Topochemical Factors in Potentiation of Contraction by Heavy Metal Cations

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ABSTRACT In addition to the previously studied Zn^{2+}, low concentrations (about 0.5 mM) of Be^{2+}, Ba^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Pt^{4+}, and, outstandingly, 0.5 μM of UO_2^{2+}, potentiate the twitch of frog sartorius and toe muscles by prolonging the active state of contraction. The degree of potentiation is a roughly S-shaped function of $p(\text{metal}^{2+})$, suggesting that each metal binds to a ligand of the muscle fiber, representative apparent affinity constants being: UO_2^{2+}, 5×10^6; Zn^{2+}, 2.8×10^5; and Cd^{2+}, 2×10^4. UO_2^{2+} potentiation effects are rapidly reversed by PO_4, and Zn^{2+} and Cd^{2+} effects by EDTA, PO_4, and cysteine. The rapidity of these reversals by the nonpenetrating EDTA and PO_4, and the fact that heavy metal ions evidently potentiate by prolonging the action potential, indicate that the metal potentiators exert their primary action at readily accessible (i.e. plasma and T tubular) membrane sites. The relatively slow kinetics of development of potentiation, and the even slower reversal of it in pure Ringer's solution, indicate that the metal ions are bound to connective tissue, as well as to muscle fibers. The binding effects at the readily accessible membrane sites evidently impairs delayed rectification and thus modifies the action potential and excitation-contraction coupling so as to cause potentiation. SH is excluded, and PO_4 and imidazole are possibilities, as the membrane ligand binding the potentiating metal ions.

Contraction of skeletal muscle is potentiated by many chemically diverse substances which include a group of divalent heavy metals, exemplified especially by zinc (Isaacson and Sandow, 1963; for general review of potentiation, see Sandow, 1964). All these agents typically augment the isometric twitch by as much as 2 to 3 times, but do not significantly change the peak tetanus tension (for certain exceptions see Brust, 1965). In our previous work we attempted to locate the site of action of Zn by using techniques which involved the kinetics of development and reversal of twitch potentiation and located the primary site of action of the anionic potentiators NO_3^- and I^- at either the plasma, or T tubular, membrane (Kahn and Sandow, 1950, 1955; Hill

and Macpherson, 1954; Hodgkin and Horowicz, 1960). But these investigations left unsettled the question of the locus of the primary effect of zinc, since both superficial membrane as well as intrafiber sites were conceivably involved. Furthermore, we left unexplored the nature of the chemically reactive groups of the muscle fiber with which zinc possibly combined. We initiated the present research in the attempt to determine the topochemistry, i.e. the location and the nature of the chemical reactions involving ligands of cell components, characterizing the first step of the action by which zinc causes potentiation. This research has led us to discover several new potentiating agents of considerable interest, the uranyl ion, in particular, and to obtain further information about the action of zinc as a potentiator. By the use especially of agents that reverse potentiation, e.g. EDTA acting on zinc-treated muscles, we can now conclude that the sites involved in the binding of the heavy metal ions that leads to potentiation are located in plasma and/or T tubular membranes. In addition, we present results that rule out involvement of sulfhydryl groups in this binding reaction. Some of these findings have already been briefly reported (Isaacson and Sandow, 1961; Sandow and Isaacson, 1963).

METHODS

We used sartorius and extensor longus digiti IV muscles from the frog, *Rana pipiens*. The sartorii were exised with pelvic bone attached, equilibrated for about 1 hr in 100 ml of oxygenated Ringer's solution, and then vertically mounted in 50 ml of Ringer's solution in a Lucite chamber provided with massive stimulating electrodes. A fine stainless steel chain connected the tibial end of the muscle to an isometric lever, the initial tension being generally 2 g. The toe muscles, after excision and equilibration, were mounted horizontally in a massive electrode chamber containing 25 cc of Ringer's solution. One end of the muscle was fixated by being tied to a plastic rod and the other was connected to the isometric lever under a tension of 0.1 g.

Tension developed by the sartorii was generally detected by a Statham Instrument strain gage, 4 oz model Gl-4-250, and after amplication recorded on a Sanborn Recorder Model 150. An RCA 5734 transducer tube, connected to an isometric lever, was used to record toe muscle responses. The amplified transducer signal was likewise displayed on the chart recorder. The transducer tube myograph, but with cathode-ray oscilloscope display, was also used for some of the experiments on the sartorius muscle, as indicated, for example, in the results of Fig. 1.

Our standard Ringer's solution, prepared with deionized water, contained the following reagent grade chemicals: NaCl, 108.4 mM; KCl, 1.6 mM; $CaCl_2$, 1.0 mM; and 2 mM tris(hydroxymethyl)aminomethane-HCl buffer at pH 7.3. Curare, 2×10^{-5} gm/ml, was routinely added to the solution before use. Stock solutions, 0.1 or 0.05 M in deionized water, were made of the following salts: UO_2 acetate, $ZnCl_2$, $CdCl_2$, $NiCl_2$, $CuCl_2$, $Be(NO_3)_2$, $AgNO_3$, $PbCl_2$, $PtCl_4 \cdot 2HCl \cdot 6H_2O$, $SrCl_2 \cdot 6H_2O$. Aliquots of these stock solutions were added to the Ringer solution to prepare fresh dilute solutions for each experiment.

The massive stimulating electrodes of our muscle chambers were of standard type (Sandow, 1947) except that they were made of platinum. In some early experiments chambers with similar Ag-AgCl electrodes were used. Upon switching to the platinum electrode chambers, we noticed a considerable improvement in the ability of the muscle to maintain tetanic tension output for prolonged periods of time. Thus, muscles exposed for several hours to the Ag-AgCl electrodes would frequently show as much as 30% loss of tetanus tension. But with the new Pt-electrode chambers, the tetanic tension usually remained within 5% of its initial value when tested at about 15 min intervals over a period of as long as 5 hr. We will discuss this difference later in connection with some results involving the action of silver ion.

We stimulated our experimental muscles by square wave shocks of 0.3 msec duration and slightly supramaximal strength. (For some comment on the use of such stimuli, see footnote 2.) Tetanic stimulation was at a frequency of 20 and 120 shocks/sec for unfused and fused responses, respectively. Tetanic durations of 0.2 and 0.5 sec were generally used for sartorius and toe muscles, respectively.

All tests were made at room temperature of about 24°C. In some experiments, muscles were placed in a refrigerator at 6°C for overnight soaks in experimental solutions.

We always obtained a control series of twitches and tetani for a period of at least 30 min prior to placing a muscle into a modified Ringer solution. This provided a baseline for determining subsequent changes in a muscle tension output.

RESULTS

In connection with our aim to determine the nature of the topochemical binding reaction presumably involved in the immediate action of zinc on muscle fibers, we postulated that the key ligand might be phosphate and that blockade of this group by the uranyl ion would prevent zinc from causing potentiation. Our initial test, however, immediately showed that uranyl was itself a strong potentiator. Some preliminary studies of isometric twitches had also shown that cadmium was a stronger potentiator than had been previously indicated in isotonic studies (Bien, 1961; Isaacson and Sandow, 1963). These results led us to question our previous view that among the divalent heavy metal ions zinc was highly specific as a potentiator. It therefore became important to study the potentiating actions of UO_2^{2+}, Cd^{2+}, and related ions in some detail. In the following, we start with the results of such studies and then later consider the question of the specific topochemical binding involved in the systems of interest.

I. *The Main Mechanical Effects of Uranyl and Cadmium Ion*[1]

Fig. 1 shows that both UO_2^{2+} and Cd^{2+}, acting at optimal concentrations produce typical potentiation effects: (*a*) severalfold increase in twitch ten-

[1] Although we refer to the active forms of the metals in our experimental solutions as if they are simple ions (e.g. UO_2^{2+}, Zn^{2+}, Cd^{2+}), it should be noted that in general each exists at neutral pH in the form of various hydrolysis complexes (in regard to uranyl, see, e.g., Ahrland, 1949).

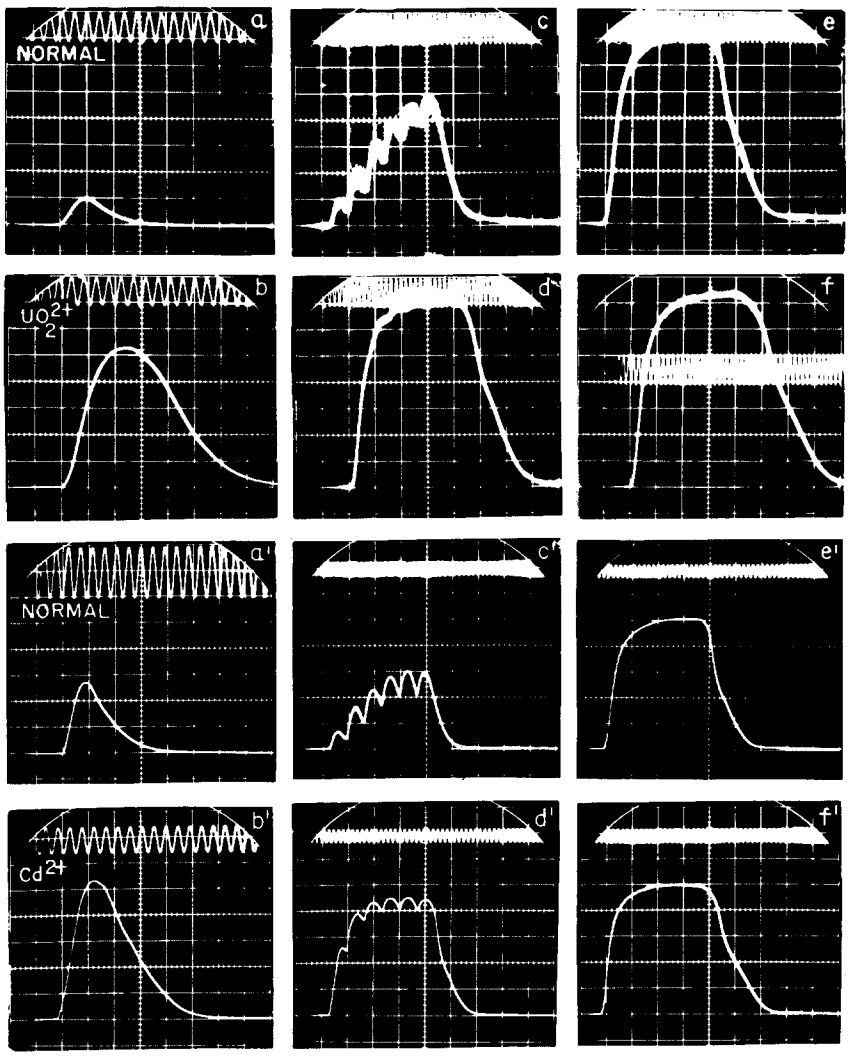

FIGURE 1. Effects of 1 μM uranyl acetate on frog sartorius muscle isometric twitch (*b*), 30 CPS tetanus (*d*), and 120 CPS tetanus (*f*) responses (following corresponding controls in *a*, *c*, and *e*). Time of exposure to uranyl: (*b*) 64 min; (*d*) 90 min; and (*f*) 144 min. Tension calibration for uranyl series (*a* to *f*) given by amplitude of 100 CPS sine wave signal equal to 10 g. Effects of 0.5 MM cadmium chloride on twitch (*b'*), 30 CPS tetanus (*d'*) and 120 CPS tetanus (*f'*) responses, corresponding controls in *a'*, *c'*, and *e'*. Time of exposure to cadmium: (*b'*) 42 min; (*d'*) 17 min; and (*f'*) 43 min. Tension calibration for cadmium series given by amplitude of 100 CPS sine wave signal equal to 11 g.

sion, especially pronounced in the case of UO_2^{2+} evidently because of the very low, normal twitch to tetanus ratio of the muscle used in this particular test (see Isaacson, 1962); (*b*) considerable increase in duration of both the contraction period and the entire twitch; (*c*) increase in degree of fusion in

a tetanus originally made considerably unfused by a stimulus of only 30 shocks/sec (and thus an indication that these ions reduce the tetanus fusion frequency); (*d*) no effect on the peak tension of the fused tetanus; and (*e*) a prolongation of the duration of the tetanus relaxation period. These changes are all in essence precisely like those produced by Zn^{2+}. Furthermore, also as in the case of zinc, diphasic action potential recording from maximally stimulated sartorii treated with either uranyl or cadmium ions demonstrates that only a single, though modified, action potential is produced. And from this laboratory (Sandow et al., 1964) other work involving internal microelectrode recording demonstrates the same result, and, moreover, clearly shows that the action potential under treatment with the uranyl ion, as well as with the zinc ion, is modified principally by having a very decelerated falling phase (for a similar effect of cadmium as well as zinc and other heavy metals, see Kobayashi, 1962). Thus, the absence of multiple firing in the responses of these treated muscles to single stimuli, proves that the augmented mechanical outputs are not some sort of tetanus, but simple twitches. It is also important that our use of supramaximal shocks for both normal and treated muscles ensures that the cause of the increased output in the twitch is not recruitment of fibers but a true potentiation of the twitch of each individual fiber of the muscle.[2]

[2] The general rationale of our procedure for determining potentiation of the twitch has been questioned recently in a series of papers by Kelly and coworkers (1964*a*, 1964*b*, 1965*a*, 1965*b*). These authors find that the peak mechanical output of an uncurarized frog sartorius muscle in normal Ringer's solution at room temperature is greatly increased when the massive, square wave shock used as maximal stimulus is prolonged from 0.2 to 0.3 msec to, e.g., 6 to 10 msec. Such increases are not obtained if the muscles are curarized, in fact such muscles produce decreased output as the shock duration is prolonged. It is therefore claimed that the output of the normal muscle stimulated by a maximal (or supramaximal) shock of only 0.3 msec duration does not provide the "maximum response capability of the muscle" (see Kelly and Fry, 1964*b*, Summary) and it should therefore not be used as a reference output for measuring the potentiating effects of, e.g., NO_3 and thus, presumably, of our presently discussed heavy metal cations.

Work in progress in our laboratory confirms the above observations, in general, and we find it particularly interesting that curarized muscles yield diminished outputs as the shock duration is increased; but we shall discuss fully our detailed results elsewhere. We stress here, however, that the inferences and conclusions which the Kelly group draw from their observations, and the attempt they make to apply them to the type of potentiated responses we study, are highly debatable because no proof is given that the augmented contractions resulting from prolonged electrical stimuli are twitches, i.e., it is not shown whether the fibers of the uncurarized muscles each produce only a single action potential when stimulated with a long shock. In fact, we note that, since these muscles are not curarized and therefore can be indirectly stimulated, it may be expected that multiple action potentials would occur in them. For it is known from the work of Katz (1936) that long, constant current shocks applied to the motor nerve fibers of a frog nerve-muscle preparation cause multiple firing of the nerve fibers which produce, in turn, repetitive action potentials in the associated muscle. Thus, there is a possibility that the enhanced contractions of the Kelly experiments are in effect short tetani and not twitches, and they must therefore be ruled out as reference contractions for gauging the augmentative effects of potentiators on the twitch.

In any case, in studying the effects of potentiators, the crux of the matter is not whether the enhanced output activated by a very long shock is a sort of tetanus or an augmented twitch, but whether the

Thus, in all essential features of these effects, the augmented mechanical responses due to UO^{2+} and Cd^{2+} are identical with those caused by Zn^{2+} and, in fact, by all of the many other potentiators (Sandow, 1964). And, consequently, we can conclude, as has been generally done on the basis of similar effects of other potentiators, that the main mechanical change produced by these cations is a prolongation of the active state of the contractile component. In the development of the research reported in this paper, it might have been desirable to use the actual degree of prolongation of the active state as a quantitative measure of the effectiveness of the action of a potentiator under a given set of conditions. But such determinations are difficult to make and to quantify precisely in respect to both the extension in time of the plateau and the delay in relaxation of activity as factors expressing prolongation of the active state. We have therefore used the easily measured and clearly defined increase in peak twitch tension as a measure of potentiation. In any case, our main interest in the present study is not in the changes in active state mechanics produced by the potentiation, but in the site and topochemis-

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contraction evoked under normal conditions by a short shock of, e.g., 0.3 msec duration can be used as a proper standard for determining the degree of potentiation. As indicated in the text above, and as more fully explained elsewhere (Sandow, 1964), such a brief shock, if slightly supramaximal, excites a twitch in all of the fibers of the muscle and thus produces a maximal twitch of the muscle. The size of this maximal twitch evidently varies considerably, relative to that of the corresponding tetanus. Jewell and Wilkie (1958) report twitch/tetanus tension ratios, i.e., values of our parameter $(P/Po)_i$, as high as 0.9 for a *Rana temporaria* sartorius, provided every precaution was used to eliminate extraneous compliances in the structures connecting the muscle to its recording lever. We have used such precautions in our experiments with *Rana pipiens* sartorii, in general, but our values of $(P/Po)_i$ are considerably lower than Jewell and Wilkie's and this is especially noteworthy in the results of Fig. 1 illustrating the effects of UO_2^{2+}. It is possible that we are dealing here with a species difference. For a general discussion of variability in $(P/Po)_i$ and its bearing on the degree of twitch potentiation obtained especially under the action of zinc, see Isaacson (1962). In any case, even if $(P/Po)_i$ is small, as it certainly is in the aforementioned UO_2^{2+} experiment, the essential thing is that the twitch tension output (P) be maximal for the muscle in question. For, then, such a response provides an adequate standard for determining what we call potentiation because, if an agent that does not evoke repetitive firing causes augmentation of the twitch output, it cannot do so by recruiting additional fibers to respond but only by increasing the intrinsic contractile output of each fiber. Thus we have clearly defined operations for determining the capacity of various agents to increase the tension output of the twitch, and it is these operations that provide the basic methods by which we have studied the various effects of potentiators in this and in past research. It is true that, if one's interest is in the basic mechanical changes produced in the active state by potentiators, then the presence of a very low value of $(P/Po)_i$ is undesirable because this indicates the presence of much extraneous series compliance which would seriously mask the changes in essential active state parameters. But in the current work, in which the degree of twitch potentiation is used merely as an indicator of the extent and possible nature of the immediate effects of the potentiators resulting from their primary binding to membrane ligands, such low values of $(P/Po)_i$ are not embarrassing but, indeed, helpful, in that they increase the degree of potentiation of twitch tension output and thus tend in general to make measurements of potentiating effects more reliable (see, e.g., Ritchie and Wilkie, 1955). To return to the question of the effects of prolonged shocks, it may be noted, finally, that, if it can be firmly established that the augmented contractions activated by prolonged maximal shocks are true twitches (and not some sort of tetani), then we would have the interesting finding that prolongation of an electric shock acts as an agent that potentiates the twitch.

try of the immediate action of these agents which enables them to induce alterations in the response to stimulation that are finally expressed as a potentiation of the twitch.

II. *Potentiation as a Function of Concentration of Metal*

In these experiments we exposed a single muscle successively to increasing concentrations of the particular ion under study. As previously described (Isaacson and Sandow, 1963), adequate time was allowed for potentiation to plateau at each concentration. Fig. 2 illustrates the variation in twitch potentiation (increment in twitch tension as a per cent of initial twitch tension, normalized to 100% for each metal) with the logarithm of the concen-

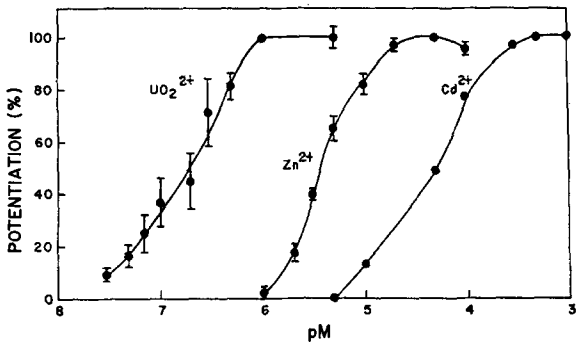

FIGURE 2. Twitch potentiation, expressed as per cent of maximum increment in twitch tension, taken as 100%, as a function of pM (the negative logarithm of the metal concentration) for uranyl, zinc, and cadmium. Standard error shown for points on uranyl and zinc curves, which are based on an average of from two to seven experiments, while points for cadmium curve are based on data from either one or two experiments.

tration of the metal. As previously shown for zinc (Isaacson and Sandow, 1963), these curves are roughly S-shaped and they therefore have a certain similarity to the sort of curve found for the binding of a metal, e.g., to some ligand. The thresholds for potentiation with these metals may be read from the figure as UO^{2+}, 3×10^{-8} M; Zn^{2+}, 10^{-6} M; and Cd^{2+}, 10^{-5} M. Maximal potentiation is obtained with about 10^{-6} M uranyl ion, 5×10^{-5} M Zn^{2+} and 5×10^{-4} M Cd^{2+}, and the absolute value of the maximal potentiation obtainable with these various metals is roughly the same, about 100% for muscles whose values of $(P/Po)_i$ (the ratio of the initial peak twitch tension to the initial peak fused tetanus tension) are about 0.25. (Variability of potentiation as a function of $(P/Po)_i$ in general is discussed by Isaacson, 1962).

Some early cadmium experiments involving silver electrode chambers and muscles with $(P/Po)_i$ values of about 0.4 to 0.5 gave evidence of only slight potentiation and indeed for antagonism to subsequent potentiation by zinc

(Sandow and Isaacson, 1963). More recent cadmium studies with Pt electrode chambers and muscles having a variety of $(P/Po)_i$ values give reasonably consistent potentiation values of about 100% for $(P/Po)_i = 0.25$ and, furthermore, do not show any specific antagonism to zinc.

We made a brief study of the effects of a number of other metal ions and found that the following, in concentration of at least 0.1 mM, all increased both twitch tension and the degree of fusion of an unfused tetanus: $BaCl_2$, $NiCl_2$, $CuCl_2$, $Be(NO_3)_2$, and $PtCl_4 \cdot 6H_2O$. Strontium chloride was also tested and the results were variable: of four muscles studied, two showed potentiation, but two others showed only deleterious effects. The research with Ba^{2+} was developed somewhat and showed that the threshold concentration for potentiation by this ion was at 0.1 mM and its maximum effect occurred at 1 mM. At still greater concentrations, 2 to 5 mM, the Ba^{2+} caused decreases in both twitch and tetanus tensions. The effects of copper were aberrant in that the twitch potentiation initially produced by 0.1 mM Cu^{2+} began to be lost after the muscle had been exposed to the copper for 40 min, and the tetanus output was progressively reduced from the start of the exposure of the muscle to the metal. These results indicate, in general, that all the above heavy metal ions in relatively low concentration prolong the active state and that copper, in addition, exerts some harmful effect that causes a reduction in intensity of the active state.

We also tested $PbCl_2$, $AgNO_3$ (in nitrate-Ringer's solution), and $HgCl_2$ in concentration of 0.1 mM or less and found that they caused only deleterious effects. The results with the silver ion are especially interesting because of their possible bearing on the previously mentioned depressive effects produced in a muscle by massive Ag-AgCl electrodes. Thus the twitch and tetanus outputs of muscles were greatly decreased after exposure to $AgNO_3$ in concentrations as low as 0.5×10^{-6} M, or even to a silver plate, having about the same area as that of massive electrodes, when placed in the normal chloride Ringer solution bath of a chamber fitted with Pt electrodes. It should be noted that the area of such electrodes is about 4 cm^2 and therefore provides a large surface from which Ag ions in considerable number could conceivably escape into the medium. The full details of these experiments will not be presented here, but it seems clear that the Ag^+ greatly depresses the mechanical response of muscle and that the deleterious effects of Ag-AgCl electrodes may be attributed to the free Ag^+ released from such electrodes into the Ringer media in which the muscles are immersed.

Despite the harmful effects of Ag^+ and Pb^{2+}, we found that evidently normal potentiation by both Zn^{2+} and NO_3^- occurred in their presence. This is an important result in respect to the Ag^+ for it assures that the many experiments that have been done on potentiation in the presence of Ag-AgCl electrodes

are not invalidated by any harmful effects caused by a possible release of free Ag^+ from such electrodes.

III. *Kinetics of Development and Reversal of Potentiation by UO^{2+}, Zn^{2+}, and Cd^{2+}*

Previous experiments with frog sartorii have shown that potentiation by 0.05 mM zinc develops with a kinetics having a half-time of about 6 min, after an initial lag period during the first minute (Isaacson and Sandow, 1963). Current results with zinc are generally similar and have been extended to the toe muscle. In this work, after the usual Cl^- control series, we exposed toe muscles

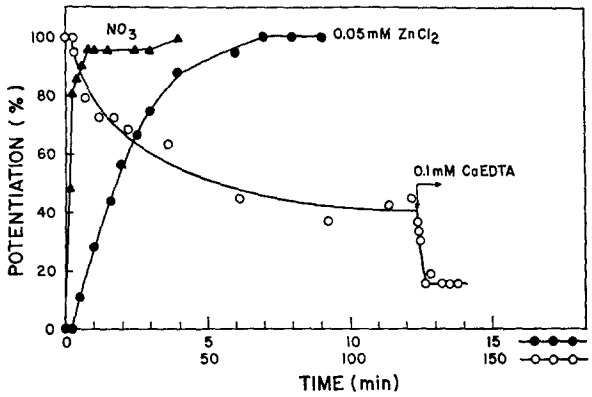

FIGURE 3. Kinetics of development and reversal of twitch potentiation (expressed in per cent of maximum potentiation) in frog toe muscle produced by 0.05 mM zinc. The development of potentiation in nitrate Ringer's solution is shown for another toe muscle with similar dimensions (0.4 mm diameter, 1.6 mg wet weight, 1.8 cm length). At 123 min 0.1 mM CaEDTA was added to reversal Ringer's solution. Note the tenfold difference in the two time scales.

to NO_3^- Ringer's solution so that we used the kinetics of NO_3^- action as a control for the studies with the heavy metals. After NO_3^- potentiation plateaued, it was reversed in Cl^- Ringer's solution and then the muscles were exposed to Zn^{2+}, UO_2^{2+}, or Cd^{2+}. Thus in a single preparation we compared the kinetics of action of the metal ions directly with that of NO_3^-.

A. DEVELOPMENT These experiments demonstrate that the ions of the metals potentiate the twitch much more slowly than NO_3^- does. For example, as shown in Fig. 3, the NO_3^- effect had a half-time of about 6 to 12 sec for toe muscles of about 0.4 mm diameter; while the 0.05 mM zinc potentiation, after an initial lag phase of about 12 sec, developed with a half-time of 96 sec. Often, the nitrate effect reached an apparent plateau after 1 min, but, as time increased, further potentiation slowly developed so that after 2 or 3 min

there was generally a further 15% increase in twitch tension, and maximal increase occurred about 10 min after exposure to NO_3. Toe muscles exposed to uranyl ion in concentration of 1 μM (Fig. 4) exhibit a lag phase similar to that found with 0.05 mM zinc, and thereafter develop potentiation with a half-time of about 10 min (6, 12, and 13 min in three separate experiments). Cadmium in concentrations of 0.05 mM does not exhibit a lag phase and potentiation develops with kinetics faster than that of 0.05 mM zinc.

B. REVERSAL IN RINGER'S SOLUTION In general, the potentiation effect of these cations reverses upon restoration of a treated muscle to normal Ringer's solution, but at a much slower rate than that of development of potentiation. Previous work, (Isaacson and Sandow, 1963) and see also Fig. 3

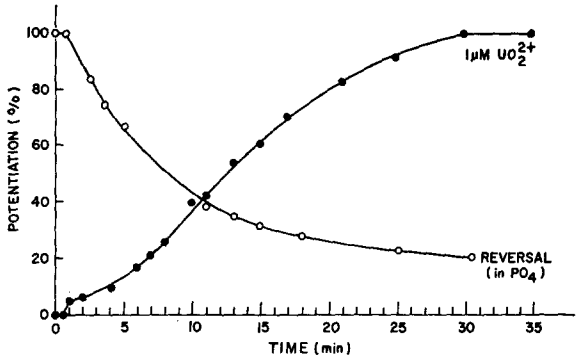

FIGURE 4. Kinetics of development and reversal of twitch potentiation (expressed in per cent of maximum potentiation) in frog toe muscle produced by 1 μM uranyl. Reversal Ringer's solution contained 4 mM phosphate. Muscle length, 2.1 cm; diameter, 0.4 mm; and wet weight, 2 mg.

shows that 0.05 mM zinc potentiation reverses at about 1/10 the rate of its development. Current studies with 0.5 mM Cd^{2+} likewise exhibit reversal kinetics at about 1/10 that of development.

Uranyl potentiation, produced, e.g., by a concentration of 0.3 μM, does not significantly reverse within 100 min in normal Ringer's solution. However, an overnight soak in Ringer's solution at about 6°C does reverse uranyl potentiation.

IV. *Reversal of Heavy Metal Potentiation by Chemical Agents*

In the previous experiments reversal of a potentiation depends on reversal of the processes causing the original potentiation, and thus it occurs at a rate determined by the kinetics of the reaction reversing the binding of the potentiating agent and the speed of diffusion of the agent away from the site of

binding. In the following experiments we used as reversing agents substances to which the metal ions in question can bind. Thus the speed of reversal depends practically entirely on the speed of diffusion of the reversing substances to the sites in the muscle at which the potentiating agents are bound and on the capacity of the reversing agents to compete with the ligands of such sites for the binding of the heavy metal ions. We chose as the reversal agents EDTA (as the Ca-salt so as to avoid chelation of the Ca of Ringer's solution), phosphate, and cysteine (in regard to its sulfhydryl ligand), since, in general, these substances form complexes, though of greatly varying binding strengths, with the various heavy metal potentiators of our experiments.

Fig. 3 illustrates that 0.1 mM CaEDTA, added to normal Ringer's solution, greatly hastens the reversal of 0.05 mM zinc potentiation in toe muscle. A similar result with sartorius muscles has already been mentioned in a preliminary report (Isaacson and Sandow, 1961). After reversal in 0.1 mM CaEDTA has been attained, reexposure of a muscle to zinc Ringer's solution produces a second potentiation. This confirms (Isaacson, 1961) that 0.1 mM CaEDTA does not in itself damage the muscle during the times of exposure used, as can also be concluded from the maintenance of tetanus tension at essentially its initial value during such an experiment. Additional experiments show that 0.5 mM CaEDTA speeds the reversal of 0.5 mM Cd^{++} potentiation, while 0.1 mM CaEDTA does not reverse 1 μM uranyl potentiation.

Inorganic phosphate (2 to 4 mM) added to Ringer's solution (as a buffer mixture of NaH_2PO_4 and Na_2HPO_4 at pH 6.8) causes essentially complete reversal of potentiation by 0.3 to 1 μM uranyl. Fig. 4 illustrates the effect of 4 mM PO_4 on reversing 1 μM UO_2^{++} potentiation of a toe muscle. An unexpected observation was that uranyl-treated toe muscles which had been reversed in PO_4 Ringer's solution redeveloped potentiated twitch responses when they were simply replaced in normal Ringer's solution. This redevelopment of potentiation occurred with more rapid kinetics (half-time of 1.5 min) than that obtained originally in uranyl Ringer's solution (half-time of 5.4 min).

Higher concentrations, 5 to 10 mM, of inorganic phosphate can hasten the reversal of zinc potentiation. However, in lower phosphate concentrations, e.g., 2 mM, reversal is not speeded up and, furthermore, muscles can be potentiated by 0.05 mM zinc in the presence of 2 mM phosphate.

Cysteine (0.5 mM) added to Ringer's solution hastens the reversal of twitch potentiation produced by either 0.05 mM zinc or 0.5 mM cadmium. Thus, the half-time of reversal in both cases (in experiments done on sartorii) was about 3.5 min, whereas in the absence of the cysteine, this half-time is about 55 min. However, exposure of a muscle for 1 hr to 0.5 mM or even 5 mM cysteine does not appreciably reverse 1.0 μM uranyl potentiation.

V. *Ligands Possibly Involved in Binding of Metals to Muscle*

To test the possibility that zinc potentiation ensues as a consequence of binding the metal to sulfhydryl groups of muscle, we pretreated sartorii for about 1 hr with low concentrations (0.01 mM) of sulfhydryl reagents. These muscles were then immersed in Ringer's solution containing equimolar (generally 0.01 mM) amounts of sulfhydryl reagent and zinc. Twitch and tetanus responses recorded during the muscle's exposure to either the sulfhydryl reagent alone or combined with zinc, showed essentially normal potentiation. We have previously reported (Sandow and Isaacson, 1963) that neither mersalyl nor

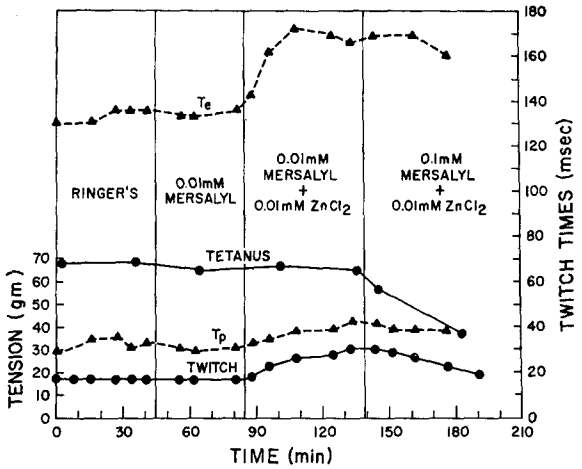

FIGURE 5. Test for effect of mersalyl on zinc twitch potentiation in frog sartorius muscle. Twitch and tetanus tensions are scaled on left ordinate. Time to peak of twitch (Tp) and time to end of twitch (Te) are scaled on right ordinate.

iodoacetamide block zinc potentiation. Since then, our work shows that NEM (N-ethylmaleimide) and PCMB (*p*-chloromercuric benzoic acid) likewise do not block zinc potentiation. Furthermore, increasing the concentration of the sulfhydryl reagent to 0.1 mM in the reversal Ringer solution, i.e. to 10 times the concentration of zinc previously used, did not speed the reversal of potentiation.

Results of a typical experiment involving mersalyl are presented in Fig. 5. The concentration of Zn^{2+} in this experiment, 0.01 mM, produced much less potentiation than that maximally obtainable at 0.05 mM (see Fig. 2). But we used this low concentration since it was desirable to keep the equimolar concentration of mersalyl sufficiently low to avoid deleterious effects. That this aim was actually achieved in this experiment is indicated by the constancy of

the tetanus and the twitch output so long as the muscle was exposed to 0.01 mM mersalyl. The results indicate, however, that 40 min pretreatment of the muscle with the 0.01 mM mersalyl did not prevent 0.01 mM Zn^{2+} from producing typical potentiation effects as is manifested not only by the increase in twitch tension, but also by the increase in times taken by the muscle to reach both the peak and the end of the twitch. It is also apparent in this figure that increasing the mersalyl concentration tenfold did cause a decrease in the zinc-potentiated twitch output. However, this is not a reversal of potentiation but a generally harmful effect since the tetanus output also suffered a decrease which was even more pronounced than that of the twitch. Fig. 6 presents

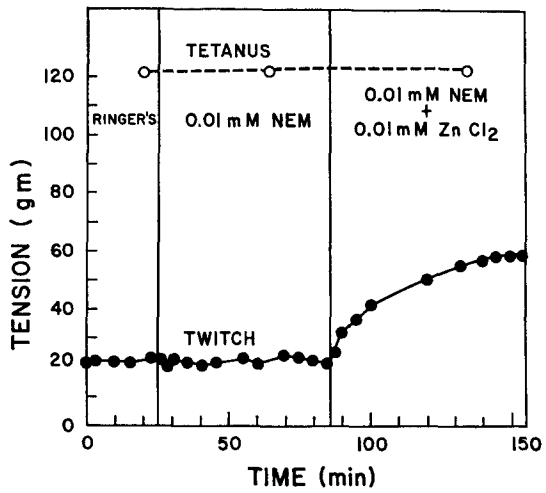

FIGURE 6. Test for effect of NEM (*N*-ethylmaleimide) on frog sartorius muscle twitch potentiation produced by zinc.

results of a similar experiment involving NEM as the sulfhydryl reagent, and it is clear that this substance does not inhibit potentiation of the twitch by zinc.

Essentially similar results were obtained with uranyl and cadmium as the potentiating agents, i.e., equimolar amounts of any of the four sulfhydryl reagents tested did not inhibit potentiation by these metals. We conclude from all these findings that SH groups are not involved as ligands in the binding of the heavy metal ions which determines potentiation.

An incidental observation in this study was that 1 mM NEM-treated muscles rapidly develop an increase in resting tension.

We attempted to determine whether imidazole groups may be involved in zinc-induced potentiation by first equilibrating muscles in Ringer's solution buffered to pH values below and above that of the pK_a of imidazole, 6.95 (Edsall and Wyman, 1958), and then trying to obtain the threshold concentration of zinc required to produce potentiation in each of these media. These attempts, however, have been inconclusive since it proved to be practically

impossible to control the incidental variations in muscle output owing to the pH variations themselves.

In another approach to the possibility of imidazole involvement we planned to test the effects of exogenous imidazole on zinc potentiation. As a preliminary control experiment, we first exposed muscles to imidazole in concentrations of 2, 5, and 10 mM at pH 7.2. All such treated muscles, however, developed typical potentiation effects. The potentiation of the twitch was graded with concentration, and in a test made after exposure to 2 mM imidazole the potentiation could be easily reversed in about 50 min of exposure to normal Ringer's solution.

As for the possibility that phosphate ligands are involved in the primary reaction leading to potentiation by the heavy metal ions, our only experiment in this regard was the one already mentioned involving use of the uranyl ion, which unexpectedly presented us with the very striking result that it is itself a very strong potentiator, in fact, the most powerful discovered to date. The possible topochemical significance of this result will be discussed later.

DISCUSSION

Our work shows that the ions of beryllium, cadmium, barium, nickel, copper, platinum, uranyl, and imidazole (whether in its charged or uncharged form is still undetermined), must be added to the already long list of substances (Sandow, 1964) that potentiate skeletal muscle contraction by prolonging the active state. Not all metallic ions are potentiators, however. Thus, strontium produces variable effects, and the ions of silver, lead, and mercury are all toxic to muscle and cause decreases in contractile output. The results with Ag^+ are especially interesting because they suggest that the progressive fall in output of a muscle exposed for a long time in a massive stimulating bath containing Ag-AgCl electrodes is due to Ag^+ released from the electrodes. It is particularly striking that supernormal external concentrations of Ca^{2+} and Mg^{2+} do not potentiate but decrease contraction (Paul, 1960; Sandow and Preiser, 1965), for these ions are both fairly abundant in myoplasm and, furthermore, evidently physiologically essential in the normal responses of muscle.

Our studies of the reversal of potentiation by means of the chemical binding agents strongly indicate that the potentiators involved in our tests act at readily accessible sites. Thus, CaEDTA does not penetrate the muscle fiber membrane (Bianchi, 1965) and yet it very rapidly causes a great increase in rate of reversal of potentiation due to zinc and cadmium. In the typical instance of this increase (given in Fig. 3), which involves the action of zinc on the toe muscle, the effect of the CaEDTA is very pronounced at only 1 min after its application to the muscle, and this suggests that the immediate action of the chelator must have occurred at readily accessible surfaces of at least the

superficial fibers of the muscle. And the course of the reversal as a whole is so rapid (half-time of about 2 min) that it most likely depends on only the time for diffusion of the CaEDTA to these surfaces of the fibers throughout the muscle. These reversal effects cannot be ascribed to disturbances in a possible role of Ca in muscular response, since use of the calcium salt of the chelator precluded chelation of the Ca involved in normal activity. Nor are they due to a direct action of EDTA, as such, since its effect on Zn-treated muscles is merely to reverse the potentiation and thus restore essentially normal activity. The EDTA evidently acts by means of its great binding capacity for zinc. This is much greater than it is for Ca^{2+}, or for Mg^{2+}, as is indicated by the respective affinity constants of these metals for EDTA, 3.2×10^{16}, 5.0×10^{10}, and 5.0×10^{8} (Bjerrum et al., 1958). Thus, we conclude that the zinc causing the potentiation had been adsorbed at surface membrane sites and that reversal was accomplished by the ability of the CaEDTA, once it had diffused to these sites, to elute the zinc in exchange for its Ca and thus nullify the action of zinc as a potentiator. Cysteine, also, evidently causes rapid reversal of both zinc and cadmium potentiation by its capacity to bind and thus elute these metallic ions from their active sites. It is well known that both these metals tightly bind to cysteine or equivalent SH compounds, the stability constants being about 10^{7} or 10^{8} (Gurd and Wilcox, 1956; or, specifically for the Zn^{2+}-cysteine complex, $10^{9.86}$, Bjerrum et al., 1958). The fact that the reversal occurs, in the sartorius, with a half-time of 3.5 min, again suggests that the active sites are at fiber structures that are reached relatively quickly by simple diffusion of the cysteine through the extracellular space of the muscle.

Similar considerations apply to the reversal experiments involving the uranyl ion, only here the reversing agent is phosphate, not EDTA or cysteine. Our inability to reverse uranyl potentiation by means of EDTA is evidently explained by the fact that the stability constant for the complex between EDTA and UO_2^{2+} is very low, of the order of 10^{-1} or less under the conditions of ionic strength and pH of our Ringer's solution medium (personal communication from Dr. R. D. Spitz of the Dow Chemical Company, Midland, Michigan). And, as for cysteine, this does not complex with uranyl (Rothstein, 1962). In contrast, the stability constants involving phosphate are about 10^{2} to 10^{3} (Bjerrum et al., 1958) and hence this ion can combine with and thus remove the uranyl ion from the sites of the muscle at which it acts, especially since our experiments involved phosphate at a concentration (2 to 4 mM), several thousand times greater than that (1 μM) of the uranyl ion. As for the locus of this reversal process, it must also be at some easily accessible structure, since phosphate, with a very high degree of preference, accumulates at the outer surface of the muscle fibers rather than penetrating into them (Causey and Harris, 1951). And, insofar as it does enter the fiber, the rate of penetration is so low, according to Simon (1962), with a half-time of 1434

min, that it would be most unlikely that penetration of the phosphate were a necessary condition for the reversal of potentiation due to the uranyl ion, which occurs with a half-time of only some 15 min. These considerations must also apply to our result that phosphate in sufficiently high concentration also causes a rapid reversal of the potentiating action of zinc. Hence, this action of phosphate is essentially similar to that of EDTA or cysteine, and it therefore reinforces the conclusion previously drawn from the work with EDTA that the primary action of zinc occurs at easily accessible structures.

It is clear that our current findings remove the uncertainties of our previous reports (Isaacson and Sandow, 1963; Sandow and Isaacson, 1963) regarding the location of the structures involved in the primary action of the heavy metal ions. It is obvious from our new results that such structures must in the first place include the plasma membrane. But the membranous walls of the T tubules may also be involved since it is now clear that these structures are in open communication with the external medium (Franzini-Armstrong and Porter, 1964; Peachey, 1965) and that they are readily entered by substances whose particle size is so large that they do not pass across the plasma membrane (Huxley, 1964; Endo, 1964). Thus, in terms of mere accessibility of fiber structures to the potentiators and their chemical reversing agents, we can infer that the primary action of our potentiators occurs at either the plasma membrane or the T tubule, or at both of these.

Relevant to the problem of determining more definitely the site of action of the metallic potentiators, and also bearing on the question of their mechanism of action, is that Zn^{2+} and UO_2^{2+} both greatly reduce the rate of repolarization during the recovery phase of the action potential and thus prolong the spike by as much as 3 to 4 times (Kobayashi, 1962; Sandow et al., 1964; Sandow et al., 1965). This effect is discussed in detail elsewhere (Sandow and Preiser, 1964; Sandow et al., 1964, 1965) in relation to the role played generally by the duration of the spike in determining the reactions by which excitation-contraction (E-C) coupling supposedly leads to the release of Ca^{2+} from the sarcoplasmic reticulum and hence to activation of contraction. The evidence suggests that although the primary action of the heavy metal ions is the prolongation of the spike, this, in turn, causes a proportionately supernormal release of Ca^{2+} which results in a corresponding prolongation of the active state and potentiation of the twitch.

Mashima and Washio (1964), using results of their experiments on the frog semitendinosus, question whether prolongation of the spike is the only effect of Zn^{2+} which determines its capacity to potentiate the twitch. They claim (*a*) that, as the concentration of Zn^{2+} was raised beyond 50 μM, the twitch tension was not further increased even though the duration of the action potential continued to increase, and (*b*) that the increase in twitch tension with time under action of, e.g., 50 μM Zn^{2+}, lagged behind the development of prolonga-

tion of the action potential, which "occurred instantaneously after adding Zn." Work from our laboratory (Taylor and Isaacson, 1965) proves, however, (*a*) that the failure of twitch tension to continue to increase as the concentration of Zn^{2+} was increased beyond 50 μM is attributable to an increase in mechanical threshold caused by such abnormally high concentrations, and (*b*) that, in carefully controlled experiments on the toe muscle, the prolongation of the action potential was not found to occur "instantaneously" but to develop in good synchrony with development of twitch potentiation. We, therefore, conclude that the essential immediate effect of Zn^{2+}, and also of UO_2^{2+}, in causing potentiation is, as previously stated, prolongation of the action potential.

This conclusion implies that the metal ions act primarily at the membrane concerned with excitation changes and that their effect in retarding the spike's repolarization phase results from a reduction, along with a prolongation in time, of the increase of potassium conductance, i.e. of delayed rectification, which predominantly determines the falling phase of the action potential. Evidence supporting the inference that Zn^{2+} reduces the effectiveness of delayed rectification has been reported by Mashima and Washio (1964). It has long been thought that delayed rectification is one of the functions of the plasma membrane as it performs its cycle of altered conductances determining the course of the action potential (e.g. Hodgkin, Huxley, and Katz, 1949; see also, e.g., Adrian and Freygang, 1962). But the results of Freygang and coworkers (1964, 1964) and Hellam et al. (1965) concerning the late after-potential of the muscle fiber's action potential indicate that delayed rectification may occur in the wall of the T tubule as well as in the plasma membrane. And we must therefore infer that, if the heavy metal ions reduce the intensity of delayed rectification, then they must exert their primary action at sites on either or both of these membrane structures. That such an action of the metal ions is indeed possible is supported by the strong indications previously discussed that these structures are easily reached by simple diffusion of the heavy metal ions from their source in the external medium and the extracellular space of the muscle. It is also interesting that Zn^{2+} in 0.05 mM concentration considerably reduces the magnitude of the late after-potential without affecting the half-time of its decay (Taylor and Sandow, 1965). For this is to be expected if the late after-potential is produced, as proposed by Freygang, Goldstein, and Hellam (1964), by an accumulation of K^+ in the lumen of the T tubules resulting from delayed rectification in these tubules, and if Zn^{2+} does in fact tend to impair this function of the tubules.

We now consider certain kinetic aspects of the action of heavy metal ions which raise certain questions regarding the mechanism of their primary action. A minor point is that development of potentiation in the toe muscle

is, in general, some 4 to 5 times faster than in the sartorius. This is evidently due to the greater speed with which a toe muscle becomes saturated with a potentiator by diffusion from the external medium, because this muscle's thickness is less than that of the sartorius and because its cylindrical shape provides a generally faster diffusion system than does the plane sheet structure of the sartorius. More interesting, however, is the much slower rate of development of potentiation caused by the heavy metals relative to that caused by the lyotropic anions, even though both of these classes of potentiators seem to act not only at the plasma membrane but also at the more deeply located T tubule membrane (in respect to the anions, see Hodgkin and Horowicz, 1960). We discussed this disparity for the sartorius in our earlier report (Isaacson and Sandow, 1963), and it still is found in our present studies of the toe muscle. Thus, typical results are indicated in the data for the toe muscle presented in Fig. 3 where the half-times of development of potentiation by NO_3^- and by zinc are 8 sec and 100 sec, respectively. This difference is very much greater than can be accounted for by the difference in the intrinsic diffusion coefficients of the $ZnCl_2$ and $NaNO_3$ involved in our experiments (see Isaacson and Sandow, 1963). We infer, however, that these results are explainable at least in part in terms of the difference in binding of NO_3^- and Zn^{2+} to connective tissue in the muscle. Such binding would, in general, retard development of saturation of the extracellular space within the muscle in respect to the prevailing external concentration of the potentiator and thus delay development of potentiation. The influence of such binding is seen directly as a retardation of diffusion by the theoretical considerations (Crank, 1956) showing that binding reactions, in effect, reduce the diffusion coefficient. Thus, if we consider that zinc, e.g., is bound to connective tissue so that its concentration, in the bound state, S, is directly proportional to its free concentration (i.e. $S=RC$), then the diffusion coefficient of the ion in the extracellular space is reduced by the factor $(R + 1)$.

Now, no data seem to be available regarding binding of anions such as NO_3^- to connective tissue; but the fact that the entry and exit of these ions into muscle occurs at a rate commensurate with free diffusion in the extracellular space (Hill and Macpherson, 1954; Kahn and Sandow, 1955) and in the T tubules (Hodgkin and Horowicz, 1960) suggests that, if such binding exists, it plays an insignificant role in determining the kinetics of either development or reversal of potentiation. Zinc, however, binds to tendon and to the intramuscular connective tissue to the amount of about 1.0 μmole/gm (Isaacson and Bianchi, 1962, and unpublished results). It is thus possible that potentiation by zinc occurs very slowly because such binding reduces the effective diffusion coefficient within the free space of the muscle and thus slows up the accumulation of the free Zn^{2+} in this space which determines the degree of binding to, and thus the development of potentiation of, the

muscle fibers. This sort of reduction of the diffusion coefficient will also hold for the reversal of potentiation. But this cannot be the main factor determining the slow kinetics of reversal, for, in the absence of chelating adjuvants, reversal is 10 times slower than even the relatively retarded rate of development of potentiation. Hence, we suppose that the rate-limiting factor in this very sluggish reversal is the great slowness by which the binding of Zn^{2+} to its active sites is reversed when the zinc-treated muscle is replaced in Ringer's solution. Presumably the same mechanisms occur with UO_2^{2+} as the potentiator. But here both the development and reversal of potentiation are considerably slower than their respective counterparts with Zn^{2+} as potentiator, and we therefore infer that the UO_2^{2+} binds to both connective tissue and muscle fiber much more tightly than does Zn^{2+}. We find evidence in support of this inference, at least for the binding to the muscle fiber (as will be described in detail later), in the fact that the apparent binding constant for UO_2^{2+} is about 100 times greater than that for Zn^{2+}. Also relevant in this respect is that Cd^{2+} both produces and reverses potentiation effects more rapidly than Zn^{2+}, as would be expected since it has a smaller apparent binding constant than Zn^{2+}. Thus, these various results indicate that, in general, the slower the development and especially the reversal of the potentiation effects due to a given metal, the tighter is the binding of the metal to the fiber ligands.

The relatively slow development of the potentiating effects of the heavy metal ions on whole muscles is not entirely explained, however, by the binding of these ions to the muscles' connective tissue. This can be inferred from the results of experiments done in this laboratory by Dr. M. Matsumura on preparations of only two or three fibers. In such preparations choline replacement of Na^+ sets up inexcitability practically instantaneously, and NO_3^- causes potentiation to develop with a half-time of about 1 or 2 sec. Thus these preparations generate changes in their responses at essentially the same speed as do similarly treated single fibers (Hodgkin and Horowicz, 1960), and therefore the role of diffusion of the relevant agents to their reactive sites in development of the effects of interest is the same as in the single fiber systems. Furthermore, such preparations will not retard diffusion to key sites by extraneous binding since they contain negligible amounts of connective tissue. Despite these conditions favoring quick access of substances to at least the plasma membrane, the experiments show that potentiation by Zn^{2+} (50 μM as in our toe muscle experiments) develops with an average half-time of 4.8 sec. This result suggests that, even if the Zn^{2+} has already diffused to the plasma membrane, some other time-consuming process must occur before its effects become apparent in the increase of mechanical output. A possibility for this is a chemical time reaction with a half-time of the order of 5 sec, which is initiated when the Zn^{2+} makes contact with the ligand of the muscle fiber membrane involved in the reaction causing potentiation. But another possi-

bility is the need for zinc ions to make contact with sites of the T tubule in order to exert their effect. This would involve a delay due to diffusion into the tubules, which we can assume to be about double that for NO_3^- since at 25°C, the diffusion coefficient in free water medium of $ZnCl_2$, 7.1 × 10^{-6} cm^2/sec (Wang, 1954) is about half of that for $NaNO_3$, 13 × 10^{-6} cm^2/sec, (see, e.g., Kahn and Sandow, 1955). Thus, we might expect that Zn^{2+} would develop its effects in the T tubules with a half-time of as much as 4 sec. Since Dr. Matsumura's results prove that these effects do develop with an average half-time of 4.8 sec, we infer that the zinc ions exert their primary effect at least partly, if not wholly, at sites along the T tubules. Thus, this inference derived from kinetic considerations is in accord with the one previously made on the basis of the location in the T tubule of the effect of Zn^{2+} on delayed rectification.

We now consider the question of the detailed nature of the chemical reaction supposedly involved in the binding of the metal ions to the active sites of the susceptible membranes of the muscle. There does not seem to be any obvious common feature chemically characterizing the potentiating ions themselves. Thus, among the Group II elements, which are all divalent metals, Be, Zn, Cd, and Ba potentiated contraction, Mg, Ca, and Hg decreased it, and Sr had variable effects. The result that the divalent metallic potentiators act oppositely to the physiologically normal divalent Ca and Mg ions, suggests the possibility of strongly different, competitive effects especially if these various ions act at identical binding sites. Research in progress deals with such interactions and the results will be reported elsewhere. (For some further effects of increased concentration of Ca^{2+}, especially in relation to Luttgau's (1963) finding that such conditions increase the mechanical threshold of muscle fibers, see Sandow, Taylor, and Preiser, 1965). Of the Group VIII metals, we found divalent Ni and tetravalent Pt to be fair potentiators. Most striking, however, is our finding that the uranyl ion is the strongest of all potentiators tested so far, i.e. in the sense that it produces maximal effects, comparable to those of other metallic potentiators, at the least concentration which, at about 0.5 μM, is, e.g., $\frac{1}{100}$ of that at which Zn^{2+} causes its maximal effect. It is noteworthy that this ion, in common with our other outstanding metal potentiators, e.g. Zn and Cd, has the capacity to form four-coordinate complexes (Evans, 1963; for Zn and Cd, see Edsall and Wyman, 1958). One therefore wonders whether the basic chemical property of a metal ion determining its capacity to cause potentiation may not be dependent on the type of coordination complex it forms when it binds to some ligand of the cell membrane.

Certain aspects of our research suggest some detailed features regarding the primary reaction of the metals of interest. Thus, in the first place, the curves we have obtained relating degree of potentiation with concentration

of uranyl, zinc, and cadmium ions are all roughly S-shaped and embrace a range of about one and a half to two decades of concentration difference in extending from minimal to maximal effects. These results suggest that the ions exert their primary effect by means of a binding reaction to some membrane ligands. If we assume that this reaction follows first order kinetics, then the apparent binding constant for each such reaction is given by the reciprocal of the concentration of the ion yielding 50% of the maximal potentiation the ion can produce. Thus, the values of the binding constants of the ions represented in Fig. 2 are: UO_2^{2+}, 5×10^6; Zn^{2+}, 2.8×10^5; and Cd^{2+}, 2×10^4. It may be noted that these values provide a quantitative measure of the relative strengths of these ions ($UO_2^{2+} > Zn^{2+} > Cd^{2+}$) in causing potentiation.

Our attempts to determine the specific nature of the topochemical primary reaction involved in the binding of the metal ions indicate at least that SH groups, presumably of proteins, are not involved. That such groups may be important in maintaining the properties which are necessary for normal function of the muscle fiber is indicated by our results that, in relatively high concentration, the SH agents we used produced harmful effects. But, in concentrations low enough to avoid such effects and yet sufficiently high (0.01 mM) to equal that of Zn^{2+} permitting good, though not maximal, potentiation, mersalyl, NEM, PCMB, and iodoacetamide did not affect the capacity of the ion to cause potentiation. Very suggestive topochemically is our result that UO_2^{2+} is a powerful potentiator. This provides further evidence that SH groups are not involved in the binding reaction determining potentiation because UO_2^{2+} does not complex with these groups (Rothstein, 1962). As previously mentioned, we used the uranyl ion, in the first place, because we thought it would specifically complex with phosphate groups, to which we supposed Zn^{2+} might have to bind, and thus block the potentiating capacity of Zn^{2+}. Our discovery that UO_2^{2+} is itself a strong potentiator suggests, however, that, if it does indeed bind specifically to phosphate ligands, it, and also Zn^{2+}, Cd^{2+}, and possibly other divalent cations, may all cause potentiation by binding to these groups. Clear evidence that uranyl binds to polyphosphates at the surface of yeast cells has been presented by Rothstein and Meier (1951), but these experiments were done at pH 3.5 (so that the uranyl would take the form of the simple, unhydrated UO_2^{2+} ion) and it is therefore not clear whether this conclusion necessarily applies to our experiments at neutral pH. There is evidence that both Zn^{2+} and UO_2^{2+} bind to carboxyl ligands (Gurd and Wilcox, 1956; Feldman and Koval, 1962); but this possibility, for Zn^{2+} at least, would not seem to apply to our experiments since this binding requires that the Zn^{2+} be at concentrations greater than 20 mM (Gurd and Wilcox, 1956), whereas we obtain maximal potentiations by zinc concentrations as low as 0.05 mM. Another type

of complexing system is indicated, however, in that these ions bind to imidazole groups (for UO_2^{2+}, see Feldman and Koval, 1962; and for Zn^{2+} and Cd^{2+}, Edsall and Wyman, 1958). And thus it is possible that such ligands of membrane macromolecules, in addition to, or even to the exclusion of, the phosphate, may be essentially involved in the immediate action of the potentiators on the muscle fibers.

Obviously it is not possible now to identify the ligands of the muscle fiber which bind the heavy metal potentiating ions. But when our other results which indicate that this binding is evidently the cause of the observed slowing of the repolarization phase of the action potential are recalled, then our foregoing considerations suggest that some sort of complexing of the heavy metal ions such as UO_2^{2+}, Zn^{2+}, and Cd^{2+} with ligands of the plasma and/or tubular membranes suppressively affects the delayed rectification function of these membranes. It should be of the greatest interest regarding processes determining recovery of the membrane from excitation to know the specific nature of the reactions by which the ions bind to particular membrane ligands.

Whatever the actual details of these processes, it is evident from the foregoing discussion that the topochemical reactions, as such, of the heavy metal ions do not directly determine the basic changes of the active state causing twitch potentiation and the increase in early rate of tension development of the twitch (Sandow and Preiser, 1964; Sandow et al., 1965). Our results strongly suggest that it is essentially the prolongation of the action potential, which results from the specific binding of the metal ions, that modifies excitation-contraction coupling so as to produce the characteristic changes in the active state and the corresponding modifications of the twitch. Thus, we may consider that the effects of the heavy metal ions on muscle become manifest in two ways: directly, they modify the electrochemical reactions of the membrane determining the course of the action potential; and, indirectly, this effect on the action potential engenders changes in excitation-contraction coupling which culminate in the characteristic mechanical effects of potentiation. So long as it could be thought that the full course of the action potential in muscle fibers is determined by conductance changes in only the plasma membrane, we could then suppose that there was a rather clear cut separation of the excitation phenomena of interest, these being restricted to the plasma membrane, and the reactions that coupled these to the contractile material, which involved in the main an inward moving signal along the T tubules that caused release of Ca^{2+} from the sarcoplasmic reticulum and thus caused activation of contraction (for review, see Sandow, 1965). But we see now that such a sharp segregation of structures according to their particular functions in E-C coupling seems no longer possible if it is accepted that delayed rectification and the effect of heavy metal ions on it take place, even

if only in part, in the T tubules. For under such conditions, the repolarization phase of the action potential and its role in determining the mechanically effective period of the action potential (Sandow, Taylor, and Preiser, 1965; Sandow, 1965) become as much a function of the T tubule as of the plasma membrane. It is pertinent to recall, however, that the regenerative increase in Na conductance that determines the depolarization, i.e. the rising, phase of the action potential is a property of only the plasma membrane, this being indicated, e.g., by the extreme rapidity with which the muscle fiber becomes inexcitable when Na^+ is withdrawn and then becomes excitable again when the Na^+ is restored (Hodgkin and Horowicz, 1960).

Taking into account these various aspects of conductance change that produce the course of the action potential, we picture its role in E-C coupling as follows. The active process (Na-activation) determining the rise of the action potential occurs in the plasma membrane and this depolarization is spread inwardly along the T tubules as a passive electrotonic pulse which supplies the signal switching on the release of Ca^{2+} from the sarcoplasmic reticulum and thus starts the mechanically effective period of the action potential when depolarization of the plasma membrane causes the membrane potential to pass beyond the mechanical threshold. The duration of the mechanically effective period is now determined by the rate with which the action potential falls, i.e., by the speed with which delayed rectification, presumably in both the plasma and T tubule membranes, restores the resting membrane potential, switches off the release of Ca^{2+} and terminates the mechanically effective period. Looked at in this way, the role of the T tubule in E-C coupling is not merely passively to convey inward the electrical effects of the action potential at the plasma membrane, but also actively to participate, by means of its delayed rectification, in the electrochemical processes determining the mechanisms of the mechanically effective period causing activation of contraction. Or, considering these events otherwise, the evidence indicates that the action potential does not merely "initiate" E-C coupling, but by virtue of the nature of delayed rectification in the muscle fiber it, intrinsically, also participates in the inward-going coupling reactions that run their course in the T tubules.

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