Effects of Zinc on Responses

of Skeletal Muscle

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ABSTRACT Zn^{++} potentiates the twitch tension of the frog's sartorius muscle by as much as two to three times, and prolongs twitch time parameters. Tetanus tension is unchanged, but fusion frequency is reduced. Thus, the basic mechanical effect of Zn^{++} is prolongation, but not intensification, of the active state. Threshold effects appear at about 0.005 mM Zn^{++}, and maximal changes at 0.05 mM. In 0.05 mM, potentiation begins after a delay of about 1 min. and develops with half-time of 6 min.; full changes reverse in pure Ringer's with half-time of 60 min. Diffusion theory analysis of these kinetics indicates that the potentiating action of zinc involves special features not found in potentiation by anions: a primary membrane action is not excluded, but Zn^{++} may have to penetrate to and act at sarcoplasmic reticular or myoplasmic sites. Zn^{++} does induce excitatory membrane effects: the fall of the action potential is slowed and the refractory period is increased about four times; but excitability as such is not changed. Unique chemical features of Zn^{++} are discussed in relation to possible mechanisms of its action on muscle fibers.

We have developed the research of this paper in consequence of an unexpected observation made while studying certain photodynamic effects of basic dyes on muscle. The genesis of this observation, and preliminary results of the present work, are discussed in detail in a previous report (Sandow and Isaacson, 1960). In brief, we found that acridine orange caused not only photodynamic effects, but also a very large potentiation of the twitch tension of the directly stimulated frog's sartorius muscle without having any significant effect on the tetanus output. Our acridine orange contained zinc on a mole to mole basis (Beers, 1960), and special control tests proved that the twitch augmentation depended merely on the presence of the metal in the dye and it could be evoked in muscles treated simply with 1 mM $ZnCl_2$ in Ringer's solution.

Quite a large number of chemically diverse substances are known to potentiate the twitch (e.g., bromide, nitrate, and iodide (Kahn and Sandow, 1950, 1955; Hill and Macpherson, 1954); quinine and quinidine (Harvey, 1939;

Lammers and Ritchie, 1955); tetraethylammonium (Edwards *et al.*, 1956); adrenaline (Goffart and Ritchie, 1952), etc.). It was thus of general interest to add zinc to this list. But our finding regarding this ion seemed particularly significant in view of the following highly special features of zinc in biological systems. This metal, unlike other potentiators (with the exception of adrenaline) is a naturally occurring constituent of tissues, and in amounts generally exceeding trace concentrations. According to Edman (1958) the rabbit psoas muscle, *e.g.*, contains zinc in a concentration comparable to that of calcium. Zinc is clearly an essential element in nutrition (*e.g.* rats, Day and McCollum, 1940) and this is evidently a consequence of its being the specific metal of several metalloenzymes, such as lactic dehydrogenase and carbonic anhydrase (Vallee, 1959). Finally, although nothing is known regarding the function of zinc in contraction of living muscle, it remarkably influences the mechanical behavior of glycerol-extracted muscle fibers. Edman's very thorough research on this problem (1958, 1959 *a–d*, 1960 *a*, *b*) proves that, in the presence of adenosinetriphosphate and Mg in physiological concentrations, zinc in small concentrations of the order of 0.001 mM enhances the contractility of the glycerinated fiber, but in larger concentrations, *e.g.* 0.25 mM, it depresses contractility, and even more strikingly, causes contracted fibers to relax. Edman (1960 *b*) has stressed the role of zinc as a relaxing agent and has in fact proposed a mechanochemical theory of physiological contraction in which the zinc normally present in muscle fibers is given a key role in determining relaxation in the mechanical response of living muscle.

This brief summary of certain features of the biological importance of zinc suggests that its capacity to potentiate contraction of living muscle may be related to the role played by it as an intrinsic constituent in muscle in the general mechanisms of response of contractile tissue. We are currently investigating this possibility. The present paper, however, is limited to experiments concerned directly with the potentiating effect of zinc and certain possibly related effects on excitability phenomena. Some of our results have already been briefly reported (Sandow and Isaacson, 1960; Isaacson and Sandow, 1961).

METHODS

We used sartorius muscles from the frog, *Rana pipiens*, which were excised with the pelvic bone attached and equilibrated before use for at least 1 hour in 100 ml of oxygenated Ringer's solution. The Ringer's solution was made up of reagent grade chemicals in glass-distilled water as follows: 117 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 2 mM tris (hydroxymethyl)aminomethane–HCl buffered to pH 7.2. We did not use the more usual phosphate buffer, since this would involve precipitation of zinc as zinc phosphate. Curare, added as routine to the Ringer's solution at a concentration

of 2×10^{-5} gm/ml, prevented indirect stimulation of the muscle through the nerve fibers and end-plates within the whole muscle. Thus we observed in this study the effects of zinc only on the muscle fibers.

We report zinc concentrations in units of moles per liter, calculated from the addition of a 0.1 or 0.05 M zinc chloride (Fisher, reagent grade) stock solution in distilled water to the Ringer's solution just prior to use. The stock solutions were prepared as rapidly as possible to mitigate effects of the deliquescence of the $ZnCl_2$. We filtered the zinc stock solution through Whatman No. 12 filter paper to remove an insoluble suspension, presumably of $Zn(OH)_2$, that initially formed. An intercomparison, of a $ZnCl_2$ stock solution and a zinc solution prepared by dissolving 30 mesh granular zinc metal (Mallinckrodt, analytical reagent) in HCl solution, served as a control of the deliquescent property of $ZnCl_2$. A dithizone colorimetric analysis (Sandell, 1959) showed that the zinc concentrations of the two solutions agreed to within 10 per cent.

Precipitation of zinc in the form of an insoluble hydroxide limits the concentration that can be put into solution at physiological pH, as appreciated in some studies (Edman, 1958; Lallier, 1955) but not in all (*e.g.*, Woronzow, 1926). The literature values of the solubility product vary from 1.8×10^{-14} (Hodgman *et al.*, 1959) to 7×10^{-18} (Fulton and Swinehart, 1954). The corresponding upper limit of zinc in solution at pH 7 is 1.8 M using the first quoted solubility product and 0.7×10^{-3} M using the latter. Even if the smaller solubility product is accepted, our zinc concentrations were correct in general, since most of our experiments employed lower zinc concentrations (0.1 mM or less, occasionally 1 mM).

We mounted muscles under a constant resting tension of 1 to 2 gm, and recorded mechanical responses using the RCA 5734 transducer tube and an isometric lever. The output of the 5734 appeared on a channel of a dual beam oscilloscope. In general, we stimulated muscles with 0.3 msec. slightly supramaximal square wave shocks, and tetanic stimuli involved a train duration of 200 msec. and a frequency of 120 shocks/ sec. except as noted later in the special experiments on fusion frequency.

We used as stimulating electrodes either two massive Ag-AgCl plates flanking the entire muscle (Sandow, 1947) in a chamber with 100 ml of Ringer's solution, or at times, Ag-AgCl wire electrodes under moist chamber conditions. In either chamber, both sides of the muscle were freely exposed to the Ringer's solution during equilibration to either the normal or the experimental solutions. The wire electrode chamber was employed when we wished to record the diphasic action potentials. Action potentials, after amplification, appeared on a channel of the dual beam oscilloscope. An audio oscillator signal served to calibrate the oscilloscope sweep speed.

All experiments were performed at room temperature (range 21 to 25°C, average 23°C). Additional procedures dealing with certain experiments are described at relevant parts of the Result section.

Generally, a slow decline in mechanical output occurs when a muscle is studied for a period of hours in Ringer's solution. In control of this, we recorded normal twitch and tetanus responses, generally three of each at approximately equal time intervals, for about 30 min. before any experimental treatment. Thus we distinguished experimentally induced changes in tension output from this more general decline, not related to the experimental treatment.

RESULTS

I. *Mechanical Features of Potentiation by Zinc*

Fig. 1 illustrates the general effects of zinc on isometric twitch and tetanus responses of the frog sartorius muscle stimulated with slightly supramaximal shocks. Comparison of Figs. 1 *a* and 1 *c* shows that after treatment with 0.1
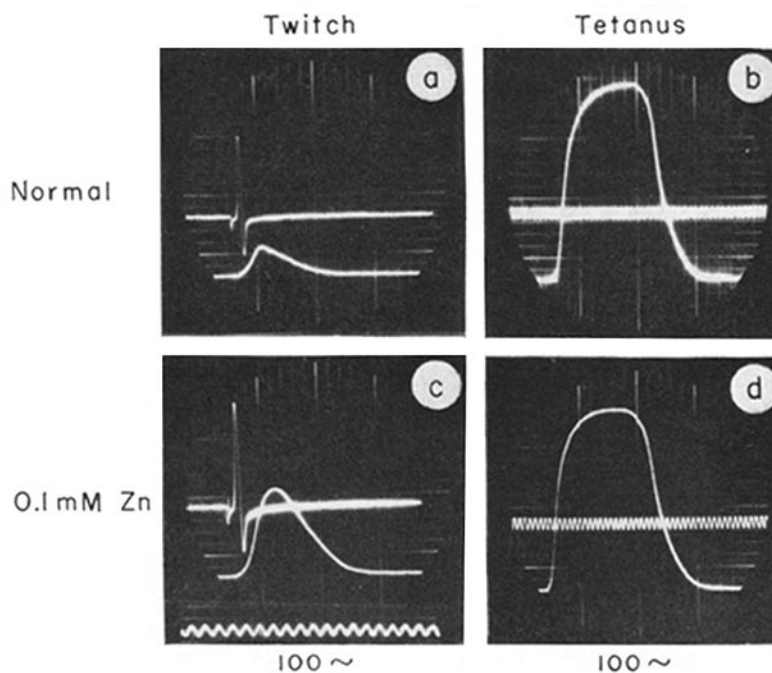

FIGURE 1. Effects of 0.1 mM zinc on isometric twitch and tetanus responses of frog's sartorius muscle. In (*a*) and (*c*) the myogram is on the lower sweep and the associated action potential on the synchronous upper sweep. The experimental responses in (*c*) and (*d*) were obtained after 30 min. exposure of the muscle to the 0.1 mM zinc solution.

mM zinc, the muscle develops tension, in response to a single shock, that is about three times greater than the normal. This increase is not due to any repetitive firing of the treated muscle, as is evident from the presence of only a single action potential in the response. Nor is it caused by recruitment of previously unresponsive fibers, since the supramaximal shocks assured that the control twitch was maximal. Hence, the augmented response activated in the presence of zinc by a single shock is a twitch whose enhanced tension develops because each fiber of the muscle produces a potentiated contraction.

The myograms of Figs. 1 *b* and 1 *d* demonstrate that the tetanus tension is slightly reduced in the test involving zinc. But this diminution, which is

typical, is not caused by the metal and is ascribable to the general progressive deterioration in tetanus output that develops with time even in the normal muscle. It will be seen later that high concentration of zinc can reduce contractility in general, and that, because of the large increase in refractoriness effected by zinc, tetanus output can be markedly impaired if the tetanus stimulus frequency is too high. These changes, however, involve special effects of zinc that will be discussed in detail later. In respect to our present interest in intrinsic mechanical effects, our results prove that zinc, acting in concentrations causing maximal potentiation (0.05 to 0.1 mM), does not reduce, nor does it increase the peak tetanus output of the muscle.

In the course of this work, we have observed that, in concentrations of zinc over a wide range (0.05 to 1 mM), the degree of twitch potentiation (expressed, *e.g.*, as the percentage increment in twitch tension relative to the normal value) varied from as low as 60 to as high as 490 per cent. Special studies (Isaacson, 1961, 1962) prove, however, that by far the greater part of this variability is a consequence, in our experiments at room temperature, of purely incidental mechanical (*i.e.*, series elastic) features involved in isometric recording. These factors directly affect the normal muscle's twitch to tetanus tension ratio $(P/P_o)_n$ and thus cause the degree of potentiation induced by zinc to vary inversely with $(P/P_o)_n$. (See also Ritchie, and Wilkie, 1955.) At a value of this ratio of, *e.g.* about 0.3, the degree of potentiation in our work averaged about 100 per cent (range: 60 to 130 per cent).

The contractions produced under the action of zinc exhibit not only (twitch) potentiation, but also alterations in time parameters. Reference to Fig. 1 shows that the zinc-potentiated twitch shows increases in both contraction and relaxation periods, and thus in total twitch time. In a series of twitches maximally potentiated by 0.05 mM zinc, the increases in contraction and relaxation periods respectively, were 74 and 42 per cent, with an average standard deviation of ±12 per cent. Even though zinc does not alter the plateau tension of the tetanus, it does somewhat hasten attainment of the plateau and prolong the time of relaxation from it.

Changes in tetanus kinetics, however, are best indicated by our studies of the effect of zinc on the fusion frequency. In these experiments tetani of 200 msec. duration were evoked by massive stimulation at different frequencies in the range from 30 to 150 shocks/sec. with the muscle first in normal Ringer's solution and then after equilibration in 0.1 mM zinc Ringer's for 1 hour. These tetani were, in general, not fused, and the degree of lack of fusion was measured by the ratio $\Delta P/P_{av}$, in which ΔP = the maximal fluctuation in tension, and P_{av} = the average tension of the unfused plateau. In Fig. 2, the records of the inset demonstrate that the degree of fusion in the response to a tetanus stimulus of 30 shocks/sec. is very much better in the presence of 0.1 mM zinc than in ordinary Ringer's solution. The curves (Fig. 2), which

relate $\Delta P/P_{av}$ to the frequency of the tetanus, clearly indicate that, in general, the degree of fusion at any frequency is much greater in the presence of zinc than it is in the control medium. Furthermore, this figure shows that for perfect fusion (*i.e.*, $\Delta P/P_{av} = 0$), the normal muscle requires a frequency of tetanus stimulation of about 130 shocks/sec., whereas for the zinc-treated muscle this is only 70 shocks/sec.
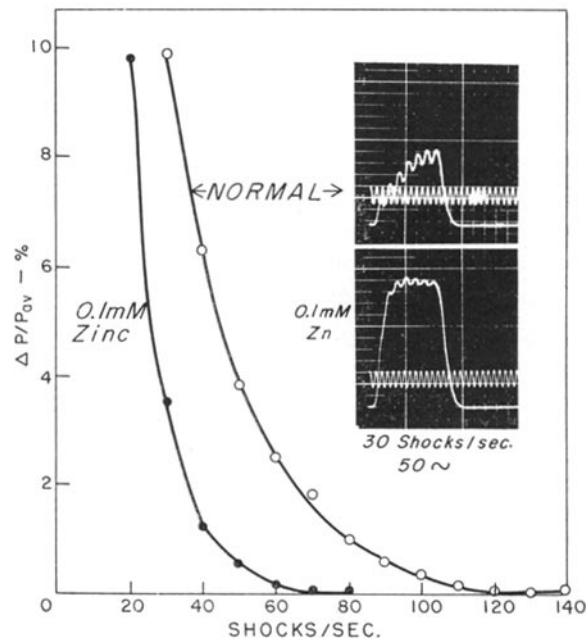

FIGURE 2.   Effect of 0.1 mM zinc on fusion frequency of tetanus responses. Average values (three experiments) of ($\Delta P/P_{av}$), per cent (a measure of the lack of fusion of tetanus responses) are plotted as a function of the frequency of stimulation (shocks/sec.) for muscles in normal and 0.1 mM zinc Ringer's solution. Inset shows typical increase in fusion of a 30 shocks/sec. tetanus response after a muscle's exposure to 0.1 mM zinc Ringer's.

All the directly observable effects described above are, in general, a function of the concentration of the zinc acting on the muscle, and they furthermore are reversible upon removal of the zinc. The details of these features of our results will be presented later.

II. *Effects of Zinc on the Active State*

The above results are in essence exactly similar to those obtained with all the potentiators mentioned in the introduction, and, in common with these, they lead to the same conclusions regarding changes in the active state (see, *e.g.*, Kahn and Sandow, 1950, 1955; Ritchie, 1954 *b*; Hill and Macpherson, 1954).

Thus, the fact that zinc does not modify the tetanus tension signifies that it has no effect on the maximal intensity of the active state. But the prolongation of various periods of both twitch and tetanus, and the large reduction in tetanus fusion frequency from 130 to 70 shocks/sec. (Sandow and Mauriello, 1953; Ritchie, 1954 a), indicate that the active state is considerably prolonged by zinc. It is known that the reciprocal of the fusion frequency measures an interval of time from the instant of application of the stimulus to the end of active state plateau. We recall, also, that the latent period takes up the first part of this interval, and that, at the temperature of our experiments, 25°C, this lasts 2.5 msec. (Sandow, 1952). Thus, our fusion frequency data show that the duration of the period from the moment of onset to end of plateau of the active state increases in 0.1 mM zinc from 5.2 msec. to 11.8 msec.; *i.e.*, by a factor of almost 2.3 times. This finding is in accord with the observation by Hill and Macpherson (1954) that nitrate about doubles the duration of the active state at room temperature. Our findings that the relaxation periods of twitch and especially tetanus are greater in the zinc-treated muscle, demonstrate that the active state under the action of the zinc not only remains at plateau longer but also decays more slowly than it does in the normal muscle. The general prolongation of the active state effected by zinc is adequate, at least qualitatively, to account for the ability of this ion to potentiate the twitch and extend the time to its peak.

III. *Potentiation as a Function of Zinc Concentration*

We studied twitch potentiation in relation to concentration of zinc by exposing individual muscles to successively increasing concentrations of zinc chloride in Ringer's solution. Adequate time was allowed to obtain an eventual maximal effect at any given concentration, about 100 minutes for the lowest concentrations, and 30 minutes for the highest, before changing to the solution of next higher concentration. The average results of the four best replicate experiments are plotted in Fig. 3.

Four features of these curves are noteworthy. (a) There is a threshold for potentiation at a zinc concentration in the range 0.001 to about 0.005 mM. Edman and Grieve (1961) state, as originally reported by us (Isaacson and Sandow, 1961), that the threshold is 0.005 mM. Evidently, the threshold may be at a somewhat lower concentration than 0.005 mM, and it would be of interest to determine this. (b) Potentiation is maximal at a concentration of about 0.05 mM. (c) At intermediate values, potentiation is approximately a linear function of the logarithm of the zinc concentration. (d) In these experiments we also found that increases in the duration of the contraction period and the total twitch time varied with zinc concentration as did the degree of potentiation.

The maximal potentiation effects that are produced in concentrations of

zinc that are optimal or only slightly superoptimal (0.05 and 0.1 mM) are maintained for prolonged periods of time without any change other than that attributable to general slight deterioration of the muscle. In experiments with greatly superoptimal concentrations, such as the 1 mM we used in most of our earliest experiments (Sandow and Isaacson, 1960), we found that subsequent to the initial quick development of maximal effects, both peak twitch and tetanus tensions declined with further time of exposure to the zinc. Further-
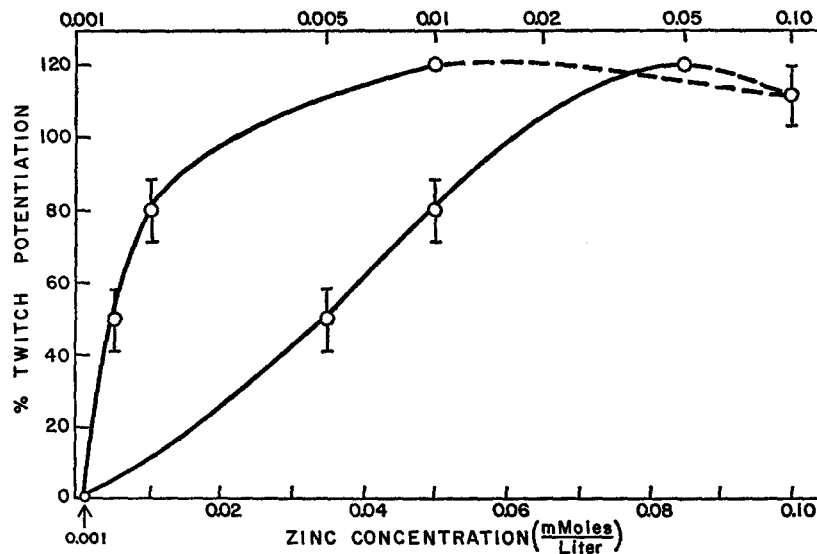

FIGURE 3.   Twitch potentiation, expressed as a per cent of normal twitch tension, as a function of the zinc concentration. The lower curve plots potentiation *versus* log of the zinc concentration, scale on top abscissa. The upper curve plots potentiation *versus* zinc concentration, linear scale on bottom abscissa. Per cent potentiation data were normalized (Isaacson, 1961) to an initial $(P/P_o)_n$ of 0.252; *i.e.*, maximum twitch potentiation was set equal to 120 per cent. Standard error of experimental points is less than ±11 per cent. Dashed lines indicate regions in which muscle's twitch tension decline is probably attributable to general decline in tension output of a muscle studied for long times; *e.g.*, 3 hours, in Ringer's solution.

more, the rate of decline was much greater when stimulation was stigmatic (*i.e.*, with the muscle in moist air) instead of massive (*i.e.*, with the muscle immersed in its experimental medium).

We obtained some elucidation of these mechanical results by determining that the externally recorded, diphasic action potential of the zinc-treated muscles, stimulated in moist air, could not be maintained in a regular, repetitive sequence if the frequency of stimulation was too high. Thus, although normal muscles could fire with perfect regularity at 150 shocks/sec., muscles exposed to 1 mM zinc were unable to do so. This effect, though to a lesser de-

gree, was obtained even when the concentration was only 0.01 mM. Evidently, the muscle's inability to maintain tetanus in 1 mM zinc is a consequence of this impairment in capacity to repeat excitatory responses rapidly. This develops, as will be seen later, because zinc greatly increases the duration of the refractory period. However, the fall in output of twitch as well as tetanus with length of exposure to 1 mM zinc signifies that other effects must develop besides impairment in capacity for repetitive firing. Further work is necessary to clarify the nature of these effects.

IV. *Kinetics of Development and Reversal of Potentiation*

Determinations of the time course of development of potentiation effects, following the moment of immersion of a muscle in a solution of a given potentiator, and of the subsequent reversal of these effects, after restoration of the muscle to pure Ringer's solution, give important information concerning the site of action of the potentiating agent. Thus, the rapidity of occurrence of these effects after exposure to the nitrate ion, clearly proves that this substance acts directly on the fiber membrane (Kahn and Sandow, 1950, 1955; Hill and Macpherson, 1954; Hodgkin and Horowicz, 1960). We drew a similar inference for the site of action of zinc from our early observation that its effects appeared so quickly that all that seemed to be required for production of these changes was the diffusion of zinc to the surface of the fibers (Sandow and Isaacson, 1960; Isaacson and Sandow, 1961). But these initial experiments involved zinc at 1 mM concentration. Since our present results show that maximal potentiation is produced by zinc in only 0.05 mM concentration, the rapidity of developing such changes in our original experiments could not be unequivocally attributed to a mere surface effect. For, in the presence of such a great excess of zinc over that required for the maximal effect, there might have been some penetration of the metal into the muscle fibers even during the rather short time involved, and hence an action of the potentiator internally as well as superficially. With these considerations in mind, we performed the following experiments in order to obtain data regarding the kinetics of development, and also reversal of the potentiation caused by zinc under conditions avoiding as much as possible any spurious complications.

We chose rather small muscles for this study (average weight, 54 mg and thickness, 0.052 cm), and after the usual equilibration in Ringer's solution and recording of normal twitches and tetani, immersed each muscle in 0.05 mM zinc and obtained twitch records at a series of successive times after immersion until maximal potentiation was clearly established; *i.e.*, generally, in about 20 to 30 min. At this time, the zinc solution was replaced with pure Ringer's, and the reversal of potentiation traced by a series of appropriately spaced test twitches until the responses were restored to normal value; *i.e.*, for about 100 or more min. (It should be noted that reversal from greatly

superoptimal (*e.g.*, 1.0 mM) solutions is extremely slow, many hours being required. This is due, at least in part, to the presence in the muscle initially of such a great excess of zinc above that just needed for maximal potentiation effects that very much time is needed for mere removal of this excess.) Of the results obtained in many experiments of this type, we have analyzed the sets of data from three muscles that behaved most consistently.

As shown by the average results graphed in Fig. 4, the general development of the zinc effect occurs with a half-time of about 6 min. At the very start,
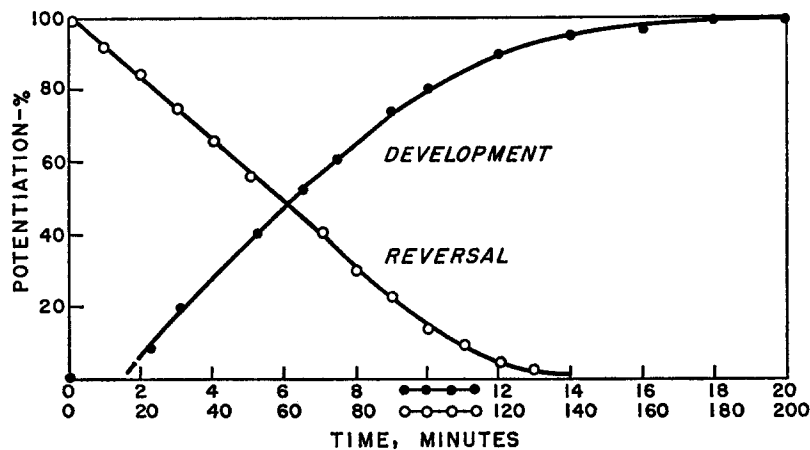

FIGURE 4. Kinetics of development and reversal of twitch potentiation (expressed in per cent of maximum potentiation). Solid circles trace development of potentiation as a function of time after a muscle is placed in 0.05 mM zinc Ringer's solution. Open circles trace reversal of potentiation after a muscle is returned to regular Ringer's solution. Note the tenfold difference in the two time scales. Curves are averages of results from three experiments.

however, the action of the zinc evidently occurs very much more slowly than is suggested by this half-time value. Accessory data obtained from other experiments providing more detailed time resolution of the earliest changes, prove that there is an initial period of about 1 min. during which the muscles actually show no effect of the zinc at all. A similar initial lag has been observed by Edman (1962). A very striking feature of these kinetic studies is that reversal of the potentiation occurs extremely slowly, *i.e.* with a half-time of 60 min., and thus at a speed only about one-tenth of that for the development process.

V. *Excitability Effects*

As previously indicated, it is important to know whether a potentiator exerts its effect by way of a direct action on the membrane of the muscle fiber. In connection with the possibility that such an influence holds for zinc, the study

of effects of this substance on excitability functions is of interest because such functions reflect properties of the membrane which, apart from their intrinsic connotations in respect to excitation, may be involved in the mechanism of potentiation.

THRESHOLD RELATIONS We determined strength-duration curves for minimal mechanical responses of massively stimulated muscles, first in normal Ringer's and then after an hour's soak in 0.1 mM zinc. Durations of shocks were provided by calibration of the stimulator, but we used as indications of threshold strengths the actual voltages, measured between the electrodes of the massive stimulation bath, just needed to produce a minimal effect. Our procedure necessarily measures thresholds of the most excitable fibers. By using relatively great amplification for recording tension, we could easily detect outputs of the order 0.1 gm; *i.e.*, less than 1 per cent of the muscle's maximal twitch tension. Assuming that a muscle, on the average, has 800 fibers, our determinations relate to the responsiveness of the eight or so most excitable of these. To ensure that the results would be at least somewhat independent of time of soaking of the muscle in its various media, we first obtained two strength-duration curves at an hour's separation in Ringer's solution, and then two similarly spaced tests in the zinc medium.

Although the curves obtained at various times under the same conditions did not superimpose, the average results indicate that zinc in a concentration causing maximal potentiation has no consistent effect on the strength-duration curve; *i.e.*, it does not significantly influence the excitability of the most excitable fibers.

In another series of tests, we obtained a measure of the distribution of thresholds among the fibers of separate muscles by massively stimulating them with shocks of 0.3 msec. duration, and increasing strengths through the range evoking responses from minimal to maximal. Zinc in 0.1 mM concentration had no effect on this distribution, and for both normal and treated muscles the strength for maximal output was about four to five times that for minimal, suggesting that a similar factor holds for the difference in excitability of the least and the most excitable fibers.

REFRACTORY PERIOD This feature holds particular interest because of Ramsey's (1960) view that refractoriness "governs" the duration of the active state. We have determined changes in refractoriness by the method involving recording of diphasic action potentials of sartorii stimulated with two maximal shocks separated by varying time intervals. The ratio of the amplitude of the first limb of the action potential evoked by the second shock to that caused by the first gave a measure at any shock interval of the degree of restoration of normal excitability present at the time of application of the second shock. Although, in this procedure, the second shock has the same

strength as the first, it must be recalled that there is a distribution of excitabilities among the fibers of the whole muscle, and thus the least interval (*i.e.*, the absolute refractory period) at which the second shock just becomes effective for the muscle involves stimulation of the most excitable fibers. Since, as shown by separate tests, these have thresholds at only about one-fourth of the maximal shock strength, our procedure determines the absolute refractory period for these fibers in respect to a test shock strength at four times threshold. At intervals greater than this absolute refractory period, our second shocks
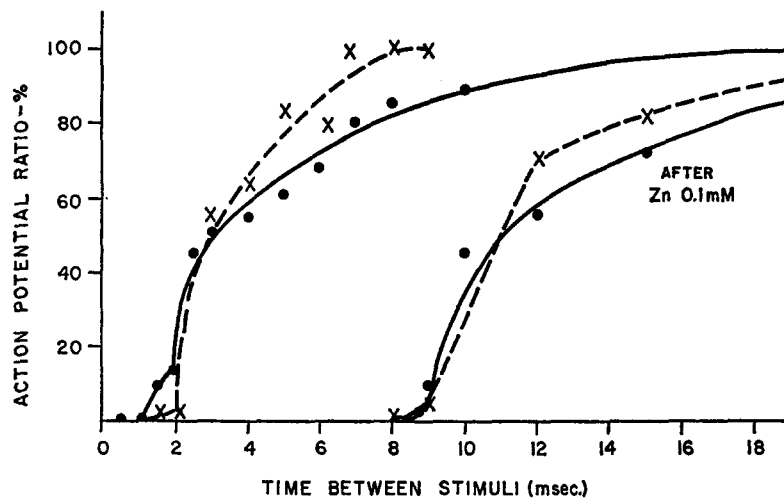

FIGURE 5. The effect of 0.1 mM zinc (1 hr.) on refractory period as measured by action potential output in dual shock responses. The ordinate plots the ratio (as a per cent) of the peak amplitude of the first limb of the second action potential to that of the first. The abscissa gives the time between the two shocks. The dots and crosses represent results from two separate muscles.

trace out effects which represent some undefined resultant of variability among the muscle's fibers in regard to both absolute and relative refractoriness.

The complete results of two experiments on two separate muscles are graphed in Fig. 5 and they prove that 0.1 mM zinc increases the absolute refractory period of the most excitable fibers by at least four times. A similar difference probably holds for other fibers and for changes in relative refractory period since the curves for a change in output for the zinc-treated muscle are in general delayed about four times in respect to those for the normal ones.

The capacity of zinc to increase the duration not only of the refractory period but also of the active state is in keeping with the previously mentioned view of Ramsey (1960). But other work from this laboratory (Brust *et al.*, 1962) proves that caffeine and nitrate do not induce any significant change in refractoriness of muscle when acting in concentrations causing considerable

twitch potentiation. This suggests that the results for zinc correlate merely fortuitously in favor of Ramsey's view, and that it is very questionable whether in general there is any causal relationship between excitatory refractoriness and contractile active state.

ACTION POTENTIAL   The diphasic recordings of action potentials we used to monitor excitation changes during the refractory period, and also to prove the absence of repetitive firing in the zinc-affected response, were not intended for study of the action potential as such. Separate investigations of
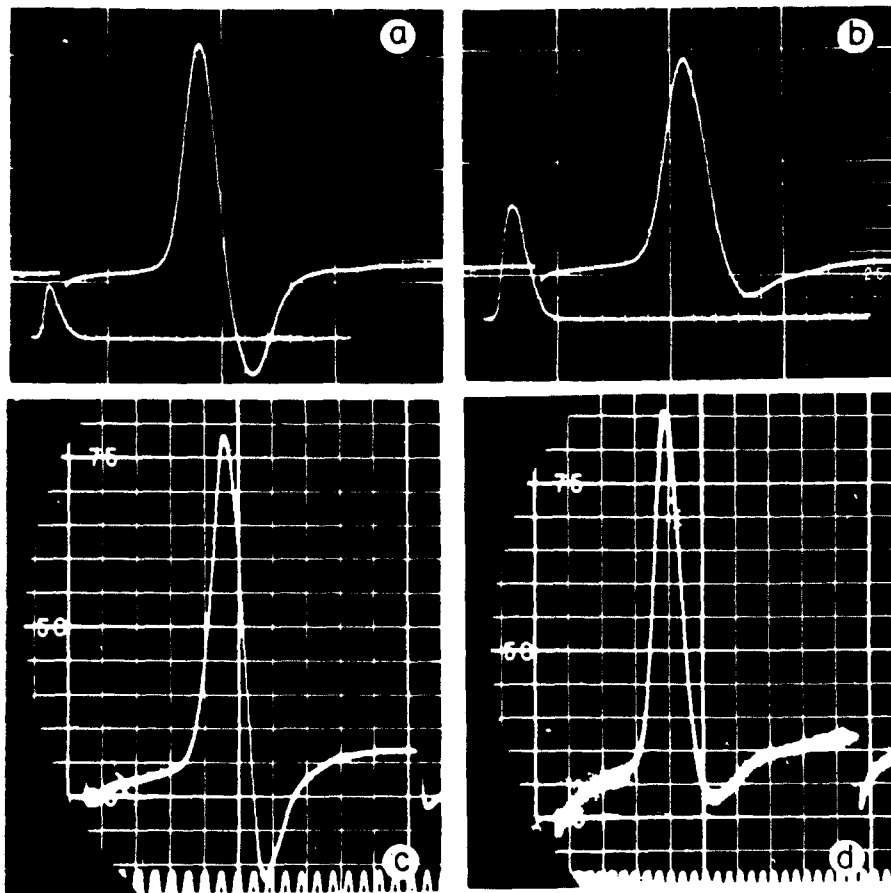

FIGURE 6.   Effects of zinc on diphasic action potential responses. (*a*) Normal diphasic action potential with accompanying twitch myogram. (*b*) Records from same muscle as in (*a*), 3.5 min. after exposure to 1 mM zinc Ringer's solution. Action potential responses in (*a*) and (*b*) were recorded at a sweep speed of 7.4 msec./inch (each large square = 1 inch) and the twitch myograms at 200 msec./inch. (*c*) Normal action potential response on upper beam. A 1000 CPS oscillator signal is displayed on synchronously swept lower beam. (*d*) Details as in (*c*), action potential response after 62 min. in 0.1 mM zinc Ringer's solution.

this type based on internal electrode techniques are under way in this labora-
tory and will be reported elsewhere. However, it became apparent, even from
the diphasic electrical records, that zinc alters the shape of the action poten-
tial (Isaacson and Sandow, 1961). Fig. 6 demonstrates that 1.0 mM zinc, acting
for only 3.5 min., causes a slight reduction in magnitude and an increase in
duration of the first limb of the action potential and a very marked decrease
in magnitude of the second limb. Also apparent in this figure are the changes
caused by 0.1 mM zinc acting for an hour: a slight increase in magnitude of
the first limb, and, again, a very pronounced decrease in the second limb. We
discuss here only the common effect, the marked decrease in the second limb.
We realize that detailed conclusions regarding the effects of zinc on the shape
of the true action potential cannot be drawn from these diphasic records. (In
fact, this is indicated by the presence in the records of Fig. 1 of only a very
slight zinc-induced relative reduction in the second limb of the diphasic poten-
tial, which, however, may have resulted from an atypically large separation
of the recording electrodes during this experiment.) Nonetheless, it seems
clear that the characteristic, large relative reduction in the second limb, that
we have observed in most of our records, signifies that zinc causes a general
slowing of the repolarization phase of the action potential. Edman and Grieve
(1961), indeed, describe such an effect of zinc in records of the action potential
obtained with the internal electrode.

ANTAGONISM TO VERATRINE   The effects of the veratrum alkaloids are
known to involve a special sort of unstabilization of the excitatory mechanisms
of the muscle fiber membrane (Shanes, 1958) which results in activation, in
turn, of the well known veratrine mechanical response. A number of potenti-
ators, quinine (Harvey, 1939), nitrate, and other anions (Sandow and Rubin,
1958) have been found to antagonize these effects of veratrine on the frog
sartorius muscle. We therefore studied the effect of zinc on the mechanical
response to a single stimulus of a muscle equilibrated to 10^{-6} gm/ml of vera-
trine. The addition of 1 mM zinc to the veratrine Ringer's solution resulted in
a considerable reduction in the duration of the entire veratrine response. The
time to peak of the mechanical response, i.e. the time during which repetitive
firing occurs, was reduced to 55 msec. from an initial value of 180 msec. Fur-
ther work is needed to fully delineate this effect and also to determine details
of the evident capacity of zinc to curtail the contracture; i.e., the prolonged
depolarization phase, of the veratrine response.

DISCUSSION

The basic finding of our study is that the zinc ion, in concentrations of the
order of 0.005 to 0.05 mM. causes reversible potentiation effects in the con-
traction of frog skeletal muscle. In other words it (a) increases the tension out-

put of the twitch but not the tetanus, (*b*) prolongs the contraction and relaxation periods of the twitch, (*c*) prolongs the relaxation period and reduces the fusion frequency of the tetanus, and (*d*) causes all these changes, as they themselves indicate, by prolonging but not intrinsically intensifying the active state of the muscle's contractile component.

In addition to potentiating contraction, zinc also affects certain excitatory reactions. A very striking change is the four times increase in the absolute refractory period (*i.e.*, a change from 2 msec. to 8 msec.) obtained; *e.g.*, in 0.1 mM concentration of zinc. This is evidently the cause of the impaired firing of uniform action potentials and consequent lack of maintenance of peak tetanic tension we have observed in zinc-treated muscles stimulated by tetanus stimuli of relatively high frequency. It is also the basis for the ability of zinc to antagonize the repetitive firing effects of veratrine in a muscle's response to a single stimulus. But it is not clear whether the increased refractoriness plays any role in the curtailment by zinc of the contracture phase of the veratrine response. Although the large increase in duration of refractoriness is in keeping with the view of Ramsey (1960) that, in general, the duration of the refractory period governs that of activity, we conclude, as already discussed in the Results section, that there is no necessary causal relation between refractoriness and active state. However, increase in refractoriness due to zinc is, itself, of interest in view of Hodgkin and Huxley's work (1952) showing that the duration of the refractory period in the squid axon is determined by the span of time following excitation during which the state of the membrane is dominated by inactivation of sodium conductance and by delayed rise in potassium conductance. If refractoriness is similarly determined in the frog muscle fiber, then our results may signify that either or both of these electrochemical processes are prolonged under the action of zinc. Our and Edman and Grieve's (1961) finding that the repolarization phase of the action potential is decelerated by zinc may reflect a retardation in development of increased potassium conductance, and this may therefore play a role in determining the great increase in duration of refractoriness induced by zinc.

Although the foregoing proves that zinc has striking actions on certain excitatory functions of the membrane, it is not clear whether the potentiation it causes is mediated by way of a membrane effect. Other potentiators do act this way; *e.g.*, nitrate and other anions (Kahn and Sandow, 1950, 1955; Ritchie, 1954; Hill and Macpherson, 1954; Hodgkin and Horowicz, 1960); tetraethylammonium (Edwards, Ritchie, and Wilkie, 1956); and methylsulfate (Hutter and Noble, 1960). This conclusion is based principally on the fact that the great speed with which each of these substances causes potentiation to appear and develop in a muscle cannot be accounted for by an intracellular locus of action, penetration of the substances would be required, and this is altogether too slow, but rather by an action at the fiber surface, since

diffusion of the potentiators to such a locus is sufficiently speedy. The experimental observations and theoretical analyses that justify this "surface contact" mechanism of potentiation are most explicit and detailed for potentiation by nitrate and iodide. It will therefore be most useful in attempting to determine the mode of action of zinc to compare the kinetics of potentiation by this substance with relevant features of the action of these anions.

The delay of about a minute before onset of potentiation in a muscle freshly exposed to zinc is not found at all for the anions, for these cause potentiation to develop at the highest rate at the instant they make contact with the surface of the fibers. The delay for zinc is not ascribable to the requirement that its concentration must exceed a threshold value of about 0.005 mm before it can act (see Fig. 3), because, in our experiments, certainly all fibers of the topmost layer of the muscle were practically instantaneously exposed to the full 0.05 mm concentration of zinc in the test solution at the moment it was applied to the muscle. In addition to this initial lag, the effects of zinc increase much more slowly than those of the anions during the period of actual development of the potentiation. Thus, for the half-time of this process we observe a value of about 6 min., but Hill and Macpherson (1954) found for potentiation of the frog's sartorius by iodide (at essentially the same temperature as for our experiments) a value of only 40 sec. We can obtain an expected half-time for the zinc effect, if only simple diffusion to the surface of the fibers is involved, by using the formula (Hill, 1928), $t = $ const. x^2/k, which gives the time, t, for a specified amount of diffusion of a substance of diffusion coefficient, k, through a distance, x. This formula is particularly useful for comparing the times, t_1 and t_2, for the same degree of diffusion in two systems whose respective parameters have the values, k_1, x_1 and k_2, x_2—in which case the relationship of interest is $t_1/t_2 = (x_1/x_2)^2(k_2/k_1)$. Here, the specified amount of diffusion is taken to be that of NaI or $ZnCl_2$ into the extracellular space of the muscle which will cause 50 per cent of the maximal twitch potentiation obtainable by the particular concentrations of each of these substances actually used in the respective experiments. We now assume that the ratio of the diffusion coefficients of NaI and $ZnCl_2$ is the same in the extracellular muscle space as it is in free water solution. Then, (a) using the values for such a solution, $k_{NaI} = 96 \times 10^{-5}$ cm²/min. (Hill and Macpherson, 1954) and $k_{ZnCl_2} = 43 \times 10^{-5}$ cm²/min. (Wang, 1954); (b) noting that the distance, x, may be taken as the thickness of the muscle (actually the half-thickness, since diffusion was two-sided; but nonetheless the ratio will be the same), 0.07 cm in Hill and Macpherson's work and 0.052 cm in ours; and (c) recalling that the half-time for the iodide effect is 40 sec., we can by application of the above relationship calculate the expected half-time for zinc, if it is involved in an action at the fiber surface like that for iodide, as $t_{1/2} = 40$ sec. $(0.052/0.07)^2$ $(96 \times 10^{-5}/43 \times 10^{-5}) = 49$ sec. It is obvious that the actual half-time for

zinc is about seven times greater than this. In view of this difference and also that involving the unique initial lag in the action of zinc, it is clear that the kinetics of development of potentiation by zinc are in general much too slow to be consistent with a surface contact mechanism like that holding for the anions.

This conclusion is reinforced by considering the kinetics of reversal of the full effects produced by the potentiators. For zinc, the half-time for reversal is some ten times greater than that for development. In small part, this must be due to a "concentration effect" (which derives from the special non-linear relation between concentration of zinc and intensity of effect, as shown in Fig. 3) like that which Hill and Macpherson (1954, see pp. 96–97) point out is responsible for the result that the iodide effects have a greater half-time for reversal than for development. Since the reversal half-time for iodide is 90 sec., application of the previously used relationship predicts a corresponding duration for zinc of 110 sec. This is more than twice the calculated half-time for development of the zinc effects, and it thus gives an approximation of what should be expected from operation of the concentration effect. But the observed half-time for reversal is actually 60 *min.*, and this proves that some process other than that causing the concentration effect is operating.

A more precise procedure for analyzing the reversal kinetics is to use the theoretical method of Hill and Macpherson (1954) (which, incidentally, automatically takes into account the concentration effect) to calculate a reversal curve giving the decrease in potentiation as a function of time. Applied to our system, this method depends on the following assumptions which are in accordance with the surface contact mechanism: (a) diffusion of zinc ions (*i.e.*, as $ZnCl_2$) out of the muscle and thus away from the surface of the fibers is the sole process determining the rate at which the potentiation tends to disappear; and (b) the degree of potentiation of any fiber is dependent on the local concentration of zinc in the neighboring extracellular space, in conformance with the data plotted in our Fig. 3. The full details of the procedure involved in making the calculation may be obtained from Hill and Macpherson's paper. In the final analysis, however, the actual fitting of the theoretical curve to the experimental one requires choice of a value for the diffusion coefficient of $ZnCl_2$ in the muscle's extracellular space. By taking this as 7.6×10^{-6} cm²/ min. we obtain the open circles shown in Fig. 7. A curve through the open circles agrees very well with the experimental results, during the latter part of the reversal, but it falls too fast during the first 30 minutes. A perfect fit to all of the experimental curve can be obtained by discarding the assumption of constancy of the diffusion coefficient throughout the reversal. Then, as presented in Fig. 7, we find that the apparent diffusion coefficient is only about 2.5×10^{-6} cm²/min. at the start of the process and gradually increases for about 36 min. until it reaches the constant value of 7.6×10^{-6}. There is, of

course, no reason to suppose that the diffusion coefficient should vary in this way with time, and thus such variation is itself evidence that the reversal of potentiation does not occur in accordance with the assumptions of the surface contact hypothesis. Furthermore, even if the coefficient were constant at its highest value, 7.6×10^{-6} cm²/min., then this would be only one fifty-seventh of the value, 43×10^{-5} cm²/min., that holds for $ZnCl_2$ in free water solution (Wang, 1954). Now, in view of the results that the diffusion coefficients of $NaNO_3$ and NaI in the muscle's extracellular space are smaller, by a factor of one-fourth to one-tenth, than their values in free solution (Hill and Mac-
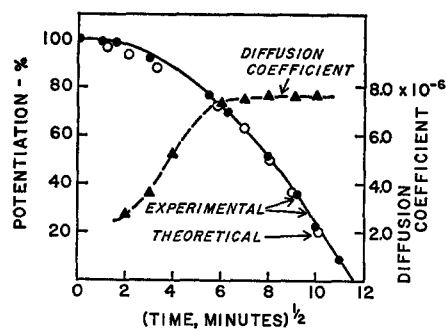

FIGURE 7. Kinetics of reversal in Ringer's solution of twitch potentiation previously developed by frog's sartorius muscles in 0.05 mM zinc Ringer's. Solid circles and continuous line giving per cent potentiation as a function of the square root of reversal time, represent the average experimental behavior of three muscles. Open circles give theoretical reversal curve, calculated as described in the text using an apparent diffusion coefficient for zinc in the extracellular space of muscle of 7.6×10^{-6} cm²/min. The triangles and dashed line give values of the apparent diffusion coefficient, as indicated on right ordinate in units of cm²/min., as a function of the square root of reversal time, calculated from theory as described in the text.

pherson, 1954; Kahn and Sandow, 1955), we would expect a corresponding reduction of the coefficient for $ZnCl_2$ by about the same factor. From the result that the theoretically determined value is very much smaller than this, we must conclude that simple diffusion away from the surface of the fibers is not the only change determining the rate of disappearance of potentiation by zinc.[1]

There are several possibilities for explaining the anomalously very slow kinetics of both development and reversal of zinc effects. In one of these we

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[1] In principle, a corresponding analysis can be made for the kinetics of development of potentiation, but it is clear that the theoretical curve cannot possibly agree with the experimental, since the theory includes nothing to account for the observed initial lag. Furthermore, any mere arbitrary shift in the zero of the theoretical time base still leaves unresolved the difficulty that any value of the diffusion coefficient for $ZnCl_2$ that seems to be valid for the reversal kinetics is altogether too small to account for the much more rapid developmental kinetics.

can retain the essential feature of the surface contact mechanism, *i.e.* that the primary site of action of the zinc is at the surface of the fiber, and account for the generally very slow kinetics of action by zinc by taking into account the finding of Isaacson and Bianchi (1962) that the Achilles tendon of the frog very strongly binds zinc. The connective tissue of the sartorius muscles of our experiments should similarly bind zinc, and this would slow up the development of the potentiation, because the concentration of the free zinc ions in the extracellular space at any time would be less than that predicted by diffusion theory as used in elaboration of the surface contact hypothesis. Furthermore, this effect should be most pronounced at the start of the exposure of the muscle to zinc when the connective tissue elements are becoming saturated with the metal, and thus we could explain the initial delay we observe. The work of Isaacson and Bianchi (1962) also shows that release of zinc from the connective tissue binding sites is a very slow process. If such a slow release occurs when the fully potentiated muscle is replaced in Ringer's solution, then this would tend to replenish the zinc ions in the extracellular space that leave by diffusion, and thus provide a mechanism which would at least qualitatively account for the extreme slowness of reversal of the zinc effects.

The available evidence regarding the role of connective tissue binding in the kinetics of the effects of zinc is only of a qualitative nature, and we therefore cannot be certain that the implication it suggests, that the immediate site of action of zinc is on the membrane, is firmly established. Further work is required on this problem, and one obvious need is to study the development and reversal of zinc potentiation in single fiber preparations, since these would be practically free of any extraneous connective tissue. For the present, however, it should be noted that the relative slowness of the various processes associated with the action of zinc may indicate that it must reach sites which are in the sarcoplasmic reticulum or the myoplasm and are therefore much less accessible than are those at the membrane. Should this be the case, we can infer from the great difference in speeds of development and reversal of potentiation that the binding of zinc at the internal sites must be very tight, as it evidently is on the tendon.

Determination of the primary site of action of any potentiator is an important first step in explaining the mechanism by which it acts. The fact that the anions act directly at the membrane necessitates the conclusion that the potentiation, *i.e.* the prolongation of the active state they engender, is a consequence of some alteration in excitation-contraction coupling, which, moreover, develops at some relatively early reaction in this sequence (Kahn and Sandow, 1950, 1955; Sandow, 1952; Hill and Macpherson, 1954). If zinc has a direct membrane action, then it must also cause some modification in a relatively early process in the coupling of excitation to contraction. It is interesting in this regard, that a kind of membrane change which is caused by

zinc, *i.e.* the slower than normal reversal of the spike potential, has been in-
ferred to play a role in general (see Etzensperger, 1961, and Falk, 1961) in
the mechanism of potentiation. There is no certainty, however, that the posi-
tive correlation that seems to exist in some cases between this sort of change
in the action potential (especially evident as an augmentation of the negative
after-potential) and twitch potentiation necessarily signifies that they are
causally related. Increase in twitch tension may be due merely to a lowering
of the mechanical threshold, as indicated for anions by the results of Hodgkin
and Horowicz (1960). Caffeine in relatively low concentrations causes po-
tentiation without any evident change in the action potential (Sandow, Brust,
and Isaacson, 1962). Much further work seems to be needed, therefore, be-
fore we can be sure that the change zinc induces in the action potential is
instrumental in determining potentiation. Furthermore, as already indicated,
we cannot, at present, rule out the possibility that zinc causes potentiation by
acting at sites which are in the deeper regions of the muscle fiber, rather than
at the membrane. In this event, zinc would exert its primary action at a later
point of the excitation-contraction coupling sequence than that at which
other potentiators, *e.g.* the anions, seem to act, and thus determine a cor-
responding difference in the mechanism of potentiation.

Another approach to the problem of the mechanism of action of zinc stems
from a consideration of certain chemical features of its effects. The question
of chemical aspects of potentiation is, of course, a very general one, as is sug-
gested by our reference in the introduction to this paper to the chemical
diversity of the various substances included in the group of potentiators. This
diversity may indicate that potentiation develops either as a common end-
point of different, converging mechanisms or in consequence of a single mecha-
nism consisting of a series of steps at each of which a particular type of
potentiator might act. Although not presently shedding any light on these ques-
tions, it is very interesting that quinine, though so different chemically from
zinc, is very similar to the metal in causing certain neuromotor effects. Thus,
besides acting as a potentiator, the drug increases refractoriness (Harvey,
1939) and augments the negative after-potential (Etzensperger, 1957; Falk,
1961). Other work from this laboratory (Sandow and Bien, 1962) proves that
zinc, like quinine (Harvey, 1939), blocks neuromuscular transmission.

But in specific reference to zinc itself, it obviously stands out, as a divalent
cationic potentiator, in distinct contrast to both the potentiating anions and
to caffeine which potentiates as a neutral molecule. Even as a divalent metal,
zinc is almost wholly unique in causing potentiation. Studies made in this
laboratory (Bien, 1961) show that of the metals Be, Mg, Ca, Sr, Cd, Mn, and
Co, only Cd and Be have potentiating effects, but very much less so than zinc,
and, as for the others, in concentrations up to 1.0 mM they either do not affect
or they depress the output of the muscle. It is also very striking that zinc

produces its effects in such extraordinarily small concentrations. The threshold for its action is at about 0.005 mM; and the concentration for maximal effect is 0.05 mM; *i.e.*, only about 1/2000 of that (100 mM) at which nitrate exerts its maximal change. Finally, our results indicate that, unlike the action of at least the anions, potentiation by zinc involves a very strong binding at some critical site. Taking into account all these various chemical features, we infer that in the primary step of the process by which it causes potentiation, zinc forms a strongly bound complex with a negatively charged ligand of some organic substance of the muscle fiber. Research dealing with the existence, specific nature, and locus of this reaction should yield results shedding light on the mechanism by which zinc alters the responses of the muscle fiber.

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