fluctuation rate. This discrepancy, however, is deduced from measurements which have not been confirmed by other workers, and is not thought reliable (*5*). No other clear contradiction of this sort is known to me.

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Bioelectric Phenomena Related to Protein-Fixed Charge in a Crab Nerve Fiber

Abstract. *The bioelectrical characteristics of a crab nerve fiber subjected to solutions containing the impermeant anions ferrocyanide, glutamate, or ethanolsulfate substituted for chloride include prolonged slow depolarizations that elicit prolonged trains of impulses superimposed on the plateau portion. Propionate and nitrate depress repetitive firing while thiocyanate has only a slight effect. Nerves treated with ferrocyanide lose potassium and gain sodium, which fact may account for their depolarization. On the other hand, studies with interference microscopy reveal shift by ferrocyanide in the interference fringe pattern of the sheath material; this suggests a reorientation of fixed charges in the protein layers by direct action of the anion on these layers. This may also account for the electrical manifestations observed.*

Although bioelectrical phenomena arising in excitable tissue have been generally related to cation concentrations and movements across cell membranes (*1*), considerable attention has also been drawn to the possibility that fixed charges in the membrane may contribute to these phenomena (*2*). We present evidence, from experiments with single crustacean axons, concerned with the effect of anions substituted for chloride in the surrounding medium; our data supports the fixed-charge hypothesis.

When the impermeant anion ferrocyanide is substituted for chloride in the medium surrounding skeletal muscle, remarkably prolonged, spontaneously occurring plateau depolarizations are recorded (*3*); these are not unlike the responses obtained from crustacean motor axons subjected to a potassium-rich medium or to one containing veratrine (*4*). Since the transmembrane characteristics of the crustacean axon are now well known (*5*), this preparation seems to be very suitable for examination of the effects of anions substituted for chloride in the medium surrounding a single nerve fiber. This is particularly so in the light of recent studies, with interference microscopy, relating structural change to the effects of potassium-rich media on these nerve fibers (*6*).

Table 1. Percentage changes of intracellular concentrations of sodium and potassium of crab nerve caused by immersion in test solutions.

Test solution	Preparations (No.)	Sodium	Potassium
Isotonic ferrocyanide	15	+58 ± 15	−31 ± 5
Potassium-free ferrocyanide	1	+75	−59
Potassium-excess (130 m*M*) ferrocyanide	1	+ 8	−56
Isotonic propionate	3	+ 4 ± 2	− 8 ± 2
Calcium-free	2	+56	−25

Single axons were isolated from the walking limbs of the crab (*Callinectes sapidus*) and mounted either for the three-electrode flow-tube method of electrical recording (*5*) or in the special chamber for interference microscopy (*6*). The chloride in *Homarus* physiological solution (*7*) was replaced by the anions ferrocyanide, glutamate, ethanolsulfate, nitrate, propionate, or thiocyanate; in the solution containing ferrocyanide, isomolarity was maintained by the addition of sucrose.

Typical dual-beam oscilloscopic recording of a single nerve fiber subjected to the ferrocyanide solution are presented in Fig. 1. The upper trace shows the pulse of the applied current; the lower trace shows the resulting membrane potential response. A slow, spontaneous depolarization occurred, indicated by the rise in the base line (Fig. 1, A1–A3), and ultimately the stimulus current (Fig. 1, A1) triggered a repetitive response not only during the duration of the pulse, but one which continued for seconds or even minutes after termination of the pulse. This response was superimposed on a prolonged plateau depolarization (Fig. 1, A3). By hyperpolarization (Fig. 1B) with a continuously applied current directed inward, the long trains of impulses and plateau could be abbreviated, the duration being inversely proportional to the amplitude of hyperpolarization. These data agree well with those on frog muscle (*3*).

The potential recordings in Fig. 1, A1, clearly show the development of the slow response with the action potentials superimposed (spikes). The S-shaped, rising phase of this prolonged depolarization is also characteristic of the slow response obtained in potassium-rich media. Nitrate and propionate simply depressed activity of the nerve fiber, increasing the critical firing level slightly and eliminating the normal repetitive firing pattern of these axons during a depolarizing stimulus. Thiocyanate caused only a slight depression of activity.

Because of the marked resemblance of the spontaneously occurring depolari-

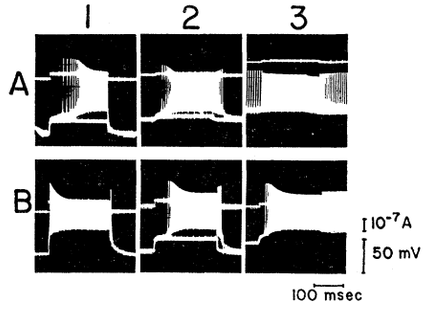

Fig. 1. Dual-beam oscilloscopic recordings from a single axon subjected to *Homarus* solution, with ferrocyanide substituted for chloride. Top trace indicates current stimulating pulse; bottom trace, membrane potential deflections. Recordings taken immediately after application of ferrocyanide (A1) and at 1-minute intervals (A2 and A3). Note development (S-shaped curve) of slow response (A1) and concomitant decline in the amplitude of the action potential with increased frequency. Row B illustrates the effect of strong (B1 and B2) and weak (B3) hyperpolarization.

zations obtained from fibers immersed in ferrocyanide to the slow response obtained in potassium-rich media (*7*), a flame-photometric study was made of the intracellular cationic content of axons subjected to these anions. Whole crab nerves were subjected to the ferrocyanide solutions or to standard *Homarus* solutions for various periods of time and then homogenized; the homogenates were then extracted with chloroform. Nerves treated with ferrocyanide had a significant (more than 30 percent) reduction in potassium concentration and a sizable (58 percent) increase in sodium concentration. (Table 1). Such variations, which were easily reversible, obviously could account for the slow, progressive depolarization (Fig. 1) produced by treatment with ferrocyanide.

To test this possibility, we subjected axons first to a ferrocyanide solution, which decreased the internal potassium concentraction, and then to potassium-free ferrocyanide solution to "increase" the relative internal potassium concentration and "restore" the transmembrane gradient in potassium concentration toward normal (Fig. 2). There was an immediate slow depolarization of approximately 20 mv indicated by the rise in the base line. When the ferrocyanide solution was replaced by the potassium-free ferrocyanide solution, repolarization took place as might be expected, the base line returning to the original level. A short time later, slow prolonged depolarization of 50 mv or more occurred spontaneously without any applied stimulus. These prolonged responses are not unlike those recorded from squid axons in which the ratio of internal to external potassium concentration is reduced (*8*). Spikes, small ripples in these recordings, are superimposed on the plateau phase. The transmembrane resistance was markedly reduced at the initial peak of these plateau responses but became progressively greater later during the plateau, as illustrated by the change and amplitude of applied hyperpolarizing pulses (Fig. 2, second trace). A depolarizing pulse had no effect when applied during the plateau phase, although a prolonged response was initiated by the pulse if it was applied during the interval between the spontaneous plateau depolarizations (Fig. 2, third trace). For example, the first and last prolonged deflections were initiated by the pulse. The pulse artifact appears superimposed

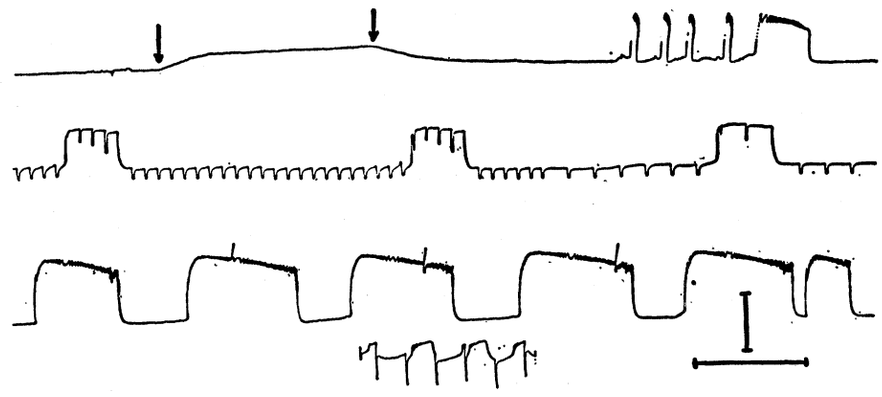

Fig. 2. Slow inkwriter recordings of spontaneous prolonged depolarization following immersion of a single axon in ferrocyanide solution—first arrow on top-left trace. The second arrow indicates substitution for this solution of a potassium-free ferrocyanide solution. Calibration: 50 millivolts vertical, 20 seconds horizontal (top trace), 10 seconds (second trace), 5 seconds (third trace), and 20 seconds (bottom trace).

during the plateau of the other prolonged responses. A strong, inwardly directed current or hyperpolarizing pulse was equally ineffective unless applied near the termination of the plateau, when the membrane resistance was high. Then the duration of the plateau depolarization was slightly abbreviated as shown (Fig. 2, bottom trace). The effects with glutamate and ethanolsulfate were almost identical to those with ferrocyanide. As already stated, nitrate, propionate, and thiocyanate caused no depolarization, but nitrate and propionate did abolish repetitive firing.

To determine just how closely the prolonged responses and spikes were related to one another and to sodium activation, we added tetrodotoxin, which specifically inhibits the sodium system, to the ferrocyanide solution. As expected, the spikes were rapidly diminished and abolished (*9*). Somewhat later, the plateau also declined and dis-

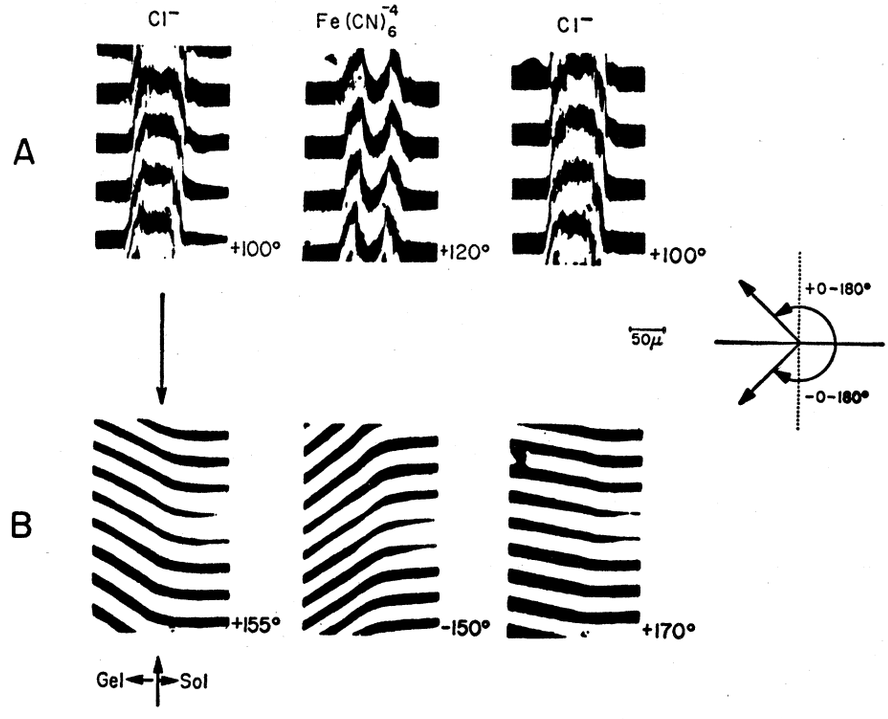

Fig. 3. Interference micrographs of a single axon (A) and interface between the solution and the protein gel (B), showing refraction of interference fringes by the vertically situated axon in A and by the interface in B.

appeared. Thus, both plateau and spike must be related to activation of the sodium system and the sodium potential. On the other hand, the slow depolarization (20 mv) caused by ferrocyanide when first applied may be the result of a direct reaction by ferrocyanide with the membrane material, resulting in changes in the transmembrane ionic concentration and equilibrium potential.

The direct effect of ferrocyanide on the axon material, as seen through the interference microscope, was striking (Fig. 3). A single axon was mounted on a specially designed microscope slide containing a tiny channel to hold the nerve fiber and to allow for a continuous flow of solution past the fiber. This slide was then placed in one beam of a dual-beam Leitz interference microscope and a second, identical "dummy" chamber, without an axon, was placed in the other beam; the microscope was adjusted until parallel fringe patterns appeared in the portion of the field containing the solution and traversed by the two monochromatic (600-μ xenon) light beams (*6*). Retardation of light by the axon material caused deviation of the fringes (Fig. 3A). Normally, the fringes bend upward at 100° where the light path traverses the material of the axon sheath. Through the diameter of the axon the maximum fringe shift was about 1.5 wavelengths (each fringe separation represents 1 wavelength or 600 mμ). When chloride was replaced by $Fe(CN)_6$ the angle for fringe refraction by the sheath material increased to 120°, and the maximum fringe shift for light through the axon diameter was reduced to zero. These increases in the refractive angle and the reductions in the maximum fringe shift, which were completely reversible, were indicative of a marked reduction in refractive index of the material, which, by definition, implies a significant decrease in material density (*6*). This most likely results from water movement into the sheath material, suggested by a pronounced thickening of the axolemma (Fig. 3A, middle picture), and from a loss of substance (potassium, for example) from the internal medium, the axoplasm.

If, instead of the nerve-fiber preparation, a thin layer of animal protein (Knox gelatin) was affixed to a microscope slide and immersed in a drop of solution and if the slide was then placed in one beam of the microscope so that the sol-gel interface appeared in the field, then a fringe pattern, as shown in Fig. 3B, was obtained. Changes in light refraction at the sol-gel interface, when chloride was replaced by ferrocyanide, were almost identical with those observed under similar conditions at the axolemma (Fig. 3A). The angle of refraction in *Homarus* solution was 155° and increased to −150° when ferrocyanide replaced the chloride. This reversed to 170° in standard *Homarus* solution again. Thus, when placed in ferrocyanide, both the material of the axon sheath and the protein gel had similar decreases in refractive index and density, indicative of water movement into the protein material.

Ferrocyanide causes very pronounced bioelectrical changes, and other anions —glutamate, ethanolsulfate, nitrate, propionate, and thiocyanate—are less effective in the order listed, thiocyanate being virtually ineffective. We found, with interference microscopy, that the effectiveness of these other anions, as substitutes for chloride, in reducing the refractive index of protein or axolemma followed the same order as their effectiveness in causing membrane depolarization, spontaneous firing, and total inactivation: $Fe(CN)_6$ > glutamate or ethanolsulfate >> NO_3 or propionate > thiocyanate (very slight effect).

To account for the very pronounced changes in fringe pattern and the simultaneous electrical events caused by $Fe(CN)_6$, one could argue that removal of calcium (inactivation) from membrane sites by the strong chelating forces of the anion was the sole cause. In fact, in numerous experiments in which calcium (five times the standard amount) was added to $Fe(CN)_6$ solution, there was a significant though not complete reversal of the fringe shift and a marked depression of the plateau phase of the prolonged response. On the other hand, solutions with only calcium removed or calcium-free solutions with chloride replaced by propionate or thiocyanate (even those containing ethylenediaminetetraacetic acid) caused only very slight and extremely slow shifts in fringe pattern from the normal and, electrically, only a rapidly transient phase of hyperactivity, followed by irreversible inactivation. Repetitive prolonged depolarizations or plateaus with spikes superimposed never appeared. Thus, even though the reactions to $Fe(CN)_6$ may be depressed by excess calcium, it seems unlikely that removal of calcium by chelation is the sole cause of the results obtained. Consequently, we believe that ferrocyanide and, to a lesser extent, glutamate and ethanolsulfate may act directly on protein structure of the membrane and may produce significant shifts in the fixed charges in this matrix, thus augmenting the effect of chelation. Since protein molecules possess many charged side groups from which high local electric fields arise, exerting attractive forces on water dipoles (*10*) in solution, the fixed-charge patterns may be reoriented by the anions, causing a marked shift in the hydration of these structures, thus accounting for the decrease in mass density. Apparently, such a change in the protein material of the nerve fiber must be intimately associated with depolarization, hyperexcitability, repetitive firing, and ultimately inactivation—possibly by an increased membrane permeability, resulting in a decreased transmembrane gradient in potassium concentration, which has been known to produce oscillatory, prolonged depolarization (*8, 11*). This conclusion necessarily implies that a fixed-charge orientation in the protein-phospholipid matrix of membrane ultrastructure is a basic prerequisite for the existence of transmembrane electrical characteristics at rest and during activity in the excitable membrane.

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