3. M. M. Lipton and J. Freund, *J. Immunol.* **70**, 326 (1953); S. H. Stone, *Intern. Arch. Allergy Appl. Immunol.* **20**, 193 (1962).
4. J. A. Bauer, Jr., *Ann. N.Y. Acad. Sci.* **73**, 792 (1958).
5. The guinea pigs came from the NIH Animal Production Center.
6. "B & B Feed A," Cooperative GLF Exchange, Waverly, N.Y.
7. P. R. B. McMaster, E. M. Lerner, E. D. Exum, *J. Exptl. Med.* **113**, 611 (1961).
8. F. Gorstein and E. M. Lerner, unpublished results.
9. We thank D. W. Alling for the statistical analyses, and E. W. McMaster for technical assistance.

8 December 1964

Localization of Calcium-Accumulating Structures in Striated Muscle Fibers

Abstract. *When frog muscle fibers from which the sarcolemma had been dissected away were perfused with a calcium solution and then treated with oxalate, electron-opaque material, probably calcium oxalate, accumulated in the terminal sacs of the sarcoplasmic reticulum. These regions of calcium accumulation were identified with the intracellular calcium sink that controls the relaxation phase of the contraction-relaxation cycle; their proximity to tubules implicated in intracellular stimulus conduction suggests that they might also be regions from which calcium is released to trigger contraction.*

Probably the most generally accepted explanation of the contraction-relaxation cycle in muscle cells is that (i) depolarization of the surface membrane leads to the release of calcium ions from a source within the cell; (ii) this calcium catalyzes interaction of the myofilaments, giving rise to contractile force; (iii) calcium is then removed by an intracellular sink, ending myofilament interaction (*1*). Although the way in which calcium is made available to the myofilaments and then removed from them is not entirely clear, structures formed by a highly differentiated system of internal membranes (Figs. 1*a* and 2*a*) have been implicated in these processes. It has, in fact, been possible to fragment these membranes and form a heterogeneous collection of vesicles and tubules that is capable of accumulating calcium (*2*). Our study was designed to localize specific regions of calcium accumulation within the cell without disrupting the internal membrane system. The general idea was to immobilize calcium *in situ* by forming oxalate deposits which could be detected by electron microscopy (see *3*).

Small bundles of fibers were dissected out of the semitendinosus muscle of the frog, *Rana pipiens*, and covered with paraffin oil. A segment of a single fiber was separated from the bundle and the sarcolemma dissected away, as first described by Natori (*4*). This technique, which did not appear to affect the internal structure of the fiber (Fig. 1*a*), made it possible to apply various solutions directly to cell components without the intervention of the surface membrane.

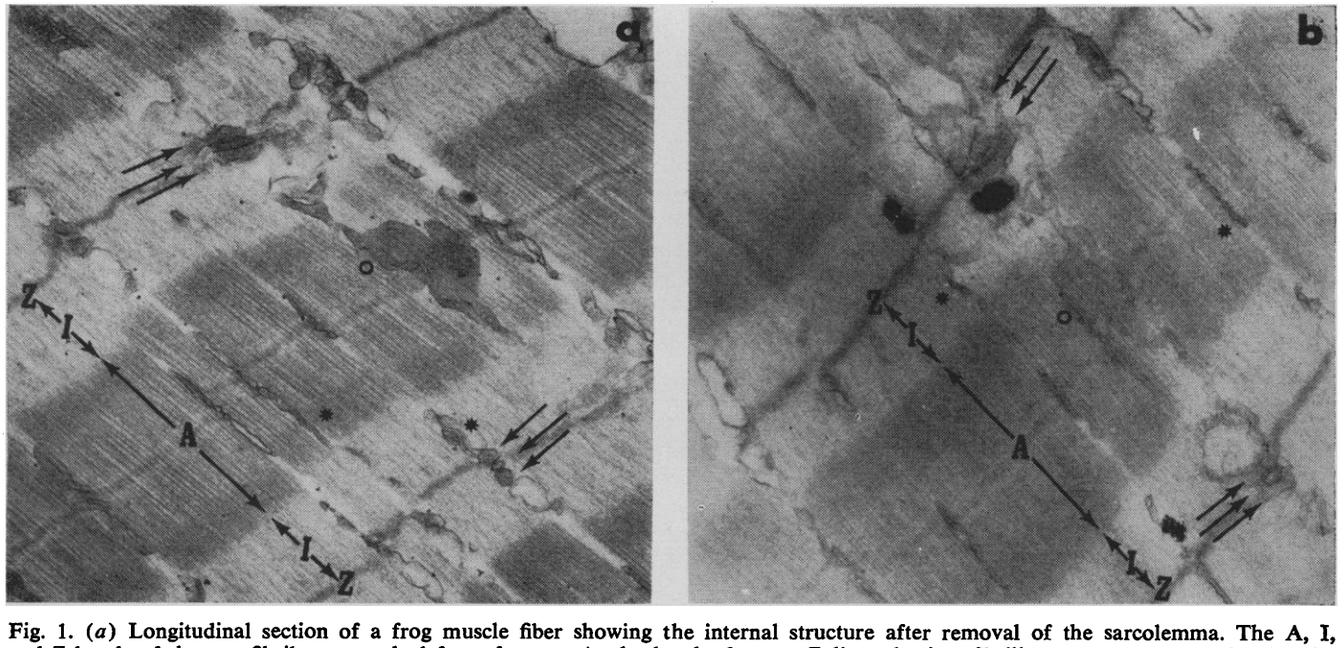

Fig. 1. (*a*) Longitudinal section of a frog muscle fiber showing the internal structure after removal of the sarcolemma. The A, I, and Z bands of the myofibrils are marked for reference. At the level of every Z line, the interfibrillar spaces are occupied by the characteristic group of three elements known as the triad (three arrows) (*13*). The central element of the triad is part of a transverse (or T) system of tubules; the lateral elements of the triad are formed by the terminal sacs of the sarcoplasmic reticulum. Along the A band are longitudinally oriented elements of the sarcoplasmic reticulum (asterisks) which connect terminal sacs at opposite ends of the sarcomere and fuse together to form a flattened cisterna in the middle of the sarcomere (o). Fixed in glutaraldehyde and osmium, stained with lead citrate. (× 24,000) (*b*) Longitudinal section of "skinned" fiber that has been perfused with calcium and then treated with oxalate. The section has not been stained, but there is sufficient contrast to distinguish the main structural features, particularly if one compares this with (*a*) where the same symbols identify similar structures. Electron-opaque material has accumulated in three areas of the sarcoplasmic reticulum, all at the level of the I band, in regions corresponding to the terminal sacs. The general features of deposition are evident: deposits are, at times, only in one corner of the larger terminal sacs (note the largest of the three deposits); some terminal sacs are empty (note lateral elements of triad in lower right corner); deposits are absent from longitudinally oriented elements of the sarcoplasmic reticulum, even though these elements are continuous with terminal sacs, and from the T system. (× 24,000)

Three types of experiments were conducted before the preparations were fixed for electron microscopy: (i) the surface of the "skinned" fiber was perfused with about 1 mμl of oxalate solution (10 m*M* sodium oxalate plus 140 m*M* KCl) over a length of about 200 μ; (ii) a similar region of the fiber surface was perfused with about 1 mμl of calcium solution (10 m*M* $CaCl_2$ plus 80 m*M* sodium citrate) and then with an equal volume of the oxalate solution; and (iii) the fiber was perfused with calcium, as in (ii), but no oxalate was added prior to fixation. The application of calcium resulted in local contraction followed by relaxation (*5*).

Fixation for electron microscopy was the same for all samples of tissue; about 5 minutes after the preparation had been exposed to the perfusion solutions, it was fixed with 6.5 percent glutaraldehyde in 0.2*M* cacodylate buffer at *p*H 7.2, washed in buffer, and fixed again in 2 percent osmium (*6*); each of these solutions also contained 10 m*M* oxalate. The fixed preparation was then rinsed with 10 m*M* oxalate, dehydrated in a series of alcohols and then in acetone, and embedded in Araldite. The embedded material was sectioned with a Porter-Blum microtome and examined in an RCA EMU 3G electron microscope. In preliminary experiments, sections were stained with lead citrate (*6*); this step was omitted in the present study because the staining solutions often dissolved away the electron-dense deposits. Although unstained preparations (Fig. 1*b*) had less contrast than stained ones (Fig. 1*a*), the fiber constituents could be clearly identified. One can recognize the myofibrils with their bands (A, I, and Z) as well as structures formed by the internal membranes: the sarcoplasmic reticulum, elements of which envelop each sarcomere, and the transverse (T) system, tubules which extend inward from the surface membrane at the Z line and are thought to conduct the electrical stimulus for contraction to the inner parts of the fiber (*7*).

In fibers to which oxalate was applied, certain areas of the sarcoplasmic reticulum showed accumulations of electron-opaque material (Figs. 1*b* and 2*b*). These dense spots were clearly within the sarcoplasmic reticulum and were almost always situated in the terminal sacs adjacent to the I band. No deposits were observed outside the membranes of the sarcoplasmic reticulum either in the myofilament region or within the mitochondria or the tubules of the T system. Similar spots were also seen in the terminal sacs when the plane of sectioning was perpendicular to the fiber axis, indicating that the deposits are roughly spherical in shape. Since the deposits were generally less than 0.1 μ in diameter, each one filled only a small portion of a terminal sac. Therefore the appearance, in a given section, of empty sacs close to those containing a deposit can be accounted for, at least in part, by the fact that the deposits are discontinuous and that only a small volume of each sac is included in the thickness of a section (500 to 1000 Å).

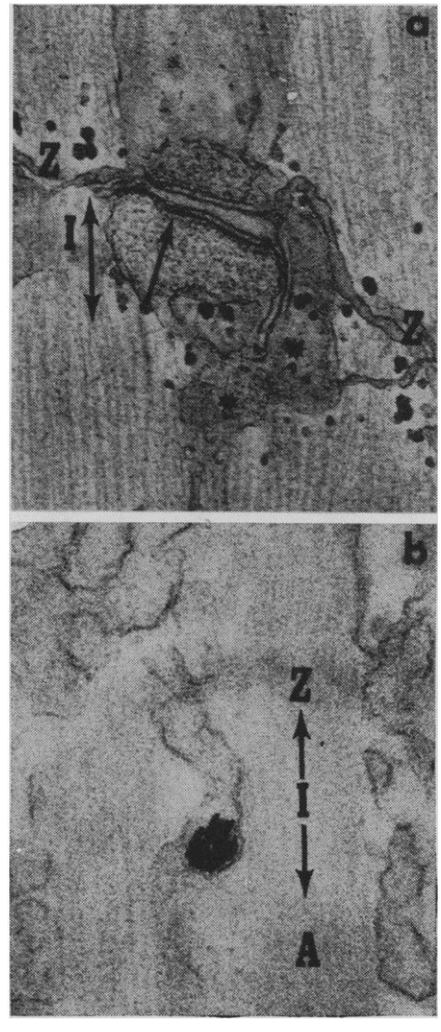

Fig. 2. (*a*) Detail of triad in a "skinned" fiber. Terminal sacs of the sarcoplasmic reticulum face opposite sides of the T system tubule. The terminal sacs have a dense granular content that differentiates them from the rest of the sarcoplasmic reticulum and the T system (*11, 14*). In this type of preparation, the terminal sacs often have protuberances that extend as far as the level of the A-I junction (asterisk). The zone of close proximity between the sarcoplasmic reticulum and T system in the triad presents a characteristic junctional structure (*10–12*); arrow marks an area where this structure is visible. (× 56,250) (*b*) Detail of a sac with enclosed deposit. Deposit fills the lower end of the terminal sac, near the middle of the I band; the membrane of the sarcoplasmic reticulum is easily seen. (× 67,500)

The dense spots were restricted to the region of the fiber that had been perfused with oxalate before fixation. In sections of preparations exposed only to oxalate, the average spot density was generally less than 1 per 250 μ^2. However, perfusion with calcium before addition of oxalate led to a considerable increase in the number of spots. On the other hand, no spots were found in sections from calcium-perfused regions when oxalate was not added prior to fixation. These results strongly suggest that the spots are due to deposits of calcium oxalate (*8*).

Since the sarcoplasmic reticulum appeared to accumulate calcium in the present experiments, it seems reasonable to identify this structure with the intracellular calcium sink that controls the relaxation phase of the activity cycle (*9*). Interpretation of the additional observation that the dense spots within the sarcoplasmic reticulum were almost always in the terminal sacs is complicated by the possibility of calcium translocation during precipitation with oxalate. The most direct explanation for this localization, however, is that the terminal sacs are differentiated regions within the sarcoplasmic reticulum in which calcium had been concentrated before oxalate was added.

The proximity of the terminal sacs to the transverse tubules, as well as the specialized connections between these two structures (Fig. 2*a*) (*10–12*), has raised the question of whether, for normal activation of the myofilaments, depolarization of the surface membrane leads to release of calcium from the terminal sacs (*12*). The finding that calcium precipitates selectively in the terminal sacs suggests that these structures have an exceptional affinity for calcium and is probably the first evidence that specific regions of the sarcoplasmic reticulum close to the transverse tubules can function as intracellular sources of calcium.

LEROY L. COSTANTIN
CLARA FRANZINI-ARMSTRONG
RICHARD J. PODOLSKY
National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland 20014

References and Notes

1. L. V. Heilbrunn and F. J. Wiercynski, *J. Cellular Comp. Physiol.* **29**, 15 (1947); A. Sandow, *Yale J. Biol. Med.* **25**, 176 (1952); A. M. Shanes, *Pharmacol. Rev.* **10**, 165 (1958); G. B. Frank, in *Biophysics of Physiological and Pharmacological Actions*, AAAS Publ. No. 69, A. M. Shanes, Ed. (AAAS, Washington, D.C., 1961), pp. 293–307; R. J. Podolsky, *J. Physiol.* **170**, 110 (1964).
2. T. Nagai, M. Makinose, W. Hasselbach, *Biochim. Biophys. Acta* **43**, 223 (1960); U. Muscatello, E. Andersson-Cedergren, G. F. Azzone, A. von der Decken, *J. Biophys. Biochem. Cytol.* **10** (suppl.), 201 (1961); S. Ebashi and F. Lipmann, *J. Cell Biol.* **14**, 389 (1962); A. Weber, R. Herz, J. Reiss, *J. Gen. Physiol.* **46**, 679 (1963); W. Hasselbach, Abstracts, Réunion Internationale de Biophysique, Paris, 22–27 June 1964 (International Organization of Pure and Applied Biophysics), abstr. B III 8.
3. We are grateful to A. F. Huxley for bringing to our attention the possibility of localizing calcium within the fiber structure by oxalate precipitation.
4. R. Natori, *Jikeikai Med. J.* **1**, 119 (1954).
5. R. J. Podolsky and C. E. Hubert, *Federation Proc.* **20**, 301 (1961).
6. D. D. Sabatini, K. G. Bensch, R. J. Barrnett, *J. Cell Biol.* **17**, 19 (1963); E. S. Reynolds, *ibid.*, p. 208.
7. A. F. Huxley and R. E. Taylor, *J. Physiol.* **144**, 426 (1958).
8. The amount of calcium detected after addition of oxalate to the "skinned" fiber accounts for at most 20 percent of the total intracellular calcium [average concentration, 1.6 m*M*; see D. L. Gilbert and W. O. Fenn, *J. Gen. Physiol.* **40**, 393 (1957)]. This estimate is based on the assumptions that (i) the electron-opaque deposits are calcium oxalate crystals of density 2.2 gm/cm^3 and molecular weight 128, (ii) each deposit is a sphere of diameter 0.1 μ, and (iii) each section of 250 μ^2 area and 0.1 μ thickness contains an average of one deposit. Although we have no direct information concerning the fate of the remainder of the calcium, the following possibilities should probably be considered: calcium could have precipitated too diffusely to be seen in the electron microscope; calcium could be lost during the fixation procedure; calcium could be inaccessible to added oxalate, either because it is firmly bound to structural elements of the cell or is contained within structures impermeable to oxalate.
9. R. J. Podolsky and L. L. Costantin, *Federation Proc.* **23**, 933 (1964).
10. J. P. Revel, *J. Cell Biol.* **12**, 571 (1962).
11. C. Franzini-Armstrong and K. R. Porter, *ibid.* **22**, 675 (1964); C. Franzini-Armstrong, *Federation Proc.* **23**, 887 (1964).
12. H. E. Huxley, *Nature* **202**, 1067 (1964).
13. K. R. Porter and G. E. Palade, *J. Biophys. Biochem. Cytol.* **3**, 269 (1957).
14. D. W. Fawcett and J. P. Revel, *ibid.* **10** (suppl.), 89 (1961).

28 September 1964

Interference between "Sex-Ratio" Agents of Drosophila willistoni and Drosophila nebulosa

Abstract. *Interference between two "sex-ratio" agents—that is, treponema-like spirochetes—of different origin, one from* Drosophila willistoni *and the other from* D. nebulosa, *was demonstrated by experiments in vivo and in vitro. When the two sex-ratio agents were combined in females of the Oregon-R strain of* D. melanogaster, *expression of the sex-ratio condition was temporarily interrupted. Several lines of evidence indicate that a substance produced by the sex-ratio agent of* D. nebulosa *may kill or incapacitate the sex-ratio agent of* D. willistoni.

A maternally transmitted condition known as "sex-ratio" (SR) has been demonstrated in a number of species of *Drosophila* (*1*). The condition is characterized by an extreme departure from the normal 1 : 1 sex ratio to give all or nearly all female offspring, the consequence of differential mortality of males, usually at an early stage in their development (*2*). The characteristics of the SR condition and the nature of the causative agents were reviewed recently in detail (*3*).

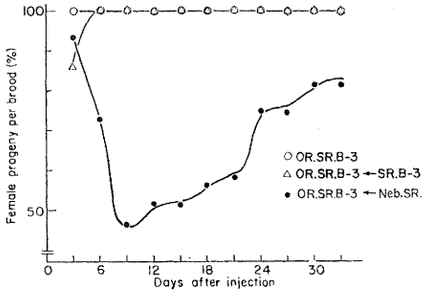

Fig. 1. Frequencies of female progeny per female per brood from eggs laid in successive 3-day periods by females injected with hemolymph of SR.B-3 or Neb.SR and by noninjected females of the OR.SR.B-3 strain.

The SR agents of *D. nebulosa* and *D. willistoni* are small treponema-like spirochetes, 10 μ or less in length and 0.1 μ wide, occurring in high concentration in the hemolymph of adult SR females (*4*). Differences between the SR agents of the two species have been demonstrated by their behavior when transferred into an inbred Oregon-R strain of *D. melanogaster*: the SR agent of *D. nebulosa* is easily transferred and the SR condition produced is very stable and persistent; that of *D. willistoni* is also readily transferrable but the SR condition produced is somewhat less stable (*5*).

We have studied the phenomenon of interference which occurs between SR spirochetes of *D. willistoni* and *D. nebulosa* when mixed in vivo and in vitro.

To test the effects of mixed infections of the two SR spirochetes in vivo, hemolymph of the SR strains PV-45 of *D. nebulosa* or of the SR.B-3 strain of *D. willistoni* was introduced into young, newly emerged adult females. The females were members of the second generation of artificially established SR strains of *D. melanogaster* derived from transfer of SR.B-3 or SR.PV-45 spirochetes into an inbred Oregon-R strain. These artificially established strains will be referred to as OR.SR.B-3 and OR.Neb.SR, respectively.

Since the SR condition in the *D. melanogaster* strains is not uniformly expressed in the first progeny, females used as hosts in these studies of mixed infections were always taken from broods produced by their mothers on days 9 to 12 or later. Methods of injection and examination of progenies from inoculated females are described elsewhere (*6*).

Results of the experiments in vivo, which are summarized in Table 1, showed that mixed infections of SR agents of different origin, SR.B-3 and Neb.SR, in the same SR host female, result in the production of male progeny, presumably as a result of some kind of interference between the two SR agents.

The proportions of female offspring in successive broods in these experiments and in the original *D. melanogaster* SR strains are shown in Figs. 1 and 2. The proportion of females in progenies of OR.SR.B-3 females injected with Neb.SR hemolymph was reduced from 93 percent in the first brood to 45 percent in the third brood

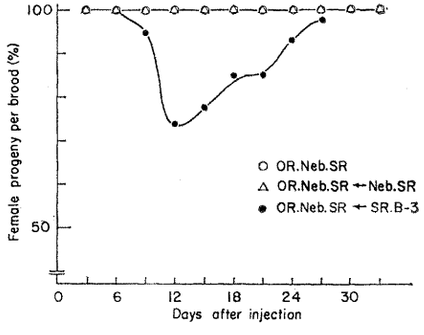

Fig. 2. Frequencies of female progeny per female per brood from eggs laid in successive 3-day periods by females injected with hemolymph of SR.B-3 or Neb.SR and by noninjected females of the OR.Neb.SR strain.