Evidence for Anion-Permselective Membrane in Crayfish Muscle Fibers and Its Possible Role in Excitation-Contraction Coupling

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ABSTRACT Under certain conditions only, isolated crayfish skeletal muscle fibers change in appearance, becoming grainy, darkening, and seemingly losing their striations. These changes result from development of large vesicles on both sides of the Z-line. The longitudinal sarcoplasmic reticulum remains unaffected. The vesicles are due to swelling of a transverse tubular system (TTS) which is presumably homologous with the T-system tubules of other muscle fibers. The vesiculations occur during efflux of water or on reducing external K or Cl, but only when KCl can leave the fiber. They never result from osmotic, ionic, or electrical changes when KCl cannot leave. Inward currents, applied through a KCl-filled intracellular cathode, also cause the vesiculations. These are not produced when the cathode is filled with K-propionate, nor by outward or longitudinal currents. Thus the transverse tubules swell only when Cl leaves the cell. Accordingly, their membrane is largely or exclusively anion-permselective. These findings also indicate that the TTS forms part of a current loop, connecting with the exterior of the fiber probably through radial tubules (RT) possessing membrane of low conductivity. Thus, part of the current flowing inward across the sarcolemma during activity can return to the exterior through the membrane of the TTS. The structure and properties of the latter offer the possibility for an efficient electrical mechanism to initiate excitation-contraction coupling.

INTRODUCTION

In the course of osmometric and electrophysiological studies on single crayfish muscle fibers (13, 31) special characteristics were noted in the membrane of organelles parallel to and lying on both sides of the Z-line. A marked change

in optical appearance of the fibers was always observed under certain experimental conditions, while it was never observed under numerous other conditions. Examination of the preparations with electron microscopy led to the characterization of organelles which appear to constitute a transverse system of tubules (TTS) that is oriented on both sides of the Z-lines, probably at each A-I junction of the fibers (7). A structure of this type was described by Veratti in the claw muscles of *Astacus* (33, Plate 3, Fig. 31). The experimental data indicate that the membrane of the TTS has distinctive anion permeability and is distinguishable by this property from that of the longitudinal sarcoplasmic reticulum. The TTS probably terminates in a series of radial tubules (RT) which are open at the surface of the fibers. The electrophysiological evidence indicates furthermore that the interior of the TTS is isopotential with the exterior of the muscle fibers.

The specific characteristics of the TTS provide the possibility for efficient inward spread of excitation. While positive identification of the TTS with the T-system tubules of other muscles (3, 10) is hindered by the absence of clearly distinguishable triadic complexes in crayfish muscle fibers (7), that homology seems likely. It is therefore possible that the excitation-contraction coupling mechanism proposed here may be operative in other muscles as well as in those of crayfish. Preliminary reports on various aspects of the data have been published (6, 11, 12, 30).

METHODS

The crayfish used in the present work were obtained chiefly from one dealer. They were probably all of the genus *Procambarus*, but of several species. Batches which were obtained and studied with various techniques over a period of about 3 years did not differ significantly in any of their properties.

Single fibers were prepared from the flexor and extensor muscles in the meropodite of the walking limb. Electrophysiological and morphological characteristics of the two kinds of fibers seem to be identical. The fibers that were used in the study were prepared from that group which comprises the largest number in the muscle, ranging in diameter between 100 and 400 μ, and with sarcomere spacings of 9 to 10 μ. Another group, much smaller in number, is composed of fibers that have not hitherto been described in these muscles, but which may be similar to muscle fibers of the stretch receptor organs (28). They are characterized by sarcomere lengths of about 2 μ.

The chitin was removed over the meropodite except for small portions of its articulation with the ischiopodite, where the muscle fuses to the exoskeleton, and with the carpopodite, where the tendon is inserted. Crayfish muscle fibers hyperpolarize when stretched. While this phenomenon has not been studied further as yet, many of the experiments described here also involved measurements of potential and volume. Usually, therefore, a small bridge of exoskeleton between the two joints was also left so as to maintain the muscle at its resting length. In some experiments the tendon was fastened to the floor of the muscle chamber. The chitin bridge could then be eliminated.

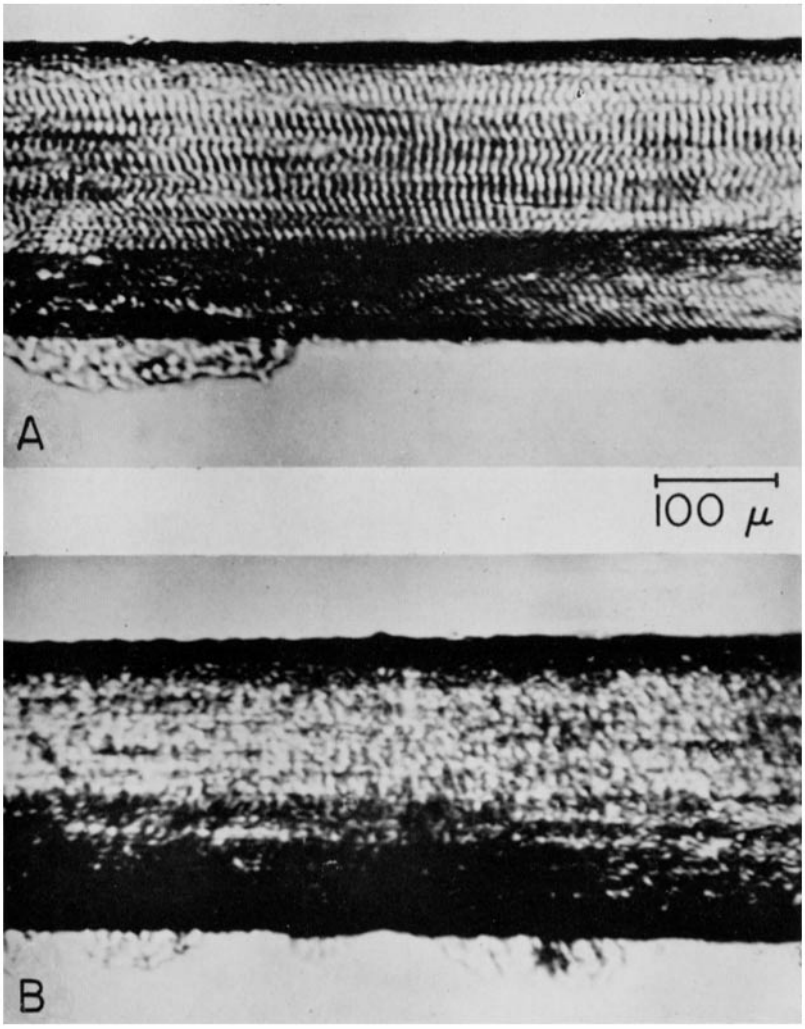

FIGURE 1. Microphotographs of a crayfish muscle fiber. *A*, in the standard saline solution (solution A, Table I), and *B*, 20 minutes after returning it from a hyperosmotic KCl-rich medium (150 meq/liter K^+).

Dissection was carried out under a microscope after the preparation was pinned down inside a lucite chamber and covered with about 5 ml of solution. By successive cuts the muscle was trimmed to a small bundle. The severed fibers could be pulled away readily after cutting the loose and relatively sparse connective tissue. The muscle fibers are very sensitive to mechanical injury and the single fiber of the final preparation could be damaged by merely touching it with the dissecting instruments. The final dissection therefore had to be done with considerable care, using microdissection instruments and sometimes a micromanipulator to clear away the connective tissue and the cut fibers from the remaining single fiber. Despite their sensitivity to mechani-

cal injury the fibers are very stable in the face of ionic or osmotic changes. Furthermore, two or three microelectrodes can be inserted without apparent damage to the cell.

The single fiber, mounted in its chamber, was subjected to various experimental procedures and was photographed at chosen intervals (Fig. 1). Fibers exposed to the same procedures were also prepared for electron microscopy. In these experiments the test solution was replaced by ice cold 1 per cent osmic acid buffered to pH 7.4 with veronal acetate. The fixation was followed under the microscope. After 1 to 3 minutes in the fixative, the fiber could be cut without visible changes in length or structure. Fixation was continued for about 1 hour. The cell was then imbedded in epon. Sec-

TABLE I

	Na	K	Cl	Ca	Propionate	HCO_3
1. Standard media used in the experiments, *meq/liter*						
A. Cl-saline	197.6	20	242	27	—	2.6
B. Propionate-saline	197.6	20	—	27	242	2.6
2. Solutions added to standard media, *meq/liter*						
C. Cl-saline, high Na	1,197.6	20	1242	27	—	2.6
D. Cl-saline, high K	197.6	1020	1242	27	—	2.6
E. Cl-saline, zero Na	2.6	20	47	27	—	2.6
F. Propionate-saline high Na	1,197.6	20	—	27	1242	2.6
G. Propionate-saline high K	197.6	1020	—	27	1242	2.6
H. Propionate saline, zero Na	2.6	20	—	27	47	2.6

tions were examined with an RCA EMU 3F electron microscope. Control fibers which were soaked in the standard solution for the same time as the treated fibers were prepared as routine.

Solutions. In the experiments in which ionic changes were made two basic media were used (Table I) which are modifications of the van Harreveld crayfish Ringer's solution (32). Both contained 20 meq/liter K, or about 4 times that of the above medium. This change reduced contractions of the muscle fibers when they were subjected to still higher levels of K. One of the media (*A*) contained Cl as the major anion, but the second (*B*) was made Cl-free by substituting propionate salts except for a small amount of $NaHCO_3$. Propionate proved to be the best available impermeant anion for a number of reasons which will be detailed elsewhere (13, 31). The omission of magnesium from the medium did not affect various parameters studied in electrophysiological experiments.

Each muscle fiber preparation was equilibrated in one of the two standard solutions for 1 to 2 hours before the experiments were begun. Changes in the ionic composition of the medium were made by mixing desired amounts of solutions high or low in Na, or high in K (C to H, Table I), into the known volume of the solution already in the chamber. When the fibers were to be returned to their control conditions the

bathing medium was aspirated off and the preparation was rinsed several times with the appropriate standard medium.

The electrophysiological data included in this paper were obtained with the techniques standard for this laboratory. The preparations for these experiments were bathed in the van Harreveld medium (32). Further relevant details will be given below.

RESULTS

Effects of Changes in the External Medium

POTASSIUM Electrophysiological measurements (13, 30, 31) indicate that the crayfish muscle fibers are permeable to both K and Cl. These two ions are probably distributed according to a Donnan equilibrium ratio ($r = \frac{Cl_i}{Cl_o} = \frac{K_o}{K_i}$) for concentrations of $K_o > 10$ meq/liter. Similar ionic permeability and distribution of KCl have been found in frog muscle (1, 5, 19). Thus different results are to be expected depending on whether (K_o) and (Cl_o) are changed with or without alteration of the $(K_o)(Cl_o)$ product. If the latter is maintained constant no ionic redistribution across the cell membrane is expected.

Increase in (K_o) was achieved in various ways: (*a*) By adding KCl to the standard Cl-containing solution, making the medium hyperosmotic; (*b*) by substituting K for Na, thus essentially maintaining the medium isosmotic;[1] (*c* and *d*) by increasing K in the Cl-free medium, keeping the latter either isosmotic (by substituting K for Na) or hyperosmotic (by adding K-propionate to the standard Cl-free medium); (*e*) by reciprocal variation of (K_o) and (Cl_o) so as to maintain a constant KCl product. Experiments in which the fibers were equilibrated in any of the above solutions and were then returned to the control medium provided data on the effects of a decrease of (K_o). The effects of these various changes are summarized in lines 1 to 10 of Table II.

Increase of KCl either in a hyperosmotic or an isosmotic medium caused changes in volume which were comparable to those observed in frog muscle under similar conditions (5). In the isosmotic conditions, in which the fiber volume could increase several fold, electron microscopy showed that the water was distributed around the myofibrils and under the sarcolemma. In the hyperosmotic conditions the cell equilibrated to a volume only slightly higher than the control. No modification of intracellular organelles was observed under either condition (Fig. 2).

However, within about 10 minutes after returning the preparation to the control solution (A, Table I), the fibers began to take on a grainy appearance and became darker. Finally, the striations seemed to disappear (Fig. 1).

[1] In the range of Na concentration used, the osmotic coefficients of NaCl and KCl are nearly identical.

Electron micrographs (Fig. 3 *A*) showed, however, that the striations were still present, but that large vesiculations had appeared which were concentrated on both sides of the Z-lines. These vesiculations, which might be as large as 4 μ in diameter, apparently disrupted the path of the light transmitted through the fibers and thereby obscured the regular ordination of the banding in the muscle fibers. Particularly noteworthy is the fact that the longitudinal sarcoplasmic reticulum was not involved in the swelling. Accordingly, the vesiculations must have occurred in an independent system of organelles whose morphology will be described in a detailed study of the electron microscopy of

TABLE II

SUMMARY OF EFFECTS OBSERVED ON CHANGING IONIC CONDITIONS

Experimental conditions	KCl flux expected across membrane	Observed E_M	Osmotic change	Swelling of TTS
1. KCl added to solution A	In	Depol.	Increase	0
2. Reversal from 1	Out	Repol.	Decrease	+
3. K substituted for Na in solution A	In	Depol.	0	0
4. Reversal from 3	Out	Repol.	0	+
5. K-propionate added to solution B	0	Depol.	Increase	0
6. Reversal from 5	0	Repol.	Decrease	0
7. K-propionate substituted for Na-propionate	0	Depol.	0	0
8. Reversal from 7	0	Repol.	0	0
9. K increased $(K_o)(Cl_o)$ = constant	0	Depol.	0	0
10. Reversal from 9	0	Repol.	0	0
11. Cl substituted by propionate	Out	Depol.	0	+
12. Reversal from 11	In	Hyperpol.	0	0
13. NaCl added to solution A	Out	Hyperol.	Increase	+
14. NaCl removed from solution A	In	Depol.	Decrease	0

crayfish muscle fibers (7). These organelles are differentiated from the longitudinal system of the sarcoplasmic reticulum not only by their transverse orientation, whence the descriptive term *transverse tubular system* (*TTS*), but also by differences in the permeability characteristics of their boundary membranes. The vesiculations occurred, as shown in lines 2 and 4 of Table II, when there was an efflux of KCl, while the fiber was repolarizing. The changes were independent of the osmotic conditions.

Structures which may be identical with the TTS have been described in the muscle fibers of the stretch receptor organ of crayfish (28). However, in the latter they have been reported as continuous with the tubules of the longitudinal system of the sarcoplasmic reticulum. It seems likely that the TTS is probably homologous with the T-system of transverse tubules which have been described in vertebrate muscle fibers (3, 10).

The vesiculations which occurred on the return of fibers from a KCl-rich medium to the standard solution A did not develop when (K_o) was increased either in a Cl-free medium or when the product (K_o) × (Cl_o) was maintained constant. As summarized in lines 5 to 10 of Table II, the common feature of these changes in ionic composition is that no flux of KCl is expected across the membrane of the muscle fiber. Osmotic conditions were varied without producing the vesiculations of the TTS. The experiments demonstrate further that the vesiculations were not produced by changes in the membrane potential.

CHLORIDE Variations of (Cl_o) could be made independently of changes in (K_o) by stoichiometric substitution of Cl with propionate and *vice versa.* Electrophysiological and volumetric data (13, 31) show that KCl is removed from the cell in the first case and that it reenters the cell in the second case. Upon replacement of Cl by propionate there was a rapid darkening of the muscle fibers which was followed by disappearance of the striations, like that shown in Fig. 1. However, in order to observe these changes in the appearance of the fibers under the light microscope it was necessary to load the fibers initially with Cl, by soaking them in KCl-rich solutions, which could be either hyperosmotic or isosmotic. Electron micrographs of the fibers which were fixed during the change in optical appearance showed that vesiculations had occurred which were indistinguishable from those illustrated in Fig. 3. These changes could be reversed on returning the fiber to a Cl-containing medium.

Removal of Cl from the medium results in a temporary shrinkage as well as depolarization of the fiber (31). Thus, the vesiculation in these experiments occurred during depolarization (line 11, Table II), whereas in the case of the conditions in line 2 and line 4 of Table II the vesiculations developed during repolarization of the fibers. The vesiculations were again independent of the tonicity of the medium, and the necessary condition again appears to be the efflux of KCl.

NACL Crayfish muscle fibers respond as osmometers for a wide range of concentrations of NaCl (31) in much the same way as does whole frog muscle (5). The swelling produced by exposing the crayfish fibers to hyposmotic NaCl media could be as much as fourfold increase in volume. This swelling was not accompanied by changes in optical appearance of the fibers, nor by alterations in the appearance of the longitudinal sarcoplasmic reticulum or of the TTS. As in the case of the swelling produced in KCl-rich media the accumulation of water was predominantly in the superficial part of the fiber immediately under the sarcolemma and between the myofibrils (7).

On increasing the osmotic pressure with addition of NaCl to the standard solution (A, Table I) there was a transient darkening of the muscle fibers which diminished within 10 minutes. The striations seen in the light microscope were not obscured. Fibers which were fixed during the period of darken-

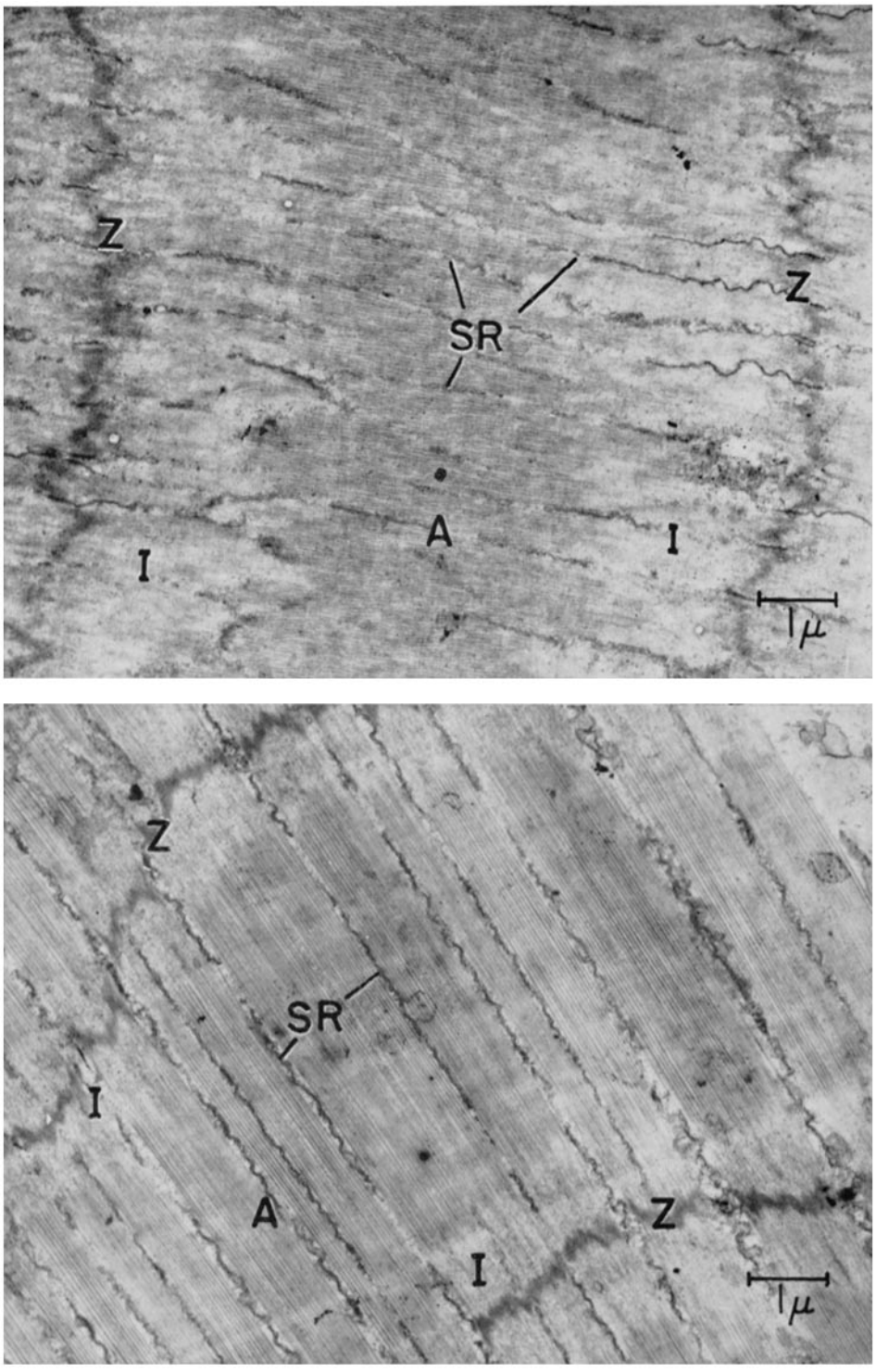

FIG. 2

ing showed swelling of the TTS, but the vesiculations were relatively small (Fig. 3 *B*).

Shrinkage of the fibers in the hypertonic NaCl medium is accompanied by hyperpolarization which results from increased intracellular concentration of K (31). Thus, the vesiculations were produced during hyperpolarization, again under a condition in which KCl efflux was expected because of the relative increase in $(K_i) \times (Cl_i)$ product. However, these conditions represent small displacements from equilibrium and the efflux could not have been large, although it was apparently sufficient to cause small and temporary vesiculations in the TTS.

SUMMARY In the foregoing experiments a number of factors were modified in various combinations. Changes in membrane potential, tonicity of the medium, or volume of the fiber appeared to have no direct effect on the TTS (Table II). The vesiculations which denoted swelling of the TTS always occurred when there was efflux of KCl from the fiber, and only then.

Effects of Applied Currents

That the optical change and vesiculations were, indeed, produced by a flow of ions across the cell membrane was also shown by the effects of applied currents. Experiments of this type had the further advantage that they permitted a clearer identification of the nature of the flux which caused the optical and morphological changes. The currents were applied either through one intracellular microelectrode and another electrode external to the fiber, so that they flowed into or out of the cell across the membrane, or they were applied through two intracellular electrodes, to flow longitudinally within the fiber.

Volume changes indicative of electroosmotic movements of water were observed with transverse currents, the fiber swelling with inward currents and shrinking with outward currents (31). A swelling such as is shown in the inset photograph of Fig. 4 was produced no matter whether the microelectrode (cathode) was filled with KCl or K-propionate. When the electrode was filled with a KCl solution there was also an optical change in the region of the microelectrode. The change became quite evident after a current of 2×10^{-7} A was applied for 5 to 10 minutes. It developed more rapidly with larger current and the opacity became more marked as the current was passed for longer times. These optical changes were accompanied by vesiculations

FIGURE 2. Electron micrographs. *Above*, a muscle fiber kept in the standard Cl-saline as a control. Longitudinal section. The Z-lines take a tortuous course. The sarcoplasmic reticulum (SR) does not show any readily observable modification at the level of the Z-lines, or at the A-I junctions. *Below*, a fiber which had been equilibrated in the hyperosmotic KCl medium. Except for some swelling of the whole fiber the appearance did not differ markedly from that of the control preparation.

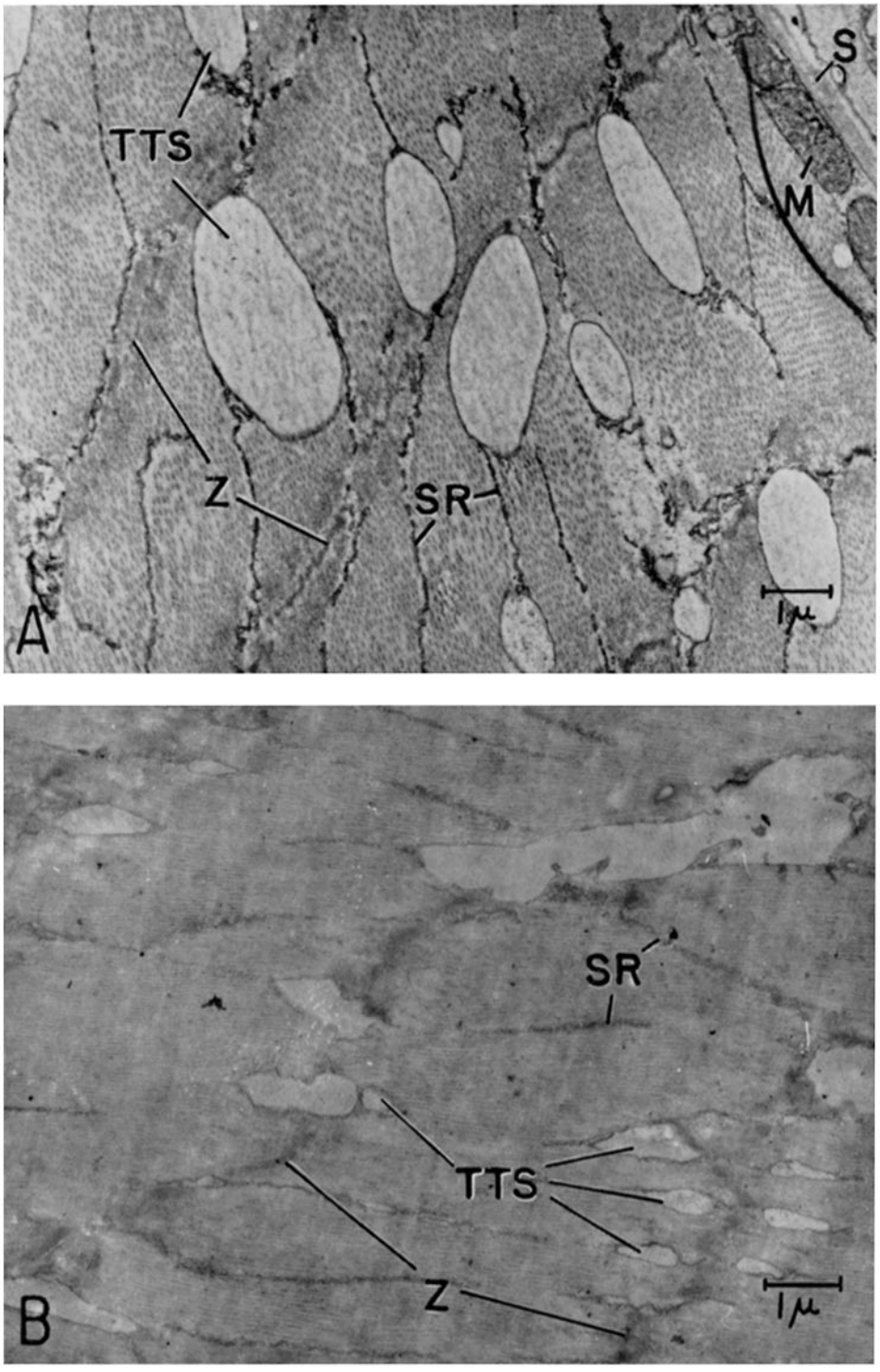

FIG. 3

(Fig. 4 *A*) which were even larger than those shown in Fig. 3 *A*. When the current was stopped the opacity slowly disappeared. However, we have not yet carried out detailed studies of the reversal with the requisite examination of numerous electron microscopic preparations.

Outward (depolarizing) currents applied through an intracellular microelectrode filled with KCl did not cause a change in optical appearance of the fiber. When the microelectrode was filled with K-propionate neither outward nor inward currents produced the changes in optical appearance, nor did they cause vesiculations (Fig. 4 *B*). Thus, flux of K in either direction did not bring about the changes. The latter occurred only when a diffusible anion, Cl, was made to leave the cell during passage of an inward current.

Furthermore, the current had to flow across the cell membrane; when longitudinal currents were applied no optical change occurred at either the cathode or the anode, and it made no difference whether the electrodes were filled with K-propionate or KCl. No contractions were observed with longitudinally applied currents of 5 to 10 μa, which were the largest amounts that could be passed through the microelectrodes. The results of the foregoing experiments suggest that the sites for K and Cl fluxes are spatially separated. The membrane of the TTS is at least predominantly Cl-permselective, while movement of K takes place chiefly or exclusively across the plasma membrane. Other evidence for the existence of separated areas permselective for K and Cl respectively was obtained by electrophysiological and pharmacological methods (13, 30, 31).

Permeability of the TTS to Other Anions

A few experiments were done to test the permeability of the TTS to NO_3. Single fiber preparations which had been equilibrated in a NO_3-Ringer solution were exposed to isotonic KNO_3 (as in condition 3, Table II) until the volume had reached its equilibrium level. Reintroducing the $NaNO_3$ in substitution for KNO_3 produced a marked optical change like that of Fig. 1 *B*.

Several experiments were performed applying currents through an intracellular microelectrode filled with KNO_3. However, no changes were observed under the light microscope when applying inward current, although the quantity of current applied was at least twice as great as that which produced the darkening and vesiculations shown in Fig. 4 *A*. The absence of an

FIGURE 3. Electron micrographs of fibers showing vesiculations. *A*, this fiber had been exposed to the hyperosmotic KCl medium and then returned to the standard Cl-saline, as in the case of the fiber of Fig. 1. The vesiculations (TTS) appear to be in a system of organelles which are independent of the SR. M, mitochondrion; S, sarcolemma. *B*, this fiber was exposed to a hyperosmotic NaCl medium for 3 minutes before fixation. The vesiculations are smaller and are clearly associated with the regions of the Z-lines. The bases of the vesicles start at the A-I junctions (lower right).

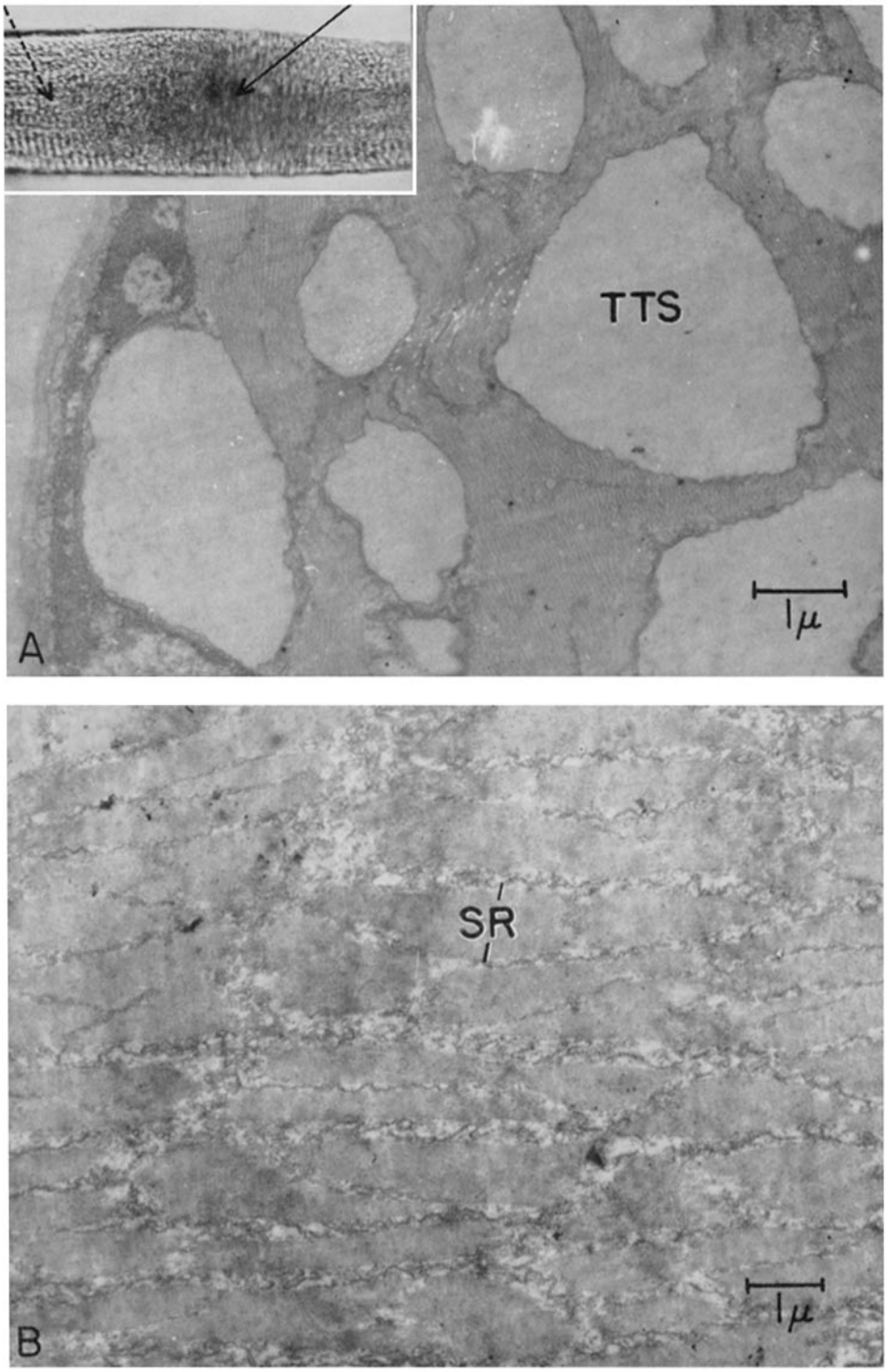

FIG. 4

effect with the applied current, although there was a marked effect when the fiber was loaded with NO_3, is presumably related to the smaller amount of this anion transported by the current, indicating a lower permeability of NO_3 than of Cl, as is the case in frog muscle (1, 16).

Connection between the TTS and the Exterior

MORPHOLOGICAL DATA The results presented above show that movements of ions between the interior of the cell and the external medium, whether by changing ionic conditions or by applied currents, also result in ionic movements across the membrane of the TTS. These findings indicate that the TTS must also connect with the external medium. Channels which probably form this connection are shown in electron micrographs (Fig. 5) in the form of well defined radial tubules (RT) about 200 A in diameter which run across the mitochondrial layer at the periphery of the fiber and toward the sarcolemma. The tubules become highly convoluted near the cell surface, but their membrane is continuous with the plasma membrane, and openings to the exterior have been observed in many preparations (7). The membrane of the tubules becomes particularly prominent in solutions containing high Ca, but this finding has not been pursued further as yet.

Electrophysiological Evidence for the Existence of RT's

Measurements of various electrophysiological properties of crayfish muscle fibers (12, 13) provided evidence for the existence of RT even before the latter structures were observed in electron micrographs. These data, on the effective resistance (R_{eff}) and the length constant (λ), will be described in detail in another paper (13).

The measured values of R_{eff} showed a marked scatter for different fibers of the same muscle, whereas the measured value of λ showed rather little dispersion. The scatter of R_{eff} values was correlated with the variation in the diameters (D) of the individual fibers, and this correlation thus implied a relative lack of correlation between λ and D. The measured values of the latter two parameters and a value of the sarcoplasmic resistance (R_i) calculated to be

FIGURE 4. Effects of intracellularly applied inward currents, using a KCl-filled microelectrode (*A*) and one filled with K-propionate (*B*). *Inset*, shows a microphotograph of the fiber. The electrode for applying current is indicated by the solid line arrow. The broken line arrow indicates the location of another electrode used for monitoring the membrane potential. Note the disappearance of the striations at the site of the stimulating electrode and the swelling of the fiber. The electron micrograph was made from a section of this fiber close to the current electrode after a hyperpolarizing current of 10^{-7} A had been applied for 10 minutes. Note extreme vesiculations. *B*, another fiber. A hyperpolarizing current of 10^{-7} A was applied for 30 minutes but through a microelectrode filled with K-propionate. This fiber swelled markedly, water having accumulated in the interfibrillar spaces. Note absence of vesiculations.

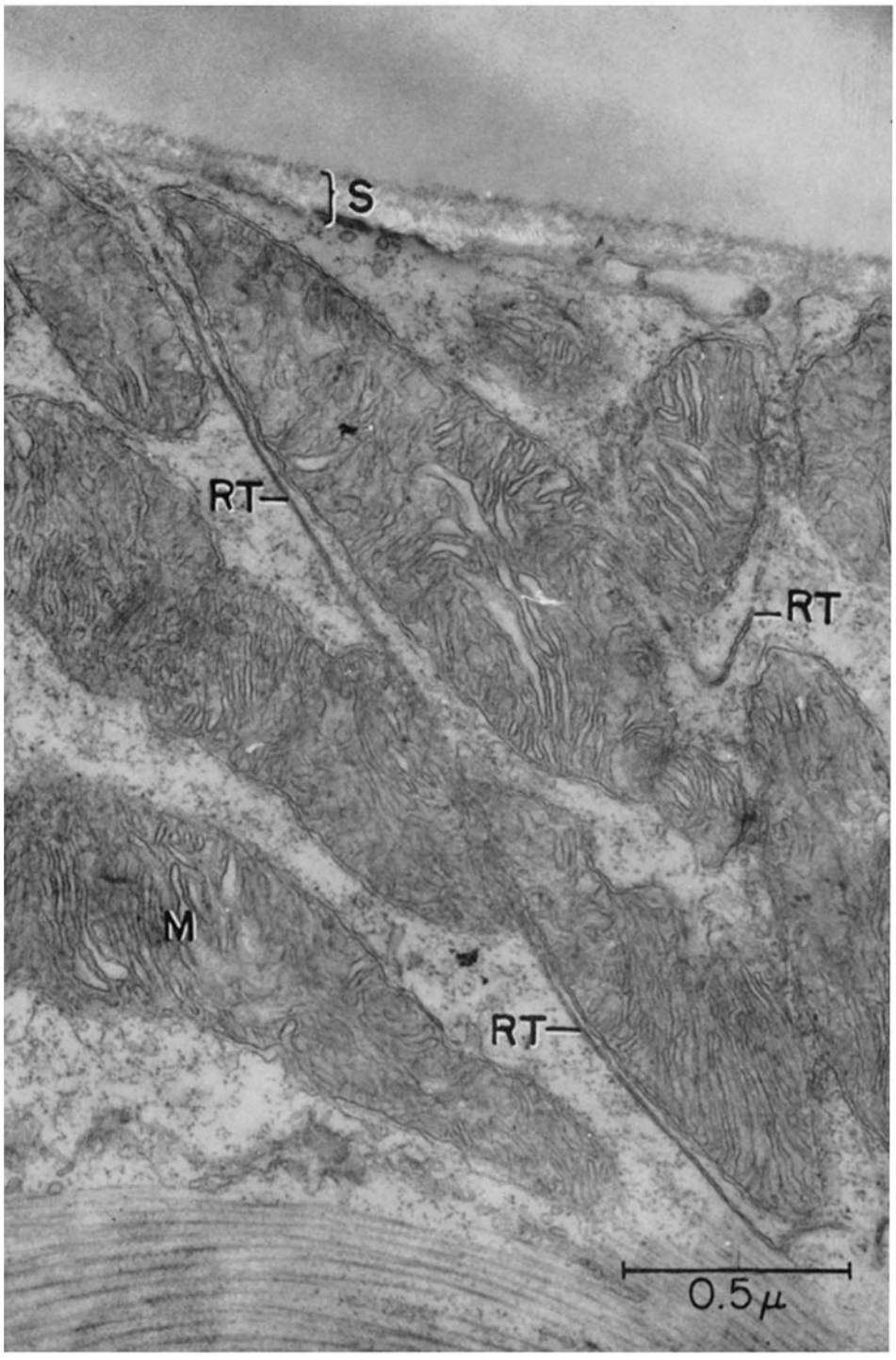

FIG. 5

310 ± 50 Ω cm provided a means for estimating the "membrane" resistance (R_{MP}) according to an approximation form of the cable equation (21, 26):

$$R_{MP} = \frac{4R_i\lambda^2}{D} \quad (1)$$

Unexpected, and previously undescribed, was the finding of a positive correlation ($r = 0.90; P < 0.05$) between D and R_{MP}. That relation was found to be expressed by the regression equation:

$$R_{MP} - 7893 = 72(D - 199) \quad (2)$$

with R_{MP} expressed in Ω cm² and D in μ.

This relation suggested that in crayfish muscle fibers there exist other conductive radial pathways in parallel with the resistance of the plasma membrane. If these pathways are radially distributed tubules, the RT, in aggregate having a low resistance in comparison with that of the cell membrane, their length and resistance would increase with the diameter of the muscle fiber to give the relation described in Equation 2.

The electrophysiological data (13) as well as some of the morphological observations on the RT (7) permit a few deductions regarding the properties of the network formed by the parallel channels and the plasma membrane. For convenience the numerical calculation shown here will be given in terms of the 10 μ sarcomere as a unit length of the muscle fiber. In a 180 μ fiber the surface area of a sarcomere is about 6×10^{-5} cm² and R_{MP} is about 6.5×10^3 Ω cm². The resistance accordingly is 10^8 Ω. In a 360 μ fiber R_{MP} is about 19.5×10^3 Ω cm² and the surface area is about 12×10^{-5} cm². Thus, on doubling the diameter the resistance increased about 1.6-fold. As an approximation it is assumed that only the length (not the number) of the RT increases when D increases, and that other properties of the system remain unchanged. The resistance of the TTS membrane is assumed to be negligible, in comparison with the resistance of the RT. If the total resistance of a unit length increases 1.6-fold when the resistance of the RT doubles, the parallel resistance of the plasma membrane must have a value about eleven times higher than the resistance of the RT. On the basis of the values of resistance (10^8 Ω) and the surface area (6×10^{-5} cm²) of a sarcomere, the specific resistance of the plasma membrane should be about 7×10^4 Ω cm².

The electrophysiological data also permit an estimate of the number of RT

FIGURE 5. Electron micrograph of radial tubules (RT) in a fiber which had been soaked in an isosmotic Cl-saline enriched with 10 × $CaCl_2$. A radial tubule crosses the mitochondrial layer (M) diagonally. It becomes convoluted before reaching the sarcolemma (S). The RT approaches the Z-line at the lower right hand corner. In part of another RT (seen in upper right) the convolutions are particularly prominent.

in each sarcomere. As an approximation it may be assumed that the membrane forming the walls of the RT has a higher resistivity than does the fluid inside the tube. The lumen has a diameter of about 200 A and the fluid has a resistivity of about 50 Ω cm.[2] Thus, the linear resistance of the RT is about 2×10^{13} Ω/cm. If their average length is about 20 μ about 400 RT would be required to constitute the resistive path of 10^8 Ω of a single sarcomere of a 180 μ fiber. Estimates on the basis of the density of RT observed in electron micrographs gave a value of 1200 RT/sarcomere.

A lower limit can also be estimated for the membrane resistance of the RT. It is likely that the resistance of the wall of the tubule is at least as high as is the resistance of the fluid in the lumen; *i.e.*, that the "length constant" of the RT is at least as great as the length of the RT. For a 20 μ tubule the specific membrane resistance of the RT would then be about 500 Ω cm^2.

On the basis of other electrical measurements Falk and Fatt (9) have also concluded that frog and crayfish muscle fibers have radial tubules which contribute a component in parallel with the membrane resistance. According to their data the AC resistance of the tubules is about one-tenth that of the cell membrane in frog muscle fibers.

DISCUSSION

The Nature of the Organelles and Their Characteristics

The data presented above demonstrate that there are present on both sides of the Z-line of crayfish muscle fibers organelles with anion-permselective membrane characteristics. Under the conditions which exist in living cells, where the diffusible intracellular anion is chiefly or entirely Cl, the anion-selective sites are permeable exclusively to Cl, although the occurrence of some component of cation-selective membrane in the TTS is not ruled out. The precise structure of these organelles is in some doubt, however, since they might be discrete vesicles, each connected to an individual RT, or they might be interconnected as a system of anastomosing tubules opening to the exterior through the RT. Definitive morphological evidence would require study of serial electron microscopic sections.

The electrophysiological evidence is decisive on the existence and properties of the TTS. Only currents which carried Cl out of the fiber produced the swellings. If the swellings had occurred in a system of intracellular vesicles or vacuoles, longitudinal as well as inward current could have produced them. However, if the organelles are a system of tubules transversely oriented in the neighborhood of the A-I junctions the same evidence requires that the TTS have some connection with the exterior of the fiber. The occurrence of

[2] The resistivity of the van Harreveld saline solution as determined with a conductivity meter was about 40 Ω cm at 20°C.

radial tubules, both as morphological and as electrophysiological entities, suggests that these tubules form the channels between the TTS and the periphery. However, the anatomical demonstration of that relation also would require the study of serial sections with electron microscopy.

Radial tubules of similar appearance have also been described in muscle fibers of mouse (3) and toadfish (10) and in the former their connection with the T-system has been established. Peterson and Pepe (28) have also observed tubules, but of much larger diameter, in the muscle fibers of the crayfish stretch receptor. They have identified these with the longitudinal sarcoplasmic reticulum, and apparently also regard the latter as confluent with what we have characterized as the TTS. The data presented above show clearly that the TTS and the sarcoplasmic reticulum are separate systems. If the radial tubules of the crayfish skeletal muscle fibers also form the peripheral terminals of the TTS, then these radial tubules must also be independent of the sarcoplasmic reticulum.

There are connections between the radial tubules and the plasma membrane (7), but they are normally not as wide as the invaginations described by Peterson and Pepe (28). Invaginations of the cell surface which are distinct from the RT have also been observed in the present work and the RT frequently begin at these invaginations (7). At the periphery, close to the plasma membrane, the RT develop complex convolutions (Fig. 5) and it is conceivable that these often open to the exterior by pores which are usually missed in the small number of samplings available with electron microscopy. Fawcett and Revel (10) have commented on the fact that radial tubules are seen rather frequently whereas openings are not seen in most preparations.

The electrophysiological data show that current can flow between the interior of the muscle fiber and the exterior through channels which are formed by the TTS and RT, and that the membrane of the TTS is anion-permselective. The efflux of Cl or NO_3 through the TTS, under the conditions described in Table II, can occur only if the TTS is accessible to the external solution and the interior of the channels, in the initial condition, is isopotential with the exterior of the muscle fiber. If the interior of the channels were isopotential with the sarcoplasm no electrochemical driving force could have developed to move anions out through the membrane of the TTS and to cause their swelling. However, if the interior of the TTS is at the same potential as the outside of the plasma membrane there must be a potential across the membrane of the TTS which is E_{Cl}, the "Nernst potential" for this ion (Fig. 6). Because Cl and K are distributed according to the Donnan ratio, E_{Cl} and E_K are both at, or close to the resting potential. This is confirmed by the fact that the inhibitory postsynaptic potentials which in crayfish muscle fibers involve Cl activation (4) are small.

As far as we know, the evidence presented here is the first direct demon-

stration that the membranes of intracellular organelles may differ in permselectivity from the plasma membrane. Adrian and Freygang (2) have suggested that in frog muscle fibers an intracellular compartment of "endoplasmic reticulum" is separated from the extracellular space by a membrane which is permeable to both Na and K, while the interior membrane, between the reticulum and the sarcoplasm, is chiefly or exclusively permeable to K. Both membranes are regarded as impermeable to anions. These assumed characteristics are different from those which have been demonstrated in the crayfish fibers both for the TTS and for the channels connecting this

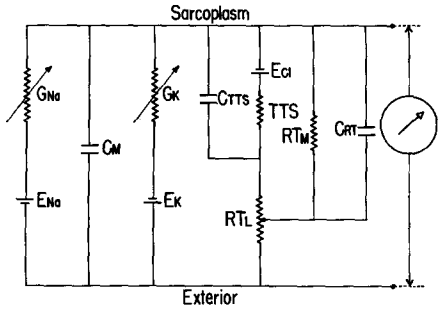

FIGURE 6. Equivalent circuit of a crayfish muscle fiber. The K and Na batteries and conductances of the plasma membrane are shown on the left. E_{Cl} is the potential across the membrane of the TTS. RT_M and RT_L are respectively the resistance of the membrane and lumen of the radial tubules, represented by lumped components, with RT_M forming a path from the sarcoplasm to the lumen of the RT. Recording conditions with an intracellular microelectrode shown on the extreme right. Three membrane capacities (across the plasma membrane and the membranes of the TTS and RT, respectively) are included in the diagram.

space with the extracellular volume. While the properties of the longitudinal component of the sarcoplasmic reticulum are not as yet characterized, they are undoubtedly different, at least in the crayfish.

The impedance locus data (9) which indicate that the frog as well as crayfish muscle fibers have a low resistance pathway in parallel with the plasma membrane have also been interpreted as evidence for the existence of radial tubules. These data do not provide information regarding the permeability characteristics of the membrane. However, the equivalent circuit suggested by Falk and Fatt (9) calls for a capacity in series with the resistance of the radial tubules. The swelling of the TTS obtained in the present work increased with the electrical and electrochemical driving forces and it developed only slowly during continued passage of current, or after a change in the external medium. Thus, it is unlikely that a physical barrier to the steady flow of ions, analogous to a capacity, exists, at least in crayfish muscle fibers.

An equivalent circuit which satisfies the data reported here is shown in Fig. 6. The plasma membrane is represented conventionally, with two reactive resistances G_K and G_{Na}, each in series with an ionic battery, E_K and E_{Na}, respectively. The membrane also has a capacity, C_M. The membranes and lumens of the TTS organelles are represented by a single resistance (which may be reactive), TTS, and which is in series with a Cl battery, E_{Cl}. The latter is oriented inside-negative like the resting potential. A capacity C_{TTS} is across the membrane of the TTS. These elements are in series with RT_L, the resistance of the interior of the RT. The membrane of the latter is shown as another resistance (RT_M) coupled between the sarcoplasm and the midpoint of RT_L. The capacity of this membrane component is C_{RT}.

The complex network shown in Fig. 6 might give rise to the impedance locus data of Falk and Fatt (9). The present work provided no information regarding the magnitudes of the respective capacities. Likewise, the resistance of the TTS could not be estimated from the present data. However, on the basis of the electrophysiological evidence described above it is inferred that the resistance of the TTS is low in comparison with RT_L or RT_M. As already noted RT_M is probably higher than RT_L.

MECHANISM OF THE SWELLING OF THE TTS The various conditions under which the swelling occurs in the TTS provide information on the properties of the system. Osmotic conditions do not determine the presence or absence of the vesiculations whereas movement of Cl in a specific direction is necessary and sufficient. This movement, an efflux of Cl, can be effected by removal of Cl from the external medium (Table II), by an inward current which is delivered through a KCl-filled intracellular microelectrode (Fig. 4 *A*), or by causing an efflux of KCl from the cell (Table II).

The mechanism of the swelling which is suggested by the present data is illustrated in Fig. 7. It includes in a continuous structure the three types of membranes whose characteristics are relevant for the discussion: the plasma membrane, the membrane of the TTS, and that of the radial tubules (RT). The longitudinal sarcoplasmic reticulum is omitted. The channels of the RT showed little or no increase in diameter when there was marked swelling of the TTS. Thus, little or no movement of water across the membrane of the RT was produced in the course of the present experiments. It seems likely therefore that this membrane component has a low conductance. The membrane of the TTS probably has a high conductance relative to the other membrane components. It is largely or exclusively anion-permeable, which means that under physiological conditions it is mainly Cl-permselective. Furthermore, as has been noted above, the majority of sites permeable to K must lie in the plasma membrane.

Evidence will be presented elsewhere (13) which indicates that the plasma

membrane is probably a fixed charge structure with predominantly negative charges. The membrane of the TTS accordingly probably has a predominance of positive fixed charges. However, whatever the nature of the respective permselectivities of the two membranes, application of a hyperpolarizing, *i.e.* inward, current with a KCl-filled intracellular microelectrode will result in an influx of K across the plasma membrane and an efflux of Cl across the

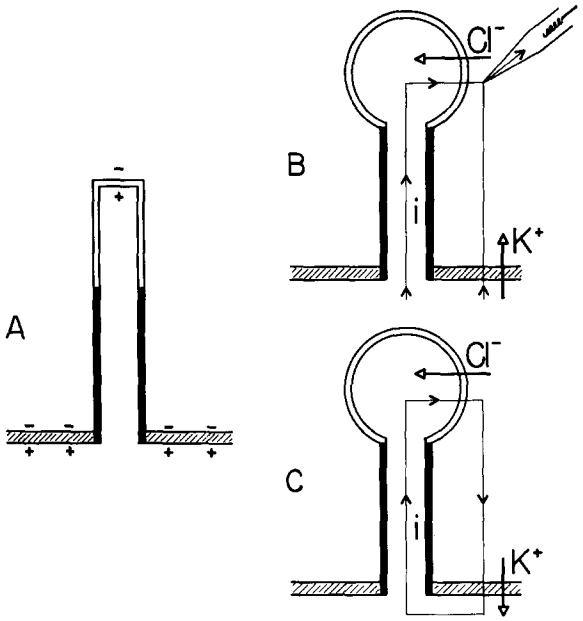

FIGURE 7. Diagrammatic models of two mechanisms for the swelling of the TTS. The plasma membrane is indicated with diagonal lines, the membrane of the RT is black, and that of the TTS is clear. *A*, initial condition. *B*, swelling is induced by an inward current delivered through a KCl-filled microelectrode. Movements of K and Cl are shown by heavy lines and of current (i) by thin lines. *C*, ion and current flows during membrane depolarization which is caused by removal of Cl from the external medium or during repolarization following removal of high external KCl.

membrane of the TTS (Fig. 7 *B*). Accumulation of water and swelling of the TTS could occur in either or both of two ways: (*a*) The inward current is carried into the tubules from the exterior by approximately equal and opposite movements of Cl and cations. From the tubules into the sarcoplasm it is carried at least predominantly by efflux of Cl. Thus there will be a net accumulation of salts within the tubules with an accumulation of water through normal osmosis. (*b*) An electroosmotic water movement can occur if the membrane is permselective because of the presence of fixed charges. The water will move under the influence of a current in the same direction as do the ions for which the charged membrane is permselective (22). According to the postulated permselectivities of the respective membranes,

when an inward current is applied the movement of water would be inward across the plasma membrane, with the K^+, and outward across the membrane of the TTS, with the Cl.

Once the water enters the TTS by either mechanism it is probably trapped within the compartment because of the high frictional (Poiseuille) resistance of the RT. Not only the TTS, but also the whole muscle fiber swells when hyperpolarizing current is applied (31, and inset of Fig. 4 *A*). It is therefore likely that both kinds of water movement occur together, but the evaluation of their relative contributions from the data at hand requires too many assumptions.

In model mosaic membrane systems with anionic and cationic components a current flows when the two sides of the membrane are exposed to different ionic conditions (27). This current can give rise to electroosmotic effects (22). The plasma membrane and the membrane of the TTS, together form such a "mosaic" structure. Since the two components are close to each other the electroosmotic effects may be quite marked (22). Data on volume changes (31) do indeed indicate that electroosmotic movement of water is a prominent feature in crayfish muscle fibers.

Upon changing the ionic medium the swelling of the TTS always occurred when (K_o) or (Cl_o) was modified (Table II) in such a way that E_K, the Nernst potential for K became more negative than E_{Cl}, the EMF of the Cl battery. The change would result (Fig. 7 *C*) in a local current flowing inward through the TTS and would cause an efflux of Cl. The local current could be achieved by decreasing an initially high level of (K_o), thus repolarizing the cell; or by decreasing (Cl_o) which causes a transient net depolarization of the cell as the potential across the tubular system E_{Cl} becomes less inside-negative than E_K. Modification of (K_o) and (Cl_o) in such a way as to leave both E_{Cl} and E_K unchanged cannot produce a local current and, indeed, in such experiments no swelling occurred. No local current would occur when (K_o) was changed in a Cl-free medium, and swelling of the TTS was absent in this condition also.

When propionate, as an impermeant anion, is substituted for Cl (or NO_3) in the medium the swelling of the TTS does not depend upon diffusion of the anions through the RT. Indeed, as noted above, the frictional resistance of the RT must be high, and diffusion through the 200 A channels would be very slow. However, a junction potential must be established at the orifices of the RT immediately upon changing the solution. This potential can provide the electrical driving force. With the efflux of Cl (or NO_3) across the membrane of the TTS there will be an electroosmotic flow of water in the same direction. The current can also transfer cations from the bathing medium into the RT and TTS, as described above, with a consequent accumulation of salts in the TTS and an osmotic influx of water.

In theory, current could also cause an electroosmotic flow of water through

the RT. The RT, however, have large diameters in relation to the "pores" of the membrane of the TTS. For a given amount of current, therefore, electroosmotic effects will be more pronounced in the TTS than in the RT. The difference is shown by the fact that current alone is not a sufficient condition to cause swelling of the TTS. It is also necessary that the current be carried by an efflux of Cl (Fig. 4).

THE POSSIBLE ROLE OF THE TTS IN EXCITATION-CONTRACTION COUPLING

The role of the sarcoplasmic reticulum and more specifically of the transverse tubules (T-system) in spreading excitation from the plasma membrane into the interior of muscle fibers has been the subject of considerable speculation by morphologists (*cf.* references 25, 29). Physiological evidence in support of this view has come from A. F. Huxley and his colleagues (23–25). However, the contractions which were produced by applied depolarizing currents were graded in amplitude. Thus, the depolarization probably spread along the tubules not by a regenerative and all-or-none propagation like that of the spike, but electrotonically, in twitch muscle fibers which have an electrically excitable plasma membrane component. The strongest current employed by Huxley and Taylor (25) could produce contractions no deeper than 10 μ inside frog muscle fibers.

It has been frequently stressed (*cf.* references 23, 25) that the cell membrane must become depolarized in order that excitation-contraction coupling can occur. Longitudinal currents, applied through two intracellular microelectrodes, do not cause contractions in frog muscle fibers (34) and, as noted above, this finding has also been obtained in the present work on crayfish muscle fibers.

As has been shown above, however, in crayfish muscle fibers there are present organelles, the TTS, which have anion-permselective membrane and which are probably connected to the exterior through the low conductance pathway of numerous RT. These conditions permit a spatial separation of differently permselective sites of the cell surface. The plasma membrane (Fig. 6) is predominantly a K-battery in the resting fiber and it tends to become a Na-battery during activity. Lying within the interior of the cell, but connected to the outside through the RT, is a Cl-battery at the membrane of the TTS. At rest, E_{Cl} and E_K are equal and opposed. During activity the plasma membrane potential moves toward inside-positivity and current can now circulate between the interior of the fiber and the external medium across the membrane of the TTS and through the channels of the RT. Indeed, it seems likely that only about 10 per cent of the action current can leave through the inactive plasma membrane in crayfish muscle fibers.

With the influx of Na and/or other cations across the plasma membrane during the action potential, an influx of Cl and/or other anions will occur from the RT and across the membrane of the TTS. However, the outward

flow of current across the anion-permselective membrane of the TTS might also lead to a local accumulation of cations (for example, Ca) in the cell interior in the vicinity of the TTS. The A-I junctions where the latter are located, presumably constitute strategic sites for triggering contraction. A local accumulation of certain cations might itself be sufficient to initiate excitation-contraction coupling and it would thus be unnecessary to postulate (*cf.* reference 23) an additional step involving secretory activity of the membrane of the TTS.

In principle the mechanism for excitation-contraction coupling outlined above does not differ from the suggestions made by other workers chiefly on the basis of studies on frog muscle (2, 20, 23, 25). It makes use of a specific property that is found in crayfish muscle fibers, the presence of organelles which appear to have a predominance of anion-permselective membrane and which connect with the exterior of the fiber. This property, in conjunction with the electrophysiological characteristics of the plasma membrane (13), establishes a condition in which there is a spatial separation of differently permselective sites of the cell surface, and that condition must involve the circulation of currents. Thus, the difficulties in obtaining excitation-contraction coupling noted by Hill (17, 18) would be overcome.

The conductance of crayfish muscle fibers is markedly increased during passage of hyperpolarizing current of sufficient magnitude. This change is caused (13) by an increased conductance for Cl (hyperpolarizing Cl activation, 15). The site of the response appears to be at the membrane of the TTS (13). It is therefore conceivable that the same membrane might also respond with increased Cl conductance to the current which flows outward across it during the action potential. A response of this type, involving depolarizing Cl activation, occurs in Rajid electroplaques (8, 15). If such activity does occur in crayfish muscle fibers it would cause an increase in the proportion of the current flowing through the transverse channels at the expense of the current flowing out through the plasma membrane. The net effect, therefore, would be to enhance the excitation-contraction coupling mechanism outlined above.

SOME OTHER POSSIBLE CONSEQUENCES OF THE SHUNTING CHANNEL FORMED BY THE TTS AND RT As a general rule, muscle fibers conduct much more slowly than do unmyelinated axons of comparable diameter. The amount of action current which can flow through the, as yet, inactive plasma membrane of a muscle fiber to excite it is diminished in proportion to the effectiveness of the shunt formed by conductive channels in parallel with the plasma membrane. Thus, everything else being similar, the rate at which the electrically excitable plasma membrane component attains its critical firing level must be diminished. Accordingly, the presence of the shunting channels would tend to slow conduction of the muscle fibers.

The effectiveness of such a shunt may be judged from the result of a similar electrophysiological condition in eel electroplaques (14). There is a high current flow during activity of these cells (*ca.* 50 ma/cm^2), the threshold depolarization to elicit a spike is only about 20 mv, and the time constant is about 75 μsec. Nevertheless, propagation of a spike along the electrogenically reactive caudal membrane of the electroplaque is only at the rate of 1 to 2 meters per second. Most of the current generated by an active region of the caudal membrane leaves through the low resistance, electrogenically inert rostral surface, and the spread of depolarization induced along the caudal membrane by a spike of some 150 mv amplitude becomes negligible within a fraction of 1 mm.

Shunting of most of the active current by the parallel channels might also account for the graded responsiveness which is exhibited by crayfish muscle fibers. Again, a somewhat similar condition is also found in eel electroplaques (14, Figs. 14 and 16). Despite the fact that one region of the excitable membrane may develop a spike of some 150 mv, another region, less than 1 mm away, may produce only a graded response. If shunting does promote graded responsiveness of crayfish muscle fibers it may be expected that substitution of an impermeant anion for the Cl of the saline medium would eliminate or minimize the shunting and might also cause these muscle fibers to respond with spikes. This is, indeed, the case when propionate is substituted for Cl (13).

Dr. Girardier was a Fellow of the Swiss Academy of Medical Science and of the American Swiss Foundation.

We wish to thank Mr. Claude Schoepf for his cooperation in developing the photographic techniques. This work was supported in part by grants (to H. G.) from the Muscular Dystrophy Associations of America, by Public Health Service Research Grants B-3728, NB 03270-02, 2B-5328(R1) from the National Institute of Neurological Diseases and Blindness; from the National Science Foundation (G-19969), and from the United Cerebral Palsy Research and Educational Foundation, and (to P. W. B.) from the National Heart Institute, United States Public Health Service on grant HE 0743301.

Received for publication, February 6, 1963.

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