

CHANGES IN THE STRUCTURE OF NEUROMUSCULAR JUNCTIONS CAUSED BY VARIATIONS IN OSMOTIC PRESSURE

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ABSTRACT

Neuromuscular junctions of the frog, *Rana pipiens*, were examined for structural modifications produced by exposure to increased and reduced osmotic pressure (π). Preparations exposed to increased π for varying lengths of time were fixed with either OsO₄-Veronal with and without calcium, glutaraldehyde-phosphate, or glutaraldehyde-formaldehyde-phosphate as primary fixatives. The greatest difference between the fixatives was seen in preparations exposed to increased π for 5 min, corresponding to the time when miniature endplate potential frequency is highest. The 5-min OsO₄ calcium-free preparations appeared comparatively normal, while those fixed with OsO₄ and 2 mM CaCl₂ or aldehyde-phosphate had wide infoldings of the presynaptic membrane and a reduced number of synaptic vesicles. Aldehyde-phosphate had the same effect on mouse diaphragm.

Another series of frog preparations were conditioned to elevated π and then returned to normal Ringer's for varying times before fixation in OsO₄-phosphate. Preparations fixed 2 min after their return to normal Ringer's showed marked disruption of the presynaptic membrane as well as apparently rupturing vesicles. If fixed after 10 min, terminals were depleted of vesicles although the presynaptic membrane had returned to its normal position and appearance.

The effects of osmotic pressure (π) on the frequency of miniature endplate potentials (MEPPs) in neuromuscular junctions have been known from the time when the MEPPs were first described in the frog (10). Subsequently, more extensive electrophysiological studies carried out on both frog (12) and mammalian (14) preparations showed that an increase in π resulting from an impermeant solute e.g. sucrose, provokes a rapid increase in MEPP frequency which then declines to a lower level that is sustained over a period of several hours.

According to the vesicle hypothesis (7), a MEPP is the result of the release of a quantum of

neurotransmitter from a synaptic vesicle. Given the observed effects of π on MEPP frequency, a nerve terminal subjected to a large increase in osmotic pressure might reveal a concomitant reduction in its complement of synaptic vesicles. Past attempts to demonstrate such a relation have been unsuccessful or inconclusive (1, 14).

More recently, a correlation was found between an increase in MEPP frequency and an unambiguous reduction in the number of synaptic vesicles in terminals treated with black widow spider venom (BWSV) (19, 4). In the course of these observations, nerve terminals subjected to osmotic stress were examined in order to see if equally unambigu-

ous evidence for depletion of vesicles could be obtained. Treatment with hyperosmotic sucrose solutions for 2 h, followed by washing with normal Ringer's for 10 min, resulted in depletion of vesicles and produced the picture seen with the venom treatment. This result was reported with the data obtained with BWSV treatment (4).

After the early events surrounding the depletion caused by BWSV had been investigated (5), the apparent depletion caused by increased π was turned to and preparations were examined at various times after the introduction of osmotic stress. In these experiments, the rinse with normal Ringer's was eliminated and hyperosmotic fixatives were used. At each of the time intervals investigated (0, 5, and 120 min after the introduction of elevated π), wide infolding of the presynaptic membrane and apparent depletion of synaptic vesicles occurred in preparations fixed with hyperosmotic aldehydes and, to a lesser extent, with hyperosmotic OsO_4 fixatives containing calcium ions. The most pronounced infolding and vesicle depletion occurred at 5 min, the time when MEPP frequency was at its peak. At 2 h, much less infolding of the presynaptic membrane was visible and substantial numbers of synaptic vesicles were present, differing from the earlier results where comparable preparations were rinsed with normal Ringer's before fixation.

Since the return to normal Ringer's appeared to bring about vesicle depletion, preparations stressed for 2 h by elevated π were rinsed with normal Ringer's for various times (0, 2 and 10 min) and then fixed with OsO_4 -phosphate to which no sucrose had been added. At 2 min, rupture of both the presynaptic membrane and the synaptic vesicles could be seen, suggesting that the depletion observed at 10 min was the result of extrusion of terminal cytoplasm as well as lytic destruction of the vesicles themselves. These results are reported both to rectify and extend the conclusions of the previous study (4) and to stress the importance of fixation conditions on establishing the relation between nerve terminal structure and function.

MATERIALS AND METHODS

Frog Neuromuscular Junctions

Cutaneous pectoris (3) and sartorius muscles from the frog, *Rana pipiens*, were used in this study. Experiments were performed at all times of the year, and no seasonal variations in the experimental results were detected. After a muscle and its accompanying nerve were dis-

sected, they were mounted in a Lucite chamber (10 ml) and bathed in a Ringer solution containing 114 mM NaCl, 2.10 mM KCl, 1.00 mM MgCl_2 , 1.91 mM CaCl_2 , 1 mM NaH_2PO_4 , and 2 mM Na_2HPO_4 ($\pi = 219 \pm 3$ mosM) at room temperature (approx. 22°C). Glass micropipettes (10–20 M Ω) filled with 3 M KCl (11) were used to impale muscle fibers near their endplate regions. MEPPs were amplified and displayed on a cathode ray oscilloscope, where they were photographed.

After an endplate with a stable resting potential of 80 mV or greater was found the resting MEPP frequency was established and the experiment was begun by flushing the chamber with enough experimental solution (20–30 ml) so that no schlieren could be observed.

With the electrophysiological response of neuromuscular junctions to elevated osmotic pressure (π) in mind, 0 time, 5 min, and 2 h were chosen as appropriate times to fix preparations after the introduction of hyperosmotic Ringer's. The solution used for this series of experiments was made by adding 250 mM sucrose to normal Ringer's, giving it a π of 480 ± 4 mosM. After this hyperosmotic Ringer's had been added to the preparation, the bath was changed again with fresh hyperosmotic Ringer's at 2–3 min for both the 5-min and 2-h preparations, and again at 60 min for the 2-h preparation. For the 0 time preparation, the normal Ringer's was flushed with hyperosmotic fixative.

In a previous paper (4), a reduction of vesicles was observed in neuromuscular junctions that had been soaked for 2 h in hyperosmotic Ringer's, and then fixed. In order to determine the mechanism of this depletion, neuromuscular junctions were conditioned for 2 h in a solution composed of frog Ringer's and 0.32 M sucrose ($\pi = 560 \pm 4$ mosM) and then returned to normal Ringer's for varying periods of time (0 time, 2 min, 10 min) before fixation. After the conditioning Ringer's was added to the preparation, it was changed again with additional hyperosmotic Ringer's at 2–3 min and again at 1 h.

Four solutions were used as primary fixatives. The first contained 1% OsO_4 and 0.05 M sodium barbital (Veronal), pH 7.4 ($\pi = 138 \pm 10$ mosM). After the muscles were fixed for 60–90 min at room temperature, they were stained *en bloc* in 2% uranyl acetate and 0.05 M sodium maleate (pH 5.0) (17) for 2–24 h at 4°C and trimmed into smaller pieces in the sodium maleate rinse.

The second fixative was like the first, except that it contained 2 mM CaCl_2 ($\pi = 143 \pm 16$ mosM). Preparations were treated in exactly the same fashion as those fixed without the added CaCl_2 .

The third primary fixative contained 4% glutaraldehyde (Ladd Research Industries, Burlington, Vt.) and 0.1 M sodium phosphate (pH 7.4) and had a π of 546 ± 21 mosM. After muscles bathed with this fixative had yellowed and stiffened, they were removed to fresh fixative for a total time of 1–2 h at room temperature. The muscles were then rinsed in several changes of plain phosphate buffer (pH 7.4, $\pi = 212 \pm 5$ mosM) for either

30 min or overnight at room temperature and then post-fixed in 1% OsO₄ and 0.1 M phosphate buffer (pH 7.4, $\pi = 253 \pm 12$ mosM) for 60–90 min. Finally, tissues were stained en bloc in 2% uranyl acetate buffered with 0.05 M sodium maleate for 2–24 h at 4°C.

The fourth primary fixative contained 1% glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.), 1% formaldehyde solution (Mallinckrodt Chemical Works, St. Louis, Mo.), and 0.1 M sodium phosphate buffer (pH 7.4) (9) and had a π of 713 ± 13 mosM. After 5–10 min in this fixative, muscles were removed to fresh solution for a total fixation time of 1–2 h. The subsequent procedures for these muscles were identical for those fixed in 4% glutaraldehyde. Junctions at rest treated with these primary fixatives were considered to be controls in the sense that observations made on these preparations were the baseline for later experimental analysis.

Junctions subjected to hyperosmotic stress were treated with the same primary fixatives described above to which 250 mM sucrose was added, the same amount added to the Ringer's in order to bring about the hyperosmotic stress. The fixative made with OsO₄ had a π of 422 ± 14 mosM while that made with OsO₄ and CaCl₂ had a π of 431 ± 29 mosM. Fixatives made with glutaraldehyde and glutaraldehyde-formaldehyde had π 's of 823 ± 35 mosM and 995 ± 7 mosM, respectively. The buffer rinse and the postfixative used after the aldehyde fixatives were also made up with 250 mM sucrose and had π 's of 448 ± 9 mosM and 484 ± 9 mosM, respectively. For the 0-time preparations, hyperosmotic fixative flushed out the normal Ringer's until schlieren could no longer be observed. The same procedure was followed for the 5-min and 2-h preparations where the hyperosmotic Ringer's was replaced with fixative.

Preparations treated with hyperosmotic fixatives were also *en bloc* stained in the same way as controls. One series of experiments was done with 0.05 M sodium maleate and 2% uranyl acetate, each made up with 250 mM sucrose. No difference in the appearance of terminals stained *en bloc* in solutions with or without sucrose could be detected.

In order to determine the effects of fixatives on the electrophysiological properties of these preparations, their MEPP frequencies and resting potentials were monitored during perfusion with solutions containing OsO₄, glutaraldehyde, or glutaraldehyde-formaldehyde. 4% Glutaraldehyde in normal and hyperosmotic Ringer's had π 's of 556 ± 15 and 816 ± 11 mosM, respectively, while 1% glutaraldehyde-1% formaldehyde in normal and hyperosmotic Ringer's had π 's of 742 ± 5 and 997 ± 6 mosM, respectively. 1% OsO₄ and both normal and hyperosmotic veronal buffers identical to those used to fix preparations for electron microscopy were also monitored electrophysiologically.

Preparations conditioned with hyperosmotic Ringer's and then returned to normal Ringer's were fixed with the same solution used to postfix aldehyde controls (1% OsO₄ and 0.1 M sodium phosphate buffer, pH, 7.4, $\pi =$

244 ± 3 mosM). For the 0-time preparations, the hyperosmotic Ringer's was flushed with fixative until no schlieren could be observed. The same procedure was followed with the 2- and 10-min preparations where normal Ringer's was replaced by fixative. Subsequent procedures were like those described above for the controls.

At least three separate preparations were made for each of the times and fixation procedures described above. Except for the controls, many more were made for most of the time periods, especially the 5-min and 2-h preparations fixed initially with OsO₄ where great difficulty was encountered in finding the proper buffer for the fixative.¹

Mouse Diaphragm Preparation

Adult white mice were killed by cervical dislocation. The diaphragm, attached to a circler of rib cage, was mounted in a deep Petri dish (30 ml) and bathed in a Locke's Ringer's solution containing 154 mM NaCl, 5.63 mM KCl, 1.0 mM MgCl₂, 2.18 mM CaCl₂, 2.38 mM NaHCO₃, and 11 mM glucose ($\pi = 284 \pm 2$ mosM). The dish was then emptied and filled with experimental solution until no schlieren could be observed (at least three times).

For the hyperosmotic stress, 250 mM sucrose was added to the Locke's Ringer's and this solution had a π of 540 ± 7 mosM. Initially, three complete changes of the bath were made very rapidly, and then once again at 2.5 min for both the 5-min and the 1-h shocks. A third change was made at 30 min for the latter preparation.

The primary fixative used for these preparations contained 3% glutaraldehyde (Ladd Research Industries), 0.1 sodium phosphate buffer (pH 7.4), and 250 mM sucrose ($\pi = 768 \pm 12$ mosM). After the Locke's Ringer's was removed from the bath, fixative was added, swirled, and then replaced with fresh fixative three times. When the muscle had stiffened and changed color (approx. 10 min), it was cut into small pieces and added to fresh fixative for a total fixation time of 1 h. The preparations were rinsed several times in hyperosmotic

¹ As in the case of Hubbard and Kwanbunbumpen (14), difficulty was experienced with osmium tetroxide, phosphate buffer, and high concentrations of sucrose. The same difficulty was encountered with cacodylate and s-collidine. Despite prolonged staining of both the tissues and the sections from these preparations, in all instances the cytoplasmic background nearly matched the density of the membranes. While the position of the presynaptic membrane and its relation to the synaptic vesicles could be made out and was the same with these buffers, reasonably satisfactory contrast could be obtained only with OsO₄, Veronal buffer, and sucrose as a primary fixative. When CaCl₂ was added to this fixative, preparations had the best appearance of all and were comparable to controls in contrast.

buffer ($\pi = 446 \pm 8$ mosM) and then postfixed for 60 min in 1% OsO₄, 0.1 M sodium phosphate buffer (pH 7.4), and 250 mM sucrose ($\pi = 465 \pm 11$ mosM). Finally, *en bloc* staining was carried out in the same way as with the frog nerve-muscle preparations. Three separate preparations were made for each of the time periods, 0 time, 5 min and 1 h.

Electron Microscopy

After *en bloc* staining in uranyl acetate, tissues were dehydrated in a graded series of ethanols and embedded in Epon 812 according to standard methods. Sections were stained with lead citrate and uranyl acetate and viewed on a Philips EM 200 electron microscope. Table 1 is included to summarize the pretreatment and fixation conditions and to summarize the appearances of the terminals.

Osmometry

The π of all solutions was determined by the freezing-point depression method, using an Advanced Osmometer. The values of osmolarity are given in terms of the sodium chloride solutions which were used as standards.

RESULTS

Resting Neuromuscular Junctions of the Frog

ELECTROPHYSIOLOGICAL RESPONSE: The MEPP frequency of resting preparations varied from 0.1 to 5 s over periods of up to 3 h. A typical record from such a preparation is shown in Fig. 1 *a*.

When resting preparations are exposed to 1% OsO₄ in normal Ringer's or in 0.05 M Veronal buffer, they react vigorously. Almost as soon as perfusion is begun, unstretched muscles begin fibrillating and, for this reason, impalements are very difficult to maintain. Because of its strength, the sartorius is unsuitable for these experiments, while the cutaneous pectoris can be stretched tightly enough to retain the micropipette. Concomitant with fibrillation, MEPP frequency increases to 300/s or more and is indistinguishable from that seen in junctions subjected to hyperosmotic shock (Fig. 1 *c*). This activity lasts for 10–15 s and then very rapidly ceases, with no further MEPP detectable. While this is going on, the resting potential (RP) of the muscle fiber falls from its normal value of –80 to –90 mV to around –40 mV and then approaches 0 mV over the next 1–3 min.

The reaction of resting preparations to 4% glutaraldehyde in normal Ringer's is much less intense and lasts for a longer time compared to OsO₄. 30 s after perfusion is begun, MEPP

frequency increases to 30–50 s (Fig. 1 *b*) and then declines over the next minute or two in both frequency and amplitude, disappearing finally into the baseline. The muscle fiber RP declines very slowly during this time, reaching –60 mV after 3 or 4 min where it can remain for as long as 8 min after the initiation of perfusion. Fibrillation of the muscle is seldom observed. 1% Glutaraldehyde-1% formaldehyde in normal Ringer's produces effects on the MEPP rate and muscle fiber RP comparable to those produced by 4% glutaraldehyde alone. Since in our hands 0.1 M sodium phosphate buffer alone provoked the firing of repetitive spike potentials in the muscle plasma membrane, it seemed pointless to record MEPPs in preparations perfused with fixatives and this buffer.

ULTRASTRUCTURE: The appearance of neuromuscular junctions fixed with either OsO₄-Veronal buffer with and without calcium or aldehyde-phosphate buffer as primary fixatives is much the same and corresponds to descriptions of this structure in the frog by other authors (11, 13). The nerve terminals contain mitochondria, elements of smooth endoplasmic reticulum, neurofilaments, neurotubules, dense-core and coated vesicles, and large numbers of synaptic vesicles (Figs. 2 and 10). Most of the synaptic vesicles are spherical with a diameter of approximately 45 nM, but a few are pleomorphic. Pleomorphic vesicles are more apparent in osmotically stressed preparations and are described in greater detail below.

Covering each terminal is a Schwann cell whose cytoplasm contains microtubules, microfilaments, mitochondria, free ribosomes, and elements of endoplasmic reticulum. The Schwann cell becomes extremely attenuated in places and also sends finger-like projections around and underneath the terminal between "active zones" (6) (Fig. 2 *f, g*). The relation of these projections to the terminal was first observed by Birks et al. (1) and described in detail by Heuser and Reese (13).

The most obvious difference in the appearance of nerve terminals fixed with the three primary fixatives is a greater loss of cytoplasmic matrix with OsO₄-Veronal. There also appear to be fewer neurotubules and dense-core vesicles when this fixative is used.

Hyperosmotically Stressed Neuromuscular Junctions in the Frog

ELECTROPHYSIOLOGICAL RESPONSE: When frog neuromuscular junc-

TABLE I
Summary of Pretreatment, Fixation Conditions, and Appearance of Terminals

	Pretreatment	Primary fixative	Secondary fixative	Appearance
OsO ₄ series without calcium	Normal Ringer's, 218 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 138 mosM	—	Spherical synaptic vesicles, normal position of the presynaptic membrane (Fig. 2)
	Normal Ringer's, 218 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 250 mM sucrose 422 mosM	—	Pleomorphic synaptic vesicles, normal position of presynaptic membrane (Fig. 3)
	Hyperosmotic Ringer's, 5 min, 481 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 250 mM sucrose 422 mosM	—	Pleomorphic synaptic vesicles, normal position of the presynaptic membrane (Fig. 4)
	Hyperosmotic Ringer's, 120 min, 481 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 250 mM sucrose 422 mosM	—	Pleomorphic synaptic vesicles, infoldings of presynaptic membrane accompanied by glial processes (Fig. 5)
OsO ₄ series with calcium	Normal Ringer's 221 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 2 mM CaCl ₂ 143 mosM	—	Spherical synaptic vesicles, normal position of the presynaptic membrane (comparable to Fig. 2)
	Normal Ringer's, 221 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 2 mM CaCl ₂ 250 mM sucrose, 431 mosM	—	Some pleomorphic synaptic vesicles, normal position of presynaptic membrane (Fig. 7)
	Hyperosmotic Ringer's, 5 min, 476 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 2 mM CaCl ₂ , 250 mM sucrose, 431 mosM	—	Pleomorphic synaptic vesicles, infoldings of presynaptic membrane (Fig. 8)
	Hyperosmotic Ringer's, 120 min, 478 mosM	1% OsO ₄ , 0.05 M sodium Veronal, 2 mM CaCl ₂ , 250 mM sucrose, 431 mosM	—	Pleomorphic synaptic vesicles, slight infolding of presynaptic membrane (Fig. 9)
Glutaraldehyde series	Normal Ringer's, 218 mosM	4% glutaraldehyde, 0.1 M sodium phosphate, 546 mosM	1% OsO ₄ , 0.1 M sodium phosphate, 251 mosM	Spherical synaptic vesicles, normal position of presynaptic membrane (Fig. 10)
	Normal Ringer's, 218 mosM	4% glutaraldehyde, 0.1 M sodium phosphate, 250 M sucrose, 823 mosM	1% OsO ₄ , 0.1 M sodium phosphate, 250 mM sucrose 484 mosM	Pleomorphic synaptic vesicles, some infolding of presynaptic membrane (Fig. 11)
	Hyperosmotic Ringer's, 5 min, 482 mosM	4% glutaraldehyde, 0.1 M sodium phosphate, 250 mM sucrose, 823 mosM	1% OsO ₄ , 0.1 M sodium phosphate 250 mM sucrose 484 mosM	Pleomorphic synaptic vesicles, marked infolding of presynaptic membrane (Figs. 12 and 13)
	Hyperosmotic Ringer's, 120 min, 482 mosM	4% glutaraldehyde, 0.1 M sodium phosphate, 250 mM sucrose, 823 mosM	1% OsO ₄ , 0.1 M sodium mM sucrose 484 mosM	Pleomorphic synaptic vesicles, normal position of presynaptic membrane (Fig. 14)
Glutaraldehyde-formaldehyde series	Normal Ringer's, 220 mosM	1% glutaraldehyde-1% formaldehyde, 0.1 M sodium phosphate 713 mosM	1% OsO ₄ , 0.1 M sodium phosphate 255 mosM	Spherical synaptic vesicles, normal position of presynaptic membrane (comparable to Fig. 10)
	Normal Ringer's 220 mosM	1% glutaraldehyde-1% formaldehyde, 0.1 M sodium phosphate, 250 mM	1% OsO ₄ , 0.1 M sodium phosphate 250 mM sucrose 485 mosM	Pleomorphic synaptic vesicles, some infolding of presynaptic membrane (comparable to Fig. 11)
	Hyperosmotic Ringer's, 5 min, 477 mosM	1% glutaraldehyde-1% formaldehyde, 0.1 M sodium phosphate, 250 mM sucrose, 995 mosM	1% OsO ₄ , 0.1 M sodium phosphate 250 mM sucrose 485 mosM	Pleomorphic synaptic vesicles, marked infolding of presynaptic membrane (Fig. 15)
	Hyperosmotic Ringer's, 120 min, 477 mosM	1% glutaraldehyde-1% formaldehyde, 0.1 M sodium phosphate, 250 mM sucrose, 995 mosM	1% OsO ₄ , 0.1 M sodium phosphate 250 mM sucrose 485 mosM	Pleomorphic synaptic vesicles, some infolding of presynaptic membrane (comparable to Fig. 14)
Hyperosmotic conditioned series	Hyperosmotic Ringer's, 120 min, 560 mosM	1% OsO ₄ , 0.1 M sodium phosphate, 244 mosM	—	Pleomorphic synaptic vesicles, intact presynaptic membrane (comparable to Fig. 11)
	Hyperosmotic Ringer's, 120 min, 560 mOs returned to normal Ringer 2 min, 219 mosM	1% OsO ₄ , 0.1 M sodium phosphate 244 mosM	—	Ruptured synaptic vesicles, ruptured presynaptic membrane (Figs. 19 and 20)
	Hyperosmotic Ringer's, 120 min, 560 mOs returned to normal Ringer 10 min, 219 mosM	1% OsO ₄ , 0.1 M sodium phosphate, 244 mosM	—	Few synaptic vesicles, intact presynaptic membrane (Fig. 21)

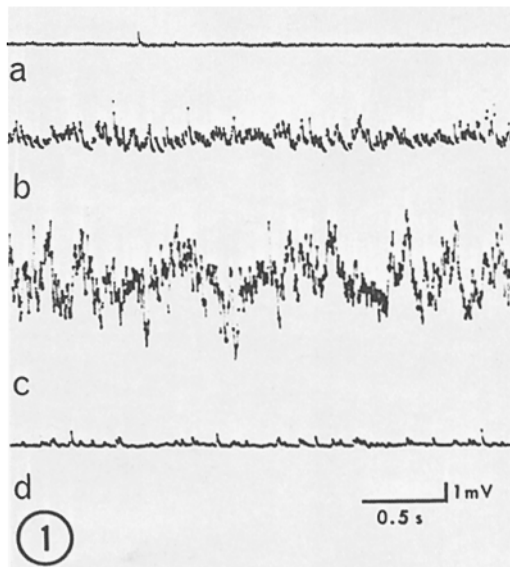


FIGURE 1 Intracellular recordings from frog neuromuscular junctions. (a) Resting preparation. (b) 30 s after the introduction of 4% glutaraldehyde in Ringer's. (c) 5 min after the introduction of 250 mM sucrose in Ringer's. (d) 2 hr after the introduction of 250 mM sucrose in Ringer's.

tions are bathed with a Ringer solution containing 250 mM sucrose, there is a rapid increase in MEPP frequency of around 300-fold. Fig. 1 *c* is a typical recording from an endplate 5 min after the introduction of hyperosmotic Ringer's. Over a period of 2 h, the MEPP rate declines to lower levels although it does not return to that of the resting terminal. Fig. 1 *d* is a typical recording from an endplate 2 h after the introduction of hyperosmotic Ringer's.

The response of hyperosmotically stressed junctions to OsO_4 initially cannot be differentiated at 0 time and 5 min from the effect of sucrose since both accelerate the MEPP rate about equally. However, like those of resting preparations, the MEPPs are abruptly terminated 10–15 s after exposure to OsO_4 and the muscle fiber RP rapidly declines. Preparations stressed for 2 h respond with a brief burst of MEPPs, an abrupt cessation of activity, and a decline of the muscle fiber RP.

The effect of 4% glutaraldehyde on stressed preparations is more rapid than on resting preparations. At both 0 time and after 5 min of stress, perfusion with 4% glutaraldehyde in hyperosmotic Ringer's brings about a cessation of the acceler-

ated MEPP activity after approximately 1 min. In the 2-h preparations, the effect has the same time-course, and any acceleration of MEPP rate provoked by glutaraldehyde is difficult to detect. Muscle fiber RP responds in the same way as that of resting preparations treated with glutaraldehyde.

The response of stressed junctions to 1% glutaraldehyde-1% formaldehyde, like that seen with 4% glutaraldehyde, also is more rapid than it is in resting preparations. In all three time periods, MEPP activity is abolished 30–40 s after the initiation of perfusion, and muscle fiber RP declines at about the same rate as it does in resting junctions perfused with the combined aldehydes.

ULTRASTRUCTURE: Resting neuromuscular junctions (0 times) fixed for electron microscopy with hyperosmotic OsO_4 -Veronal, both Ca^{2+} -free and containing Ca^{2+} (hyperosmotic OsO_4 , and hyperosmotic OsO_4 -calcium), have much the same appearance as those fixed in solutions without added sucrose (Figs. 3 and 7). Especially with the hyperosmotic OsO_4 fixative, there is an increase in the number of pleomorphic synaptic vesicles present in the nerve terminal (Fig. 3). Many of these pleomorphic vesicles are cup-shaped as though one side of the vesicle had been pushed in (Figs. 6 and 18, arrows).

Neuromuscular junctions hyperosmotically stressed for 5 min and then fixed with hyperosmotic OsO_4 have nerve terminal mitochondria with a dense matrix. In addition, the synaptic vesicles appear to be crowded together and most of them are pleomorphic (Fig. 4). Finally, the presynaptic membrane displays occasional narrow invaginations the extent of which is difficult to determine because of the low contrast encountered in these preparations (Fig. 6, asterisk).

With hyperosmotic OsO_4 -calcium, 5-min preparations have a strikingly different appearance (Fig. 8). The clarity with which structures in the nerve terminal can be seen is comparable to that in preparations fixed at rest. While the matrix of the mitochondria is similarly dense and the synaptic vesicles are about equally pleomorphic, the latter can be much more easily seen. The most striking difference, however, is in the wide infolding of extensive regions of the presynaptic membrane, away from its normal position opposite the junctional folds. When a count of 20 cross sections from each of three different preparations was made, the numbers of "lifted"

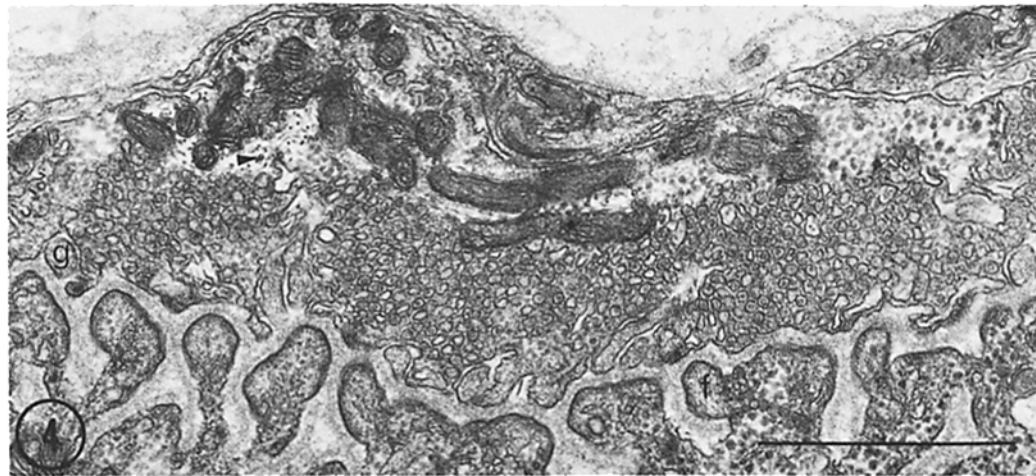
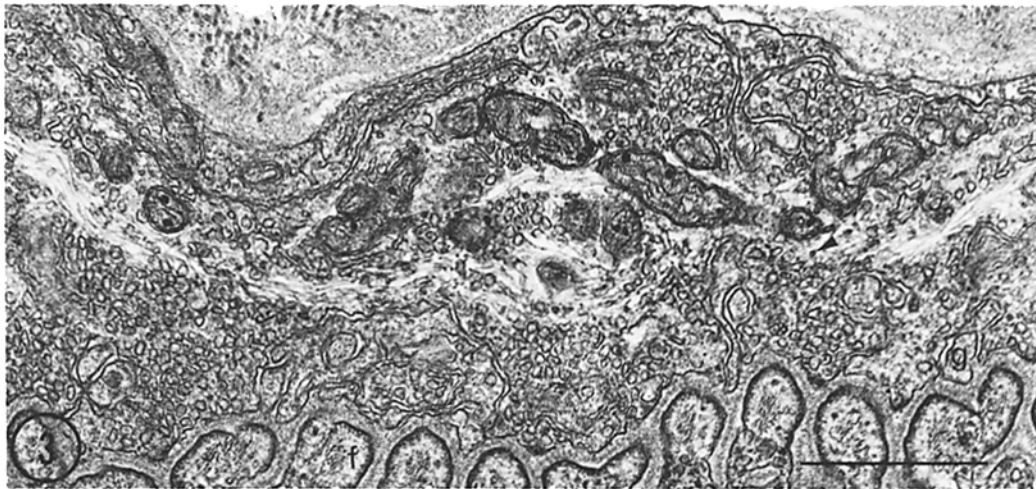
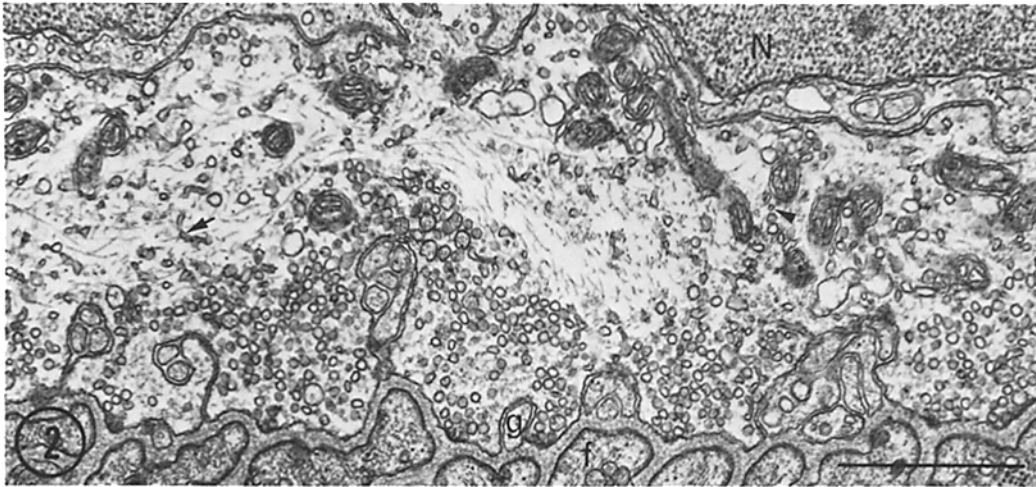


FIGURE 2 Frog neuromuscular junction fixed with 1% OsO₄ and 0.05 M Veronal (control). The nerve terminal contains numerous mitochondria and neurofilaments as well as elements of smooth endoplasmic reticulum (arrow), and a few neurotubules (arrowhead). Synaptic vesicles are located near the presynaptic membrane. The terminal is covered over with a Schwann (teloglia) cell, part of whose nucleus (*N*) can be seen. Teloglia processes (*g*) extend around underneath the terminal. The junctional folds (*f*) of the endplate membrane are also visible. Bar is 1 μ m. \times 25,000.

FIGURE 3 Frog neuromuscular junction fixed with 1% OsO₄, 0.05 M Veronal, and 250 mM sucrose (0 time). The same elements present in Fig. 2 can also be seen here except that the cytoplasmic matrices are denser and some of the synaptic vesicles have a pleomorphic shape. Bar is 1 μ m. \times 25,000.

FIGURE 4 Frog neuromuscular junction fixed with 1% OsO₄, 0.05 M veronal, and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Ringer's. Again, most of the elements visible in Fig. 2 are present here. As in Fig. 3, the cytoplasmic matrices are denser and many of the synaptic vesicles appear pleomorphic. Bar is 1 μ m. \times 34,000.

or widely infolded, slightly affected, and normal junctions were 32, 20, and 14, respectively.

When neuromuscular junctions are bathed in hyperosmotic Ringer's for 2 h and then fixed with hyperosmotic OsO_4 and hyperosmotic OsO_4 -calcium, again the changes seen are chiefly confined to the nerve terminal. The most striking difference with hyperosmotic OsO_4 is the sharp demarcation between the synaptic vesicles and the terminal cytoplasm containing neurofilaments and neurotubules (Fig. 5). Although many of the vesicles are pleomorphic, with both fixatives most of them are not so deeply indented as those seen in the preparations stressed for 5 min (Figs. 5 and 9). Furthermore, vesicles that are two or three times greater in diameter can be seen scattered among the synaptic vesicles of normal size. In many places along the presynaptic membrane, areas occupied by the enfolding arms of the Schwann cell appear to have enlarged, leading to an increase in the area of presynaptic membrane not directly associated with the endplate membrane (Figs. 5 and 9, *g*).

A comparable series of hyperosmotically stressed neuromuscular junctions fixed with 4% glutaraldehyde-phosphate buffer and sucrose (hyperosmotic glutaraldehyde) show changes in

the nerve terminal that are quite similar to those in the same OsO_4 -fixed preparations with regard to the structure of synaptic vesicles and mitochondria. However, the cytoplasmic matrix is denser in these preparations, differing in the same way as the control junctions where the two primary fixatives lacked sucrose. The glutaraldehyde series resembles terminals fixed with hyperosmotic OsO_4 -calcium in that the presynaptic membrane displays a degree of wide infolding that varies chiefly with the elapsed time of osmotic stress.

At 0 time, when a resting junction is fixed with hyperosmotic glutaraldehyde, a given nerve terminal can have regions in which the presynaptic membrane appears in its normal position opposite the endplate membrane, and it can also have regions in which the presynaptic membrane between active zones has lifted up, leaving clear areas of intercellular space. This lifting of the presynaptic membrane can also become much deeper (Fig. 16). The regions with a normally positioned presynaptic membrane differ from the controls only in the overall density of the cytoplasmic matrix and the pleomorphism of the synaptic vesicles (Fig. 11). When a count of 20 transversely sectioned nerve terminals from each of three different 0-time preparations was made, the num-

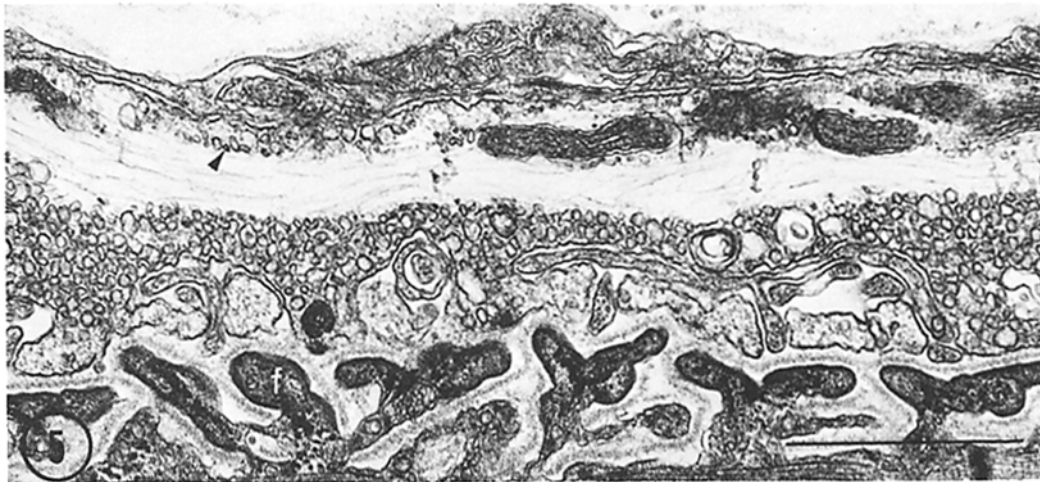


FIGURE 5 Frog neuromuscular junction fixed with 1% OsO_4 , 0.05 M Veronal, and 250 mM sucrose 2 h after perfusion with 250 mM sucrose in Ringer's. While the same elements present in resting terminals can be seen here, there is a sharp demarcation between the synaptic vesicles and the rest of the terminal cytoplasm. Some of the vesicles have diameters several times that of normal vesicles. In many places along the presynaptic membrane, areas occupied by telogial processes (*g*) have enlarged so that much of the presynaptic membrane is not directly associated with the endplate. Arrowhead, neurotubule. Bar is 1 μm . $\times 31,000$.



FIGURE 6 Frog neuromuscular junction fixed with 1% OsO_4 , 0.05 M Veronal, and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Ringer's. A higher magnification view of a preparation like that shown in Fig. 4. Two cup-shaped synaptic vesicles are shown in different planes of section (arrows). The presynaptic membrane also shows narrow infoldings, one of which is indicated by an asterisk. Bar is 1 μm . $\times 103,000$.

bers of normal, slightly infolded, and widely infolded terminals were 21, 25, and 14, respectively. Although no attempt was made to quantitate terminals cut longitudinally, approximately the same ratio appeared to hold.

The presynaptic membrane in preparations stressed for 5 min and then fixed with hyperosmotic glutaraldehyde is even more widely infolded than the hyperosmotic OsO_4 -calcium counterpart. Long stretches of the membrane have lifted away, leaving extensive cavities of clear intercellular space. Accompanying the increase in the surface area of the presynaptic membrane is a reduction in the number of synaptic vesicles (Figs. 12 and 13).

After 2 h of hyperosmotic stress, neuromuscular junctions fixed with hyperosmotic glutaraldehyde resemble those fixed at 0 time, with stretches of normally positioned presynaptic membrane next to places where the membrane is moderately to deeply infolded (Fig. 14). A similar count of 20 transverse sections from three different preparations yielded a ratio of 21, 21, and 18, respectively. As in the OsO_4 -fixed counterpart, vesicles

900–1,500 Å in diameter are scattered among the synaptic vesicles.

The appearance of a comparable series of junctions fixed with 1% glutaraldehyde-1% formaldehyde-phosphate buffer and sucrose (hyperosmotic glutaraldehyde-formaldehyde) is like that of preparations fixed with hyperosmotic glutaraldehyde, with extensive wide infolding of the presynaptic membrane 5 min after the beginning of osmotic stress (Fig. 15).

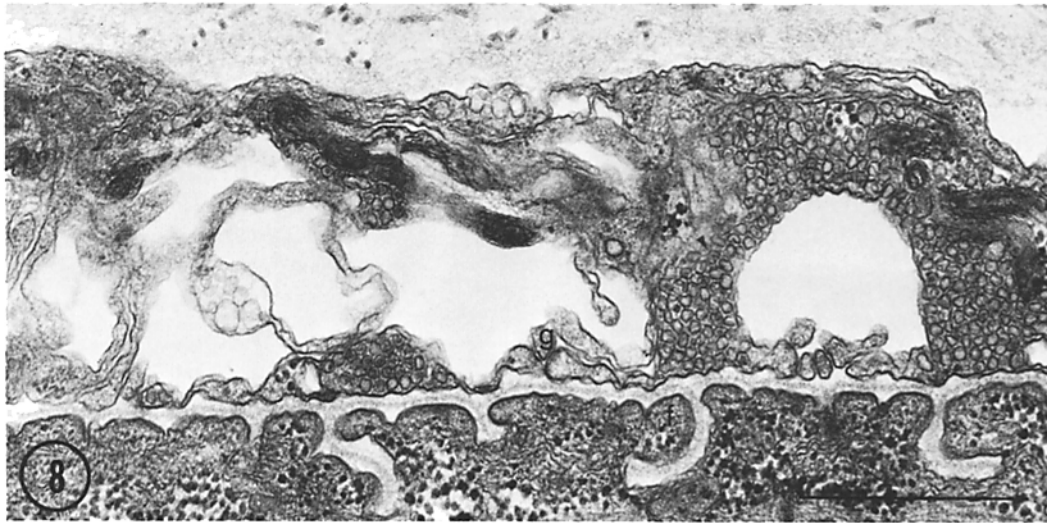
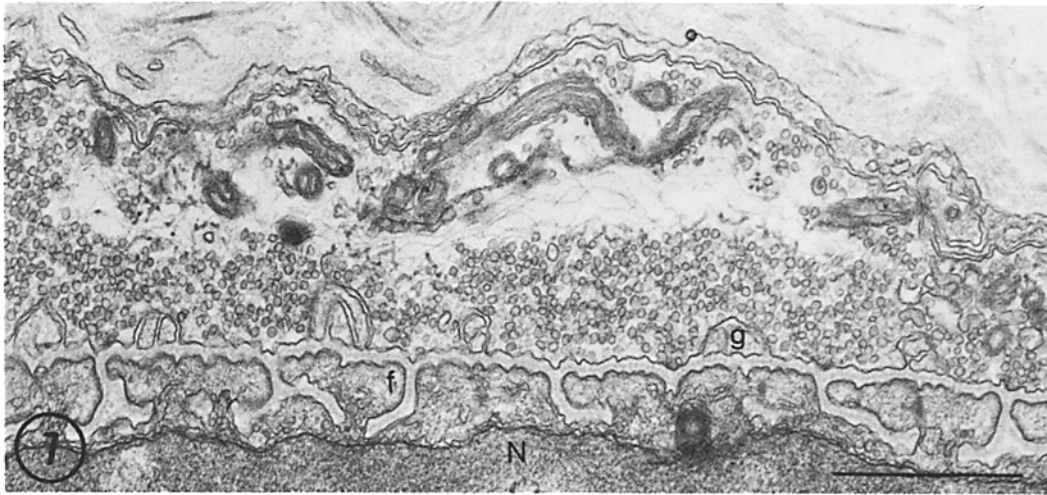
Hyperosmotically Stressed Neuromuscular Junctions in the Mouse Diaphragm

In order to extend these observations to a mammal, the mouse diaphragm was chosen for the comparative ease with which its neuromuscular junctions can be seen. As in the 0-time and 2-h frog junctions, some infolding of the presynaptic membrane is also seen in the mouse diaphragm at 0 time and 1 h when osmotically stressed preparations are fixed with hyperosmotic glutaraldehyde. As in the frog, the infolding seen after 5 min of osmotic stress is much wider and the reduction in the number of synaptic vesicles is obvious (Fig. 17). Cup-shaped synaptic vesicles are also present in terminals from each of the three time periods (Fig. 18, arrows).

Unlike comparably treated frog terminals, where wide infolding of the presynaptic membrane is visible along much of their length, indentations may be confined to only one or two of the several nerve terminal arborizations characteristic of neuromuscular junctions in the mouse diaphragm (compare Fig. 17 to Fig. 12). Consequently, nerve terminals which at first have a normal appearance can be shown by serial sectioning to be connected to regions with wide infolding in 5-min preparations. As in the 2-h frog junctions, vesicles with diameters of 900–1,000 Å are also found scattered among the synaptic vesicles in terminals from diaphragms stressed for 1 h.

Hyperosmotically Stressed Frog Neuromuscular Junctions Returned to Normal Ringer's

Neuromuscular junctions of the frog soaked for 2 h in hyperosmotic Ringer's and then fixed with OsO_4 and normal phosphate buffer (0 time) look very much like resting junctions fixed without previous hyperosmotic exposure, except for the presence of pleomorphic synaptic vesicles in the nerve terminals.



In contrast, preparations soaked for 2 h in hyperosmotic Ringer's and then rinsed in normal Ringer's for 2 min before fixation in OsO_4 -phosphate buffer have nerve terminals with regions of marked disruption. In these regions, there are breaks in the plasma membrane, and the axoplasm appears to be either clear or extracted (Fig. 19, asterisks). Bordering the regions of clear axoplasm, there are usually a few profiles with the appearance of ruptured synaptic vesicles, in that they have approximately the right dimensions and shape and they have what appear to be free ends of membrane (Figs. 19 and 20, arrows).

When rinsed for 10 min in normal Ringer's before fixation, nerve terminals appear to have intact plasma membranes that are normally positioned, but the cytoplasm of many of the junctions is remarkably clear with only a few mitochondria and synaptic vesicles remaining (Fig. 21, cf. Fig. 2). These last results are comparable to those reported previously (4).

DISCUSSION

The results of these experiments show that when a neuromuscular junction is conditioned in hyperosmotic Ringer's and then returned to normal Ringer's, both the nerve terminal and some of the synaptic vesicles undergo lysis. Further, the results also show that, during the initial stages of hyperosmotic stress, depletion of synaptic vesicles can be seen in nerve terminals when the primary fixative contains aldehydes or OsO_4 and calcium ions. Above all, the results reported here show that great care must be exercised about the fixative and the procedures of fixation used on the neuromuscular junction before conclusions can be drawn about changes in its structure.

In a previous paper (4), frog neuromuscular junctions soaked in hyperosmotic Ringer's for 2 h, rinsed for 10 min in isoosmotic Ringer's, and then fixed, were shown to be depleted of vesicles. It was concluded that their relatively clear appearance was related to the increased transmitter release provoked by 2 h of hyperosmotic stress, a conclusion that the present results have shown to be incorrect. Depletion occurs instead through a transient lysis of the terminal plasmalemma with an extrusion of terminal cytoplasm, as well as through possible lytic destruction of the vesicles themselves approximately 2 min after return of the preparation to normal Ringer's.

There were two reasons for this previous misinterpretation. The first involves the assumption that sucrose is an impermeant molecule. A terminal soaked in a solution of Ringer's and sucrose should resume its previous dimensions after returning to normal Ringer's, if sucrose is impermeant. Single cells, exposed to a comparable sequence of solutions, do behave in this fashion (20) whereas neuromuscular junctions clearly do not. One possible explanation is that, like the squid giant axon plasma membrane (24), both the terminal and the synaptic vesicles have low permeability to sucrose: it enters both in small amounts over a period of 2 h. Such a process might explain the relaxation in MEPP rate that can occur over this time (12, 15). Furthermore, with sucrose inside both the terminal and the vesicles, a sudden return to normal Ringer's would lead to the swelling and bursting of both the terminal and vesicles that has been observed.

The second reason for the previous misinterpretation was the unexpected capacity of the terminal plasmalemma to recover its normal position and appearance after lysis. The appearance of termi-

FIGURE 7 Frog neuromuscular junction fixed with 1% OsO_4 , 0.05 M Veronal, 2 mM CaCl_2 , and 250 mM sucrose (0 time). The structures and their arrangement in this terminal are comparable to those seen in Fig. 2. There are a few pleomorphic vesicles, and the cytoplasm has a density comparable to the controls (*N*, subjunctional nucleus). Bar is 1 μm . $\times 25,000$.

FIGURE 8 Frog neuromuscular junction fixed with 1% OsO_4 , 0.05 M Veronal, 2 mM CaCl_2 and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Ringer's. Notice the deep infoldings of the presynaptic membrane usually between adjacent active zones. Many of the synaptic vesicles that remain are deeply indented (arrowhead). Bar is 1 μm . $\times 29,000$.

FIGURE 9 Frog neuromuscular junction fixed with 1% OsO_4 , 0.05 M Veronal, 2 mM CaCl_2 and 250 mM sucrose 2 h after perfusion with 250 mM sucrose in Ringer's. As in the 2-h OsO_4 calcium-free preparation (Fig. 5), there are abundant vesicles some of which are larger than normal. The presynaptic membrane appears to have drawn away from the endplate, and most of the spaces are filled with telogial processes (*g*). Bar is 1 μm . $\times 25,000$.

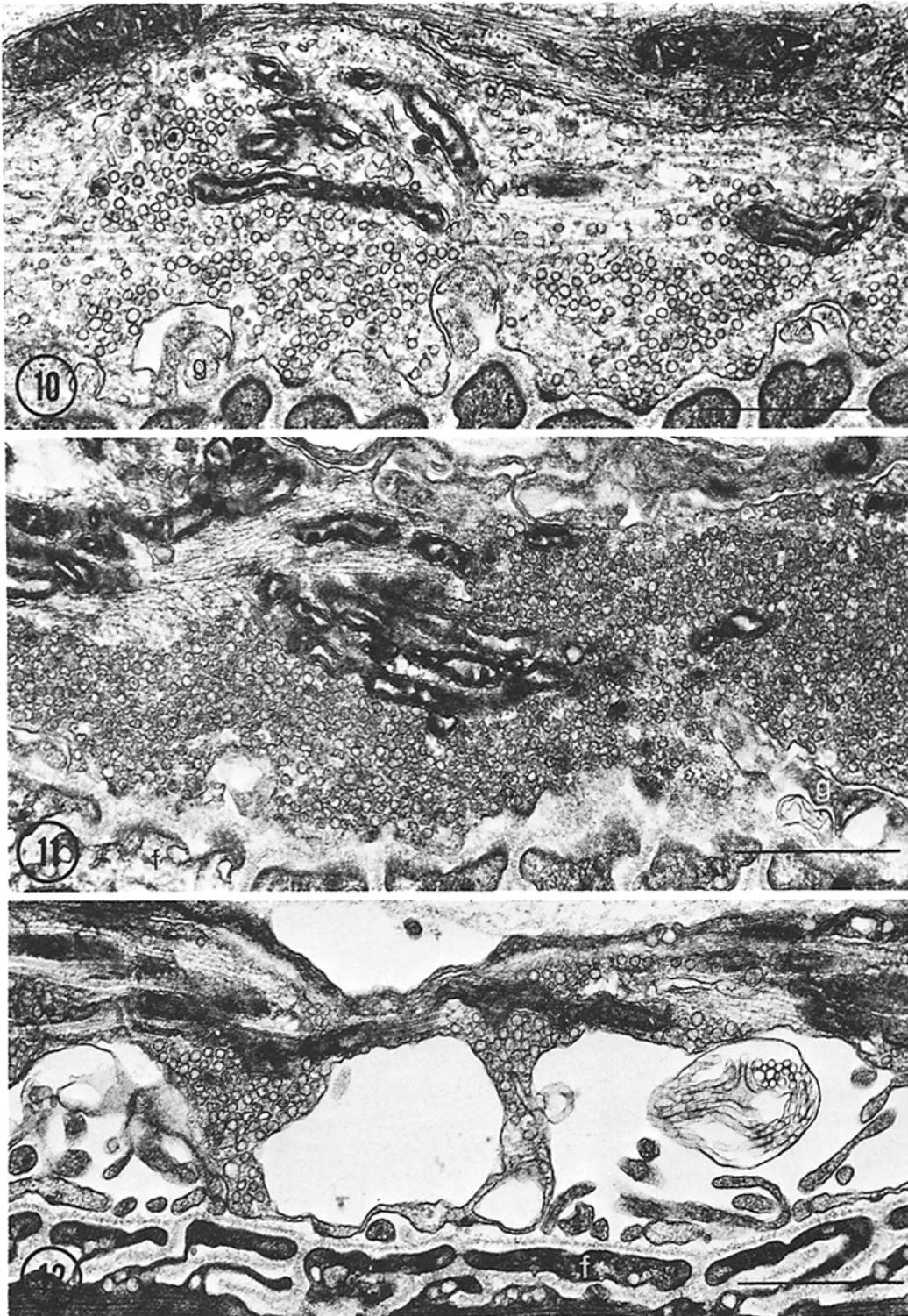


FIGURE 10 Frog neuromuscular junction fixed with 4% glutaraldehyde and 0.1 M phosphate buffer (control). The structures present in Fig. 2 can also be seen here although neurotubules in longitudinal section are more numerous. Bar is $1 \mu\text{m}$. $\times 25,000$.

FIGURE 11 Frog neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate buffer, and 250 mM sucrose (0 time). In this particular region of the terminal, the axoplasm has much the same appearance as the resting terminal except for increased density. Many of the synaptic vesicles have a cup-shaped appearance. Bar is $1 \mu\text{m}$. $\times 25,000$.

FIGURE 12 Frog neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate buffer, and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Ringer's. Notice the infolding of the presynaptic membrane and the reduction in the number of synaptic vesicles. Bar is $1 \mu\text{m}$. $\times 25,000$.

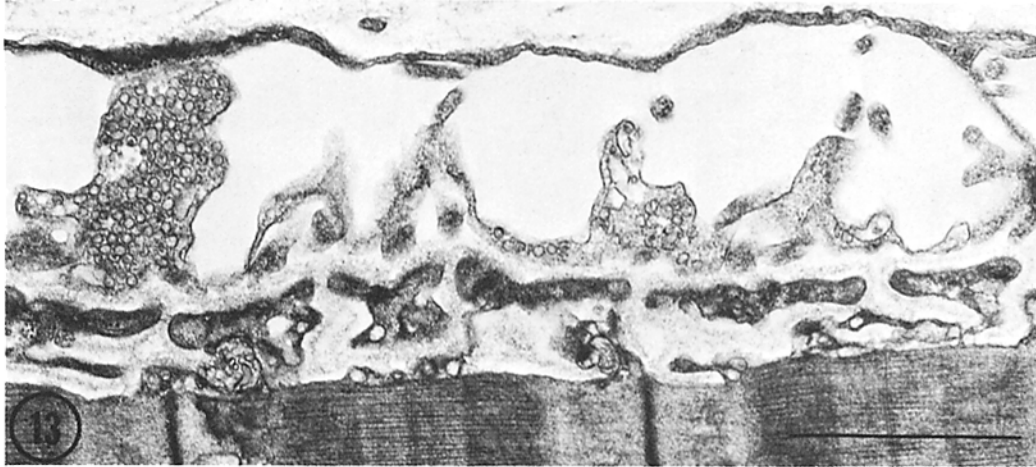


FIGURE 13 Frog neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate buffer, and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Ringer's. This demonstrates how extensive the infolding can be at 5 min of hyperosmotic stress where only a thin roof of the Schwann cell remains over spaces formerly occupied by the nerve terminal. Bar is 1 μ m. \times 30,600.

FIGURE 14 Frog neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate buffer, and 250 mM sucrose 2 h after perfusion with 250 mM sucrose in Ringer's. In this region of the terminal, there is a moderate amount of infolding of the presynaptic membrane. Some of the synaptic vesicles appear to have diameters two to three times that of the normal. Bar is 1 μ m. \times 31,000.

FIGURE 15 Frog neuromuscular junction fixed with 1% glutaraldehyde-1% formaldehyde, 0.1 M phosphate buffer, and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Ringer's. This shows a region with extensive infolding of the presynaptic membrane and a reduction in the number of synaptic vesicles. Bar is 1 μ m. \times 31,000.

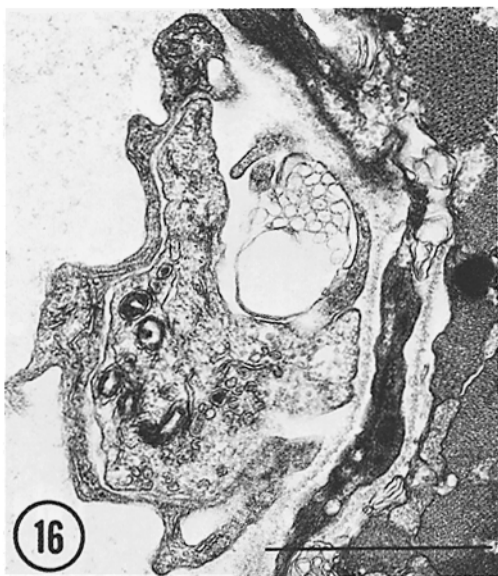


FIGURE 16 Frog neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate buffer, and 250 mM sucrose (0 time). This transverse view shows the deep infolding that occurs in places along the presynaptic membrane in these preparations. Bar is 1 μ m. \times 30,000.

nals with an intact plasmalemma and few vesicles was in agreement with our expectations, and a determination of the actual events was not made at the time.

It seems worthwhile to point out that the "hypo-osmotic shock" (going from a hyperosmotic solution to normal Ringer's) used here on neuromuscular junctions involves the same osmotic gradient commonly used to prepare synaptic vesicles from brain synaptosomes, where a pellet of synaptosomes is sedimented in 0.32 M sucrose and then resuspended in distilled water (25). From those synaptosomes that are lysed, two fractions of acetylcholine (ACh) are obtained, one associated with the terminal cytoplasm and liberated into the supernate ("labile-bound" fraction), the other remaining with those free synaptic vesicles that can be recovered ("stable-bound" fraction). Since some synaptic vesicles appear to be sensitive to hyperosmotic conditions (2, 18, 23; Figs. 6 and 18), it seems reasonable that some of them should also respond to hypo-osmotic conditions as well, even to the point of lysis. Indeed, some vesicle lysis apparently does occur in the 2-min preparations (Figs. 19 and 20). A similar phenomenon may also

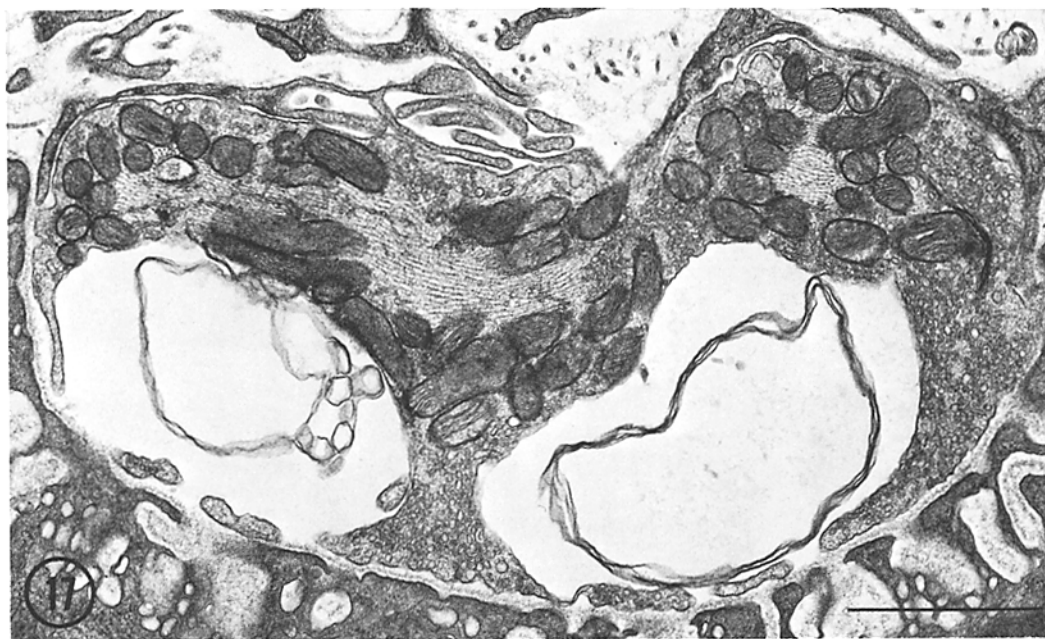


FIGURE 17 Mouse diaphragm neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate, and 250 mM sucrose 5 min after perfusion with 250 mM sucrose in Locke's solution. As in the comparable preparation from the frog, a pronounced infolding of the presynaptic membrane and a reduction in the number of synaptic vesicles are seen here. Bar is 1 μ m. \times 25,700.



FIGURE 18 Mouse diaphragm neuromuscular junction fixed with 4% glutaraldehyde, 0.1 M phosphate, and 250 mM sucrose (0 time). As in similar frog preparations fixed with both OsO_4 and glutaraldehyde, many of the synaptic vesicles are cup-shaped and two of these cut in different planes of section are indicated by the arrows. Bar is $0.1 \mu\text{m}$. $\times 103,000$.

occur in synaptosomes subjected to distilled water, and this could produce labile-bound ACh in a manner consistent with the vesicle hypothesis.

The conclusion that the rinse with normal Ringer's is the crucial step in producing vesicle depletion is strengthened by the observation that hyperosmotic fixatives as well as normal OsO_4 -phosphate immediately applied to 2-h preparations produce nerve terminals with a comparatively normal complement of vesicles. In order to determine whether vesicle depletion could still be observed with hyperosmotic fixatives, a time was chosen when quantal release provoked by elevated π is at its peak. Unfortunately, the results are complex and hard to interpret.

If it is assumed that the vesicle hypothesis is correct, and that quantal release is the result of the exocytosis of ACh, then there are at least two separate parts to the process, incorporation of the vesicle into the presynaptic membrane and vesicle membrane retrieval. These two aspects of the process may be related in several possible ways: (a) the rate of incorporation exceeds the rate of retrieval; (b) retrieval is accomplished at a site

distant from incorporation; (c) the rates of incorporation and retrieval are always equal; (d) the rate of retrieval exceeds the rate of incorporation. The early investigators, attempting to validate the vesicle hypothesis, probably based their hopes on the first two possibilities, since both would yield a terminal depleted of vesicles. The last possibility, although conceptually sound, cannot be distinguished from the third, since both would result in stimulated terminals indistinguishable from those at rest.

The use of the variations in terminal structure produced by hyperosmotic fixatives to select one of these possibilities is difficult since the kinetics of chemical fixatives are not yet fully understood. However, it is known that OsO_4 is able to fix lipids (22). From its rapid destruction of the muscle fiber resting potential and the freeze-fracture plane of other membranes (16), OsO_4 must react with and stabilize the hydrophobic region of the membrane. Given these properties, retrieval of vesicle membrane into a terminal exposed to OsO_4 is unlikely. Therefore, a terminal exposed to hyperosmotic OsO_4 could not have a deeply infolded presynaptic membrane and depleted vesicles just before the arrival of fixative. The results with hyperosmotic OsO_4 alone require that the rates of incorporation and retrieval be always the same.

Unfortunately, acceptance of the results with hyperosmotic OsO_4 requires that the hyperosmotic aldehydes and OsO_4 -calcium fix the terminal asymmetrically. Because of their relative failure to accelerate MEPP rates, it can be argued that the plasmalemma is permeable to the aldehydes. Once across the presynaptic membrane, the aldehydes could fix the retrieval apparatus and allow vesicle fusion to continue on for a time in the same way that they leave the resting potential intact. The infolding of the presynaptic membrane and the depletion of vesicles seen at 0 time, 2 h, and particularly 5 min would then be explained.

The importance of extracellular calcium in the fixative cannot involve the dependence of depolarized release on its presence (8) since the phosphate buffer used with the hyperosmotic aldehydes probably removes extracellular calcium more effectively than the calcium-free Veronal used with hyperosmotic OsO_4 . Furthermore, increased π is capable of accelerating MEPP rate in the absence of extracellular calcium (15, 21). Instead, OsO_4 and calcium may compete for the same sites on the external surface of the presynaptic membrane. Such a competition could briefly protect sites of

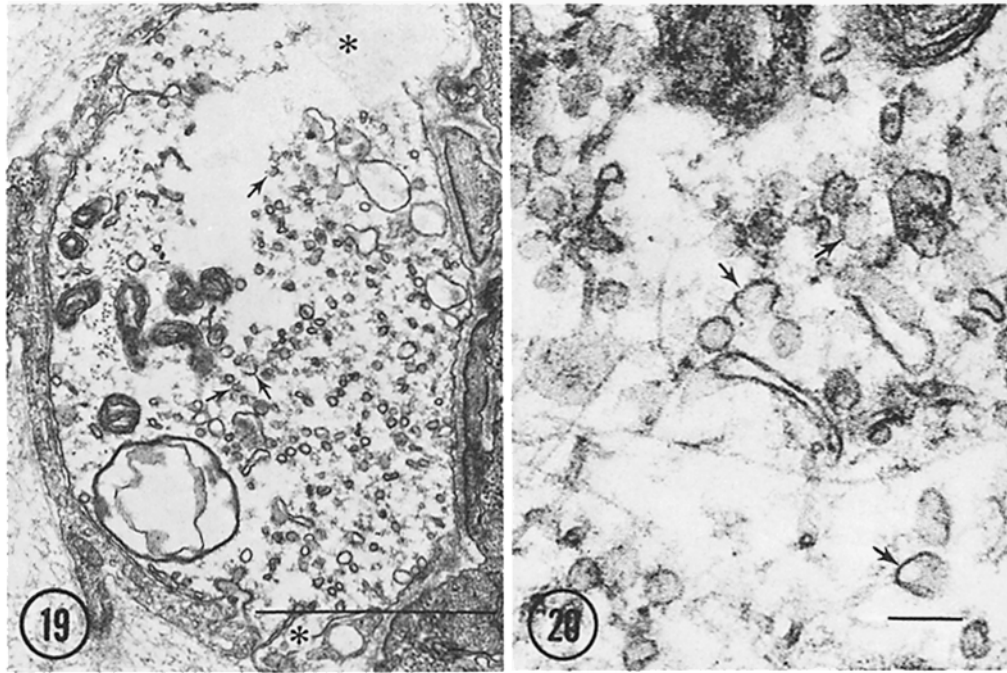


FIGURE 19 Frog neuromuscular junction soaked in hyperosmotic Ringer's for 2 h and then rinsed with isoosmotic Ringer's for 2 min before fixation. Large tears are visible in the terminal plasma membrane (*). Careful examination of the axoplasm reveals profiles of synaptic vesicles that appear to be in the process of rupturing (arrows). Bar is 1 μ m. \times 31,000.

FIGURE 20 Higher magnification view of the same sort of preparation as shown in Fig. 16. Arrows indicate profiles of vesicles that appear to be in the process of rupturing. Bar is 0.1 μ m. \times 97,000.

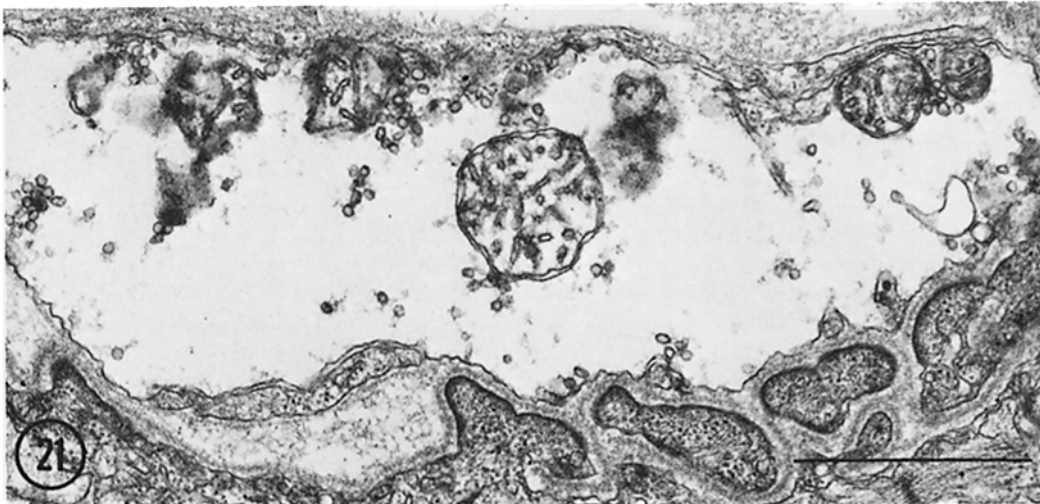


FIGURE 21 Frog neuromuscular junction soaked in hyperosmotic Ringer's for 2 h and then rinsed in isoosmotic Ringer's for 10 min before fixation. Note the clean appearance of the axoplasm with very few of the normal components remaining, as well as the normal appearance of the terminal plasma membrane. This figure is comparable to one published by Clark et al. (1970). Bar is 1 μ m. \times 31,000.

vesicle fusion and give rise to the infolding and depletion seen at 5 min with hyperosmotic OsO₄-calcium.

Birks et al. (1) did not mention adding calcium to their OsO₄-Veronal fixative or the conditions of their hyperosmotic fixation, so the present results, at the very least, confirm theirs and those of other early investigators who probably used hyperosmotic OsO₄ without calcium. Furthermore, the present results also establish that an increase in the surface area of the presynaptic membrane and a depletion of synaptic vesicles accompanies an increase in MEPP rate with the use of three other hyperosmotic fixatives. The hyperosmotic aldehydes and OsO₄-calcium probably attack retrieval first and shut down the capacity of the terminal to incorporate vesicles somewhat later, since the retrieval of vesicle membrane from a plasmalemma that has been exposed to OsO₄ is less likely than continued incorporation of unexposed vesicles. This view implies that with increased π the rates of incorporation and retrieval are approximately the same, even at very high rates of quantal release, and also that the classic OsO₄-Veronal fixative gives the most accurate representation of osmotically stressed terminals.

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