxed *Maia* fibres is presumably high enough to inhibit any magnesium-activated contraction and therefore addition of further magnesium by injection fails to cause contraction.

In the absence of calcium, ATP tends to start inhibiting the magnesium-activated ATPase of myofibrils when the ATP concentration exceeds the magnesium concentration (Perry & Grey, 1956). Higher concentrations of ATP also inhibit the ATPase of glycerol-extracted fibre models, particularly in the presence of relaxing factor, and also their tension development (Hasselbach & Weber, 1953). An increase in the ATP concentration in a relaxed *Maia* muscle fibre as a result of the injection

of ATP would not therefore be expected to cause contraction, as was in fact found experimentally.

The injection of AMP was not expected to cause contraction and failed to do so. It is possible that part of this failure may have been due to a rapid conversion of AMP to ATP immediately after the injection. EDTA is able to cause the relaxation of contracted glycerol-extracted fibres (Bozler, 1954) and the inhibition of myofibrillar ATPase (Perry & Grey, 1956), probably by binding calcium which is needed in addition to magnesium to activate these systems. The injection of EDTA therefore, as expected, failed to cause a contraction.

## SUMMARY

1. A preparation consisting of a cannulated single muscle fibre from the leg muscles of the crab *Maia squinado* is described. Solutions can be injected into the fibre and any resultant shortening measured.

2. Contractions were produced when $CaCl_2$, $SrCl_2$, $BaCl_2$ and caffeine were injected, the contraction produced by caffeine becoming weaker during a rapid series of injections of the same quantity. At lower concentrations the injection of the same quantity (in moles) of $CaCl_2$, $SrCl_2$, $BaCl_2$ and of caffeine (if this was the first injection of caffeine into the fibre) produced contractions with similar time courses.

3. Contractions were only very slight or absent after the injection of distilled water, KCl, NaCl, $MgCl_2$, ATP, AMP, EDTA, potassium phosphate and arginine hydrochloride.

4. The external application of 5 mM caffeine caused the single-fibre preparation to go into a maintained contraction without, in the early stages, causing any significant change in the membrane potential. Caffeine contractions were obtained while fibres were depolarized by high external potassium and also with previously depolarized fibres when they were simultaneously repolarizing as a result of being transferred from a solution of high-potassium concentration containing no caffeine to a low-potassium crab saline containing caffeine.

5. $CaCl_2$ and caffeine were injected into fibres depolarized by high external potassium and were found to cause contraction.

6. The results are discussed in terms of hypotheses which seem to be likely in the light of present knowledge about muscular contraction and relaxation, the important role which the release and removal of ionized calcium may play being particularly emphasized.

We should like to thank the Director and the Staff of the Laboratory of the Marine Biological Association of the U.K., Plymouth, for the facilities and help given during the course of this work. Most of the work was carried out while one of us (P.C.C.) was Johnston, Lawrence and Moseley Research Fellow of the Royal Society.

REFERENCES

AXELSSON, J. & THESLEFF, S. (1958). Activation of the contractile mechanism in striated muscle. *Acta physiol. scand.* **44**, 55–66.

BAILEY, K. (1942). Myosin and adenosinetriphosphatase. *Biochem. J.* **36**, 121–139.

BENDALL, J. R. (1953). Further observations on a factor (the 'Marsh' factor) effecting relaxation of ATP-shortened muscle-fibre models, and the effect of Ca and Mg ions upon it. *J. Physiol.* **121**, 232–254.

BOZLER, E. (1954). Relaxation in extracted muscle fibers. *J. gen. Physiol.* **38**, 149–159.

BRIGGS, F. N. & FUCHS, F. (1960). The biosynthesis of a muscle-relaxing substance. *Biochim. biophys. acta*, **42**, 519–527.

CALDWELL, P. C. (1954). An investigation of the intracellular pH of crab muscle fibres by means of micro-glass and micro-tungsten electrodes. *J. Physiol.* **126**, 169–180.

CALDWELL, P. C. (1958). Studies on the internal pH of large muscle and nerve fibres. *J. Physiol.* **142**, 22–62.

CALDWELL, P. C. (1960). Some aspects of the part played by phosphate compounds in the regulation of certain inorganic ions in cells. *CIBA Foundation Study Group*, **5**, 69–74.

CALDWELL, P. C. (1961). The use of micro-injection techniques and large nerve and muscle fibres in the study of active transport and muscular contraction. *Pflüg. Arch. ges. Physiol.* **272**, 215–222.

CALDWELL, P. C. & WALSTER, G. E. (1961). A cannulated crab muscle fibre. *J. Physiol.* **157**, 36–37*P*.

CONWAY, D. & SAKAI, T. (1960). Caffeine contracture. *Proc. nat. Acad. Sci., Wash.*, **46**, 897–903.

EBASHI, S. (1961). Calcium binding activity of vesicular relaxing factor. *J. Biochem., Tokyo*, **50**, 236–244.

FALK, G. & GERARD, R. W. (1954). Effect of micro-injected salts and ATP on the membrane potential and mechanical response of muscle. *J. cell. comp. Physiol.* **43**, 393–403.

FATT, P. & KATZ, B. (1953). The electrical properties of crustacean muscle fibres. *J. Physiol.* **120**, 171–204.

FRANK, G. B. (1962). Utilization of bound calcium in the action of caffeine and certain multivalent cations on skeletal muscle. *J. Physiol.* **163**, 254–268.

HASSELBACH, W. & MAKINOSE, M. (1961). Die Calciumpumpe der 'Erschlaffungsgrana' des Muskels und ihre Abhängigkeit von der ATP-Spaltung. *Biochem. Z.* **333**, 518–528.

HASSELBACH, W. & WEBER, H. H. (1953). Der Einfluss des MB-Faktors auf die Kontraktion des Fasermodells. *Biochim. biophys. acta*, **11**, 160–161.

HEILBRUNN, L. V. (1943). *An Outline of General Physiology*, Ch. 28 and Ch. 37. Philadelphia: W. B. Saunders Co.

HEILBRUNN, L. V. & WIERCINSKI, F. J. (1947). The action of various cations on muscle protoplasm. *J. cell. comp. Physiol.* **29**, 15–32.

HODGKIN, A. L. (1958). Ionic movements and electrical activity in giant nerve fibres. *Proc. Roy. Soc.* B, **148**, 1–37.

HODGKIN, A. L. & HUXLEY, A. F. (1945). Resting and action potentials in single nerve fibres. *J. Physiol.* **104**, 176–195.

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid giant axons by means of a microsyringe. *J. Physiol.* **131**, 592–616.

HUXLEY, A. F. & STRAUB, R. W. (1958). Local activation and interfibrillar structures in striated muscle. *J. Physiol.* **143**, 40–41*P*.

HUXLEY, A. F. & TAYLOR, R. E. (1958). Local activation of striated muscle fibres. *J. Physiol.* **144**, 426–441.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol.* **143**, 486–505.

MUSCATELLO, U., ANDERSSON-CEDERGREN, E. & FELICE AZZONE, G. (1962). The mechanism of muscle-fiber relaxation, adenosine triphosphatase and relaxing activity of the sarcotubular system. *Biochim. biophys. acta*, **63**, 55–74.

NAGAI, T., MAKINOSE, M. & HASSELBACH, W. (1960). Der Physiologische Erschlaffungsfaktor und die Muskelgrana. *Biochim. biophys. acta*, **43**, 223–238.

NAGAI, T., UCHIDA, K. & YASUDA, M. (1962). Some further properties of the muscle relaxing-factor system and the separation of the effective substance. *Biochim. biophys. acta,* **56**, 205–215.

NIEDERGERKE, R. (1955). Local muscular shortening by intracellularly applied calcium. *J. Physiol.* **128**, 12–13*P*.

PARKER, C. J. & GERGELY, J. (1960). Soluble relaxing factor from muscle. *J. biol. Chem.* **235**, 3449–3453.

PERRY, S. V. (1951). The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle. *Biochem. J.* **48**, 257–265.

PERRY, S. V. & GREY, T. C. (1956). A study of the effects of substrate concentration and certain relaxing factors on the magnesium-activated myofibrillar adenosine triphosphatase. *Biochem. J.* **64**, 184–192.

PORTER, K. R. (1961). The sarcoplasmic reticulum. Its recent history and present status. *J. biophys. biochem. Cytol.* **10**, Suppl. 219–226.

PORTZEHL, H. (1957). Die Bindung des Erschlaffungsfaktors von Marsh an die Muskelgrana. *Biochim. biophys. acta,* **26**, 373–377.

SANDOW, A. (1952). Excitation-contraction coupling in muscular response. *Yale J. Biol. Med.* **25**, 176–201.

TAYLOR, R. E. (1953). The contractile process is not associated with potential changes. *J. cell. comp. Physiol.* **42**, 103–123.