ASPECTS OF TURNOVER AND BIOGENESIS OF SYNAPTIC VESICLES AT LOCUST NEUROMUSCULAR JUNCTIONS AS REVEALED BY ZINC IODIDE-OSMIUM TETROXIDE (ZIO) REACTING WITH INTRAVESICULAR SH-GROUPS

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ABSTRACT

Retractor unguis nerve muscle preparations from the locust were subjected to the zinc iodide-osmium tetroxide reaction (ZIO) after pre-fixation in glutaraldehyde. Applied for 18 h at 4°C in the dark, ZIO reacts at pH 4.2–5.0 fairly selectively with the matrix of synaptic vesicles. ~53% of the vesicles are completely and 4% partially stained. The percentage of ZIO-positive vesicles is increased to nearly 90% and reduced to 4% or less by pretreatment with SH-protecting (dithiothreitol) or SH-blocking (*N*-ethylmaleimide, *p*-chloromercuriphenyl sulfonic acid) and SH-oxidizing (azodicarboxylic acid-bis-dimethylamide) reagents, respectively. Stimulation of the motor nerve at 20 Hz for 7 min, partially fatiguing synaptic transmission, reduces the number of vesicles per square micrometer of terminal area by ~52%; 2 min of rest restores this number to its pre-stimulation level. These changes are chiefly accounted for by changes in the number of completely ZIO-positive vesicles. 2 min after the end of stimulation, partially ZIO-positive vesicles are three times more frequent than before. With all experimental conditions, the average volume of vesicles was as follows: ZIO-negative < partially ZIO-positive < completely ZIO-positive. The average volume of ZIO-positive vesicles is almost unaffected by stimulation; that of ZIO-negative vesicles is decreased by 25% immediately after stimulation, increasing with subsequent rest to the initial level after 1 h. It is suggested (*a*) that ZIO demonstrates intravesicular protein(s) containing SH-groups and (*b*) that the completely ZIO-positive vesicles represent the mature ones ready to be used for transmitter release. How the ZIO reaction differentiates between different developmental stages of vesicles which could arise from the smooth endoplasmic reticulum is discussed.

KEY WORDS synaptic vesicles · insect neuromuscular junction turnover · ZIO reaction intravesicular proteins

The zinc iodide-osmium tetroxide method (ZIO), first introduced by Maillet (33) into the field of neurohistochemistry, has been demonstrated by

various authors to stain, rather selectively, under appropriate conditions, synaptic vesicles in both central and peripheral synapses (for recent reviews, see references 3 and 46). Not all types of synapse within a nervous system (e.g., giant fiber system of the squid [15]) give a positive reaction and, in those that do, not all of the synaptic vesicles are stained (e.g., references 1, 24, 26, 35, 56). Both cholinergic (2, 35, 42) and aminergic vesicles (41, 44, 47, 48) have been shown to react with ZIO, showing that the reaction is not specific for a particular transmitter.

Inasmuch as, in addition to synaptic vesicles, other subcellular structures can react with ZIO, e.g., rough and smooth endoplasmic reticulum, mitochondria, dictyosomes, and lysosomes in various tissues (8, 14, 16, 39, 56), it is conceivable that a variety of substances may serve as substrates for this histochemical reaction. However, from the effects of *N*-ethylmaleimide and dithioerythritol on the ZIO reactivity in the pineal gland and in rod outer segments of the rat, Pellegrino de Iraldi (44, 45) inferred that SH-groups may be essential, an inference which we have tested here in a quite different preparation.

Because only some of the vesicles within a synapse are reported to be ZIO-positive, the question arises whether this reflects functional and/or developmental differences between vesicles. In view of this possibility, we decided, during the course of morphometric investigations on the dynamics of synaptic vesicles in an insect neuromuscular synapse, to use the ZIO method.

We report here (*a*) the effects of various agents on the ZIO reaction, supporting the view that SH-groups of proteins are involved, and (*b*) differences in morphology and ZIO reactivity between rested and previously highly active nerve terminals which seem to be related to a rapid turnover and re-formation of synaptic vesicles. Some of these results have been previously published in abstract form (51).

MATERIALS AND METHODS

The experiments were carried out on adult female locusts (*Locusta migratoria*) which had moulted a few weeks previously. The femoral retractor unguis muscle of the hind leg was used, the anatomy and physiology of which resemble those reported for the same muscle in the locust *Schistocerca gregaria* (64): It consists of 13–15 fibres which are 30–80 μm thick. They are arranged into two bundles each of which is innervated by one excitatory motoneuron which is presumed to release glutamic acid as transmitter (63). After dissection, the preparation was equilibrated in a small Perspex chamber for at least $1^1/_2$ h in slowly flowing saline of the following composition: 154 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, buffered with 2 mM trismaleate buffer at pH 6.8. The claw was fixed in an extended position to keep the muscle at its maximum resting length of 7–8 mm.

After equilibration the muscle was either stimulated through its motor nerve or immediately fixed in glutaraldehyde (see below). Stimulation via a suction electrode was performed at 20 Hz for 7 min with positive-going square pulses of 0.5 ms and 1-3 V. The saline was kept flowing rapidly during stimulation to avoid partial block of axonal conduction which otherwise tended to occur towards the end of the stimulation period. Preparations that did not show a tetanic contraction throughout the period of stimulation were discarded. If fixation was carried out immediately after stimulation, it took 10–15 s for the saline to be completely replaced by glutaraldehyde.

The electrical responses of the muscle fibres to nerve stimulation were investigated in separate experiments. To minimize lesions caused by the vigorous contractions, fine-tipped KCl electrodes (20–30 MΩ) were inserted into the proximal end of muscle fibres. The signals were recorded conventionally and were processed either from film or from tape, using a Nicolet 1074 averager (Nicolet Instrument Corp., Madison, Wis.).

Fixation was carried out in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8, containing sucrose to achieve an osmolarity of 350 mosM, for 2 h at room temperature. The specimens were then rinsed in the phosphate buffer overnight at 4°C, cut into small pieces, and exposed to one of the following treatments: (*a*) Fixation in 2% OsO_4 in 0.1 M phosphate buffer for 2 h at room temperature. The osmolarity of this solution was adjusted to 350 mosM with sucrose. (*b*) The specimens were washed four times for 5 min in a tris buffer solution containing 1.13 M NaCl, 0.011 M $CaCl_2$, 0.03 M $MgCl_2$, and 0.01 M tris-aminomethane (according to Vrensen and de Groot [66]). The required pH, usually 3.8–4.5, was adjusted with 1 N HCl. The muscles were then incubated in the dark for 18 h at 4°C in the ZIO reagent, which had been prepared as follows: 6 g of zinc powder and 2 g of iodine resublimate were dissolved in 40 ml of doubly distilled water, and the mixture was stirred for 5 min. After filtering, the solution was mixed with the tris buffer solution in the ratio 1:1. Four parts of this solution were mixed with one part of a 2% OsO_4 solution in doubly distilled water 5–10 min before use. The pH of the final ZIO reagent was more alkaline by 0.3–0.6 pH units than that of the tris buffer solution. (*c*) The specimens were treated as described under *b*; however, the zinc iodide mixture was replaced by a sucrose solution of the same osmolarity (570 mosM). (*d*) For cytochemical studies several specimens were incubated for 30 min at room temperature in one of the following agents dissolved in 0.1 M phosphate buffer at pH 6.9 before incubation in the ZIO reagent: 1 mM *N*-

ethylmaleimide (NEM) (17, 52); 0.1 mM *p*-chloromercuriphenyl sulfonic acid (*p*-CMPS) (57); 1 mM dithiothreitol (DTT) (9); 0.1 mM *p*-CMPS followed by 1 mM DDT for another 30 min; 1 mM DTT followed by 0.1 mM *p*-CMPS for another 30 min; 1 mM azodicarboxylic acid-bis-dimethylamide (diamide) (29); 5% trichloroacetic acid (TCA).

The cytochemical studies are based on at least two experiments for each procedure. The physiological experiments were carried out at least four times for each of the different conditions.

After treatment with one of the procedures described under *a-d*, the specimens were rinsed in the respective buffer solution, dehydrated in ethanol, and embedded in styrene-methacrylate (30).

Thin sections of interference color gray were cut with glass knives on a Reichert Om U 3 ultramicrotome (C. Reichert, American Optical Corp., Buffalo, N. Y.) and stained with lead citrate. Pictures for the morphometric studies were exclusively taken with a Zeiss EM 9S electron microscope with the fixed magnification of 26,000. Additional studies were done with a Siemens Elmiskop 101. Counting of vesicles was carried out from positives at a magnification of 78,000, and vesicle volumes were measured from negatives projected to give a magnification of 260,000. Statistical analyses of the results were performed using Student's *t* test. Mean values are given together with standard error mean (SEM).

RESULTS

General Morphology of the Neuromuscular Synapses

The final branching of the two motor axons and the general morphology of the synapses closely resemble the pattern described for *Schistocerca* (50): The electron-transparent synaptic vesicles are often scattered over the whole cross-sectional area of the nerve terminal, but tend to be more frequent near the junctions (Fig. 1). They are partly associated with cytoplasmic densities. This sometimes leads to beadlike arrangements. Mitochondria are accumulated in the nerve terminals. In addition, profiles of smooth endoplasmic reticulum (SER) (Fig. 1, inset) and rarely neurotubules are present; both elements are more frequently found within the axons. The average distance between the junctions established by a nerve terminal is ~5 μm.

The Pattern of ZIO Reaction at pH 4.2-5.0

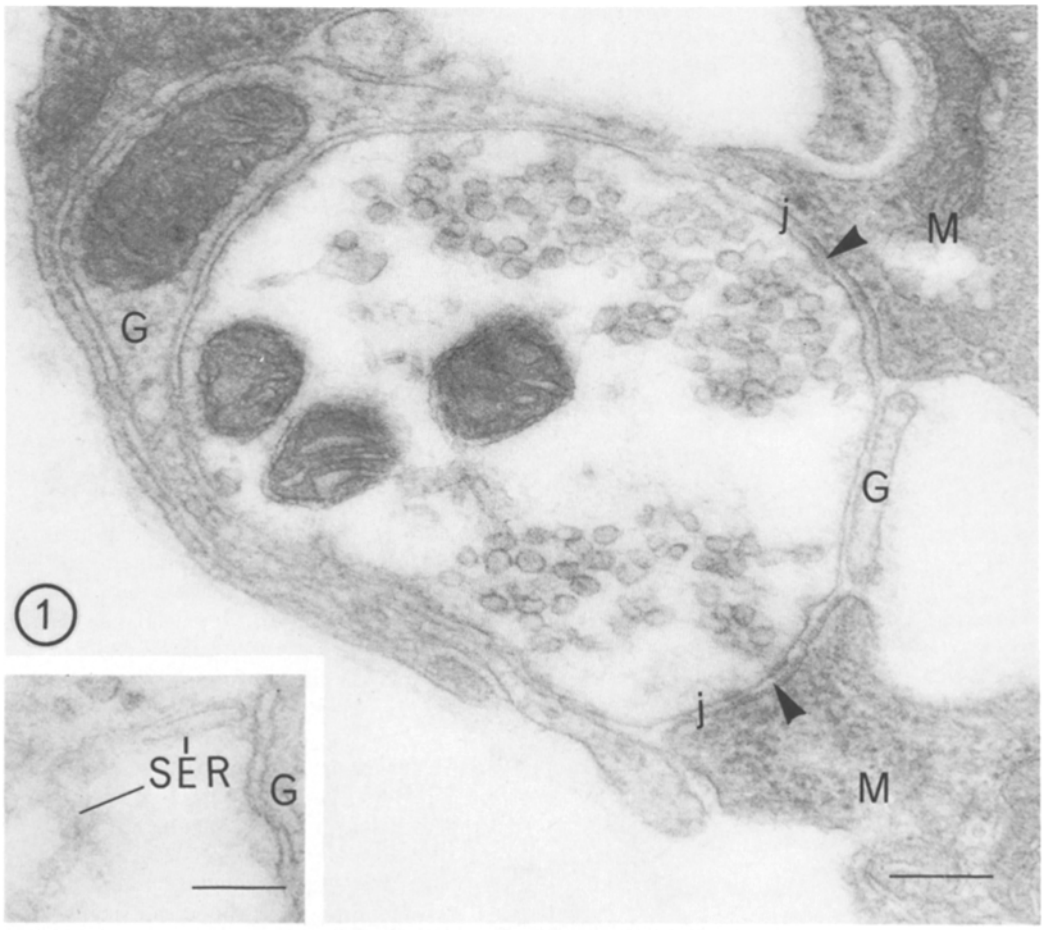
If the ZIO reaction is carried out at a pH of between 4.2 and 5.0, mainly synaptic vesicles are stained (Fig. 2). About half of them (52.8% ± 2.73; data from 51 nerve terminals) show a compact black matrix. The vesicular membrane is often clearly separate from the matrix. The ZIO-positive vesicles seem to be scattered randomly between the unstained (ZIO-negative) ones. To test this the cross-sectional areas of 39 terminals were roughly subdivided into three categories: central (i.e., 120 nm away from the terminal membrane), junctional, and peripheral minus junctional. The ratio of the number of ZIO-positive to the number of ZIO-negative vesicles in each category was found to be 1.14 ± 0.16, 1.37 ± 0.18, 1.06 ± 0.11, respectively, the differences not being statistically significant.

A small fraction of the synaptic vesicles (4.0% ± 0.5) stains only partially, usually in such a way that the black deposit is most intense close to the vesicular membrane, leaving a more or less unstained core (Fig. 2). Partial staining is not the result of an incomplete reaction due to too short an incubation time, inasmuch as in preliminary experiments with 4 h of ZIO-incubation the completely and partially ZIO-positive vesicles were found in approximately the same proportions. Apart from synaptic vesicles some cisternae of the SER are stained. In addition, short (25–75 nm), narrow (5 –20 nm) membrane profiles filled with black deposit (Fig. 7) are present in the nerve terminals, though rather infrequently. They are irregular in outline, often shaped like strokes or commas, and are probably shorter aspects of SER. Sometimes, black deposits of rather diffuse outline and granular appearance occur. These seem to be portions of cytoplasmic densities and can form "tails" to synaptic vesicles.

In the surrounding glial cell sheaths, occasionally ZIO-positive multivesicular bodies are found. Also, the inner face of the tracheae shows a positive reaction. In the muscle fibres, ZIO-positive structures are virtually absent. Rather, rarely black deposits without specific structural peculiarities, presumably artifacts, occur in the extracellular space.

The Dependence on pH of the ZIO Reaction

At a pH of 3.5 or lower, almost all synaptic vesicles give a positive reaction and are completely electron-opaque (Fig. 3). The mitochondrial matrix is stained, too, in the nerve endings and often also in the muscle (Fig. 4) and glial cells. The latter, in addition, exhibit ZIO-positive cytosomes. Profiles of the SER in the nerve terminals and the T system and the sarcoplasmic reticulum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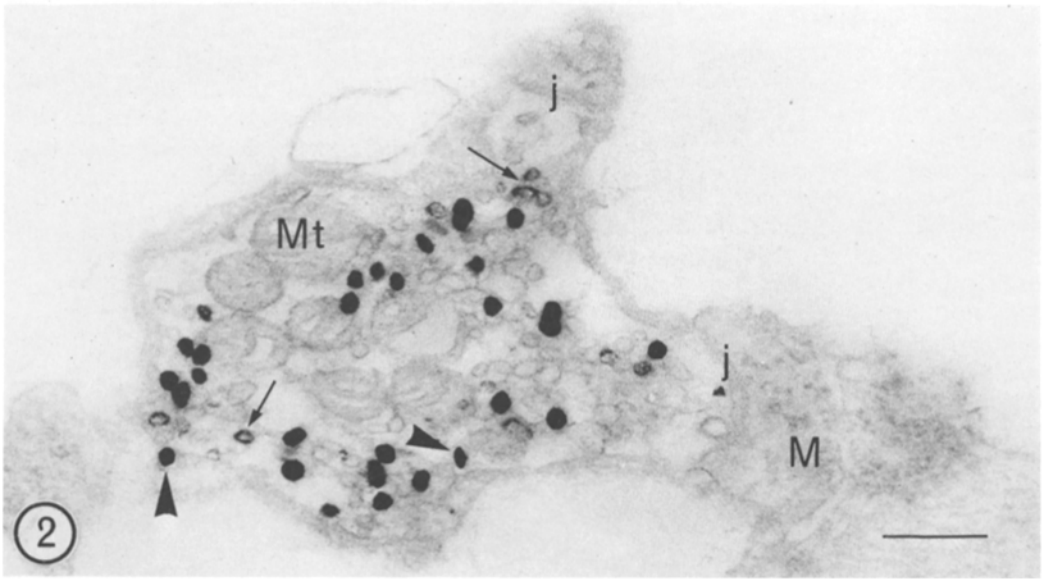
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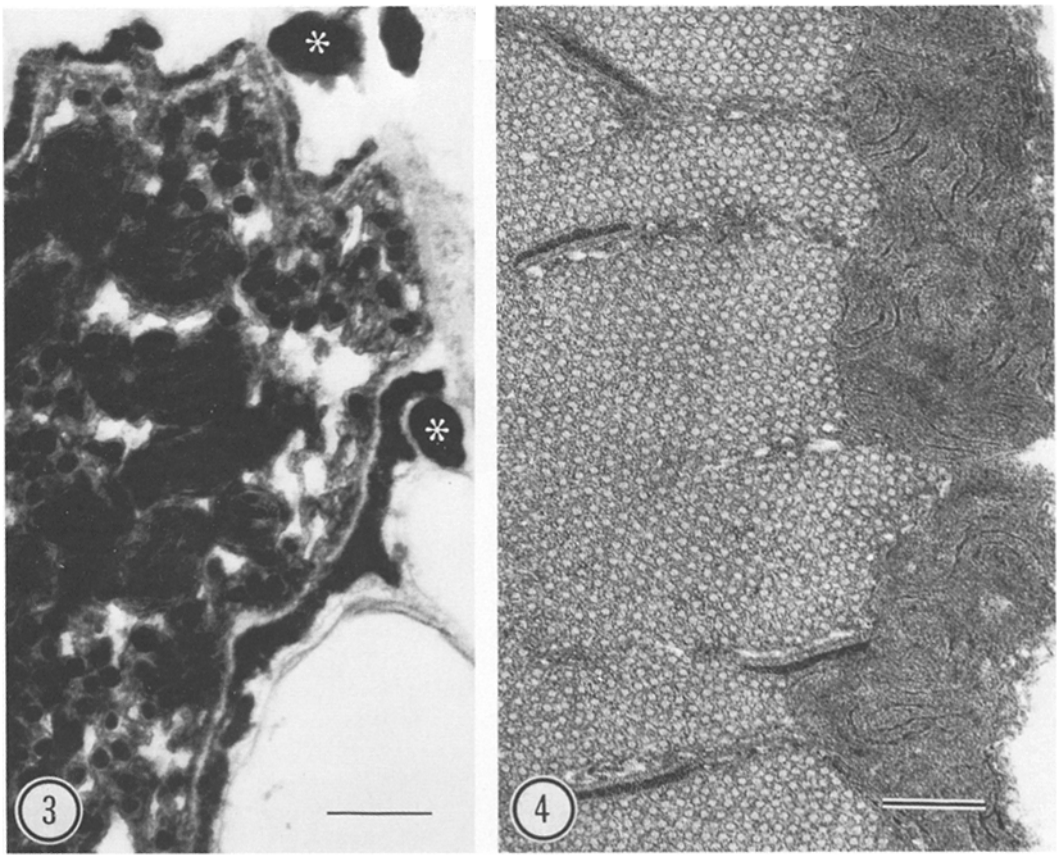


FIGURE 3 Portion of a nerve terminal, ZIO reaction at pH 3.0. The interior of all synaptic vesicles and the matrices of the mitochondria are completely ZIO-positive. Some artifacts occur (asterisks). Bar, 0.2 μm. × 78,000.

FIGURE 4 Portion of a muscle fiber in cross section, ZIO reaction at pH 3.0. Black deposits are present in the matrices of the mitochondria and in the transverse tubular system. The cytoplasm shows a weak overall reaction. Bar, 0.2 μm. × 78,000.

FIGURE 1 Cross section of a nerve terminal in an unstimulated retractor unguis nerve muscle preparation, pre-fixed in 3% glutaraldehyde and fixed in 2% OsO_4. The whole terminal is ensheathed by glial processes (*G*) except for the junctions (*J*) with the muscle (*M*). The terminal contains a large number of synaptic vesicles, which tend to accumulate near the junctions, and some mitochondria. In the synaptic clefts, granular material (arrowheads) is present adjacent to the postsynaptic membrane. Bar, 0.2 μm. × 78,000. Inset: Portion of another nerve terminal from the same preparation, showing profiles of smooth endoplasmic reticulum (SER). Bar, 0.1 μm. × 120,000.

FIGURE 2 Cross section of an unstimulated nerve terminal, ZIO reaction at pH 5.0. About half of the synaptic vesicles are ZIO-positive, the majority completely and some partially (arrows). The vesicular membrane is for the most part clearly distinct from the stained matrix (arrowheads). The ZIO-positive vesicles are intermingled with the ZIO-negative vesicles. The matrices of the mitochondria (*Mt*) remain unstained (*J*, junction). Bar, 0.2 μm. × 78,000.

are mostly filled with black deposit (Fig. 4). The interior of the neurotubules is also stained. The cytoplasm of glial and muscle cells shows a weak overall reaction, and all over the section large artifacts, both intra- and extracellular, are present. Thus at this very low pH the ZIO reaction loses any specificity.

If the pH of the ZIO reaction medium is 5.5 or higher, only the inner face of the tracheae is stained and occasional artifacts are found. Within the range of pH 4.2 to 5.0 (cf. above) the percentage of completely stained vesicles does not change. For example, at pH 4.2 57.3% and at pH 5.0 56.8% of the vesicles were ZIO-positive (data from 14 and 51 nerve terminals, respectively; difference statistically not significant). The pH of the ZIO reaction mixture was between 4.8 and 5.0 throughout the experiments reported in the following sections.

Cytochemical Studies

To obtain information about the substrate for the ZIO reaction, tissues pre-fixed with glutaraldehyde were treated with TCA or various agents affecting SH-groups before carrying out the reaction (cf. Materials and Methods). The results of the morphometric evaluation of these experiments are summarized in Table I.

TRICHLOROACETIC ACID: TCA, a protein-precipitating agent, causes extensive ultrastructural damage of the muscle fibres. In the nerve terminals, the mitochondria show considerable swelling. The synaptic vesicles remain clearly detectable (Fig. 5*b*). Of these, <3% are completely stained by ZIO. Partially stained vesicles are very rarely observed.

REAGENTS BLOCKING SH-GROUPS: *p*-CMPS and NEM are considered to block SH-groups rather specifically (17, 52, 57). The structural preservation of tissues preincubated with either of these agents is somewhat impaired but ZIO-positive and ZIO-negative synaptic vesicles can still be distinguished (Fig. 5*c* and *d*). The synaptic vesicles tend to be attached to each other and frequently appear to be associated with cytoplasmic densities. Both reagents reduce the proportion of ZIO-positive vesicles from ~57% in control preparations to <4%. Only partially stained vesicles are found under these conditions.

TABLE I

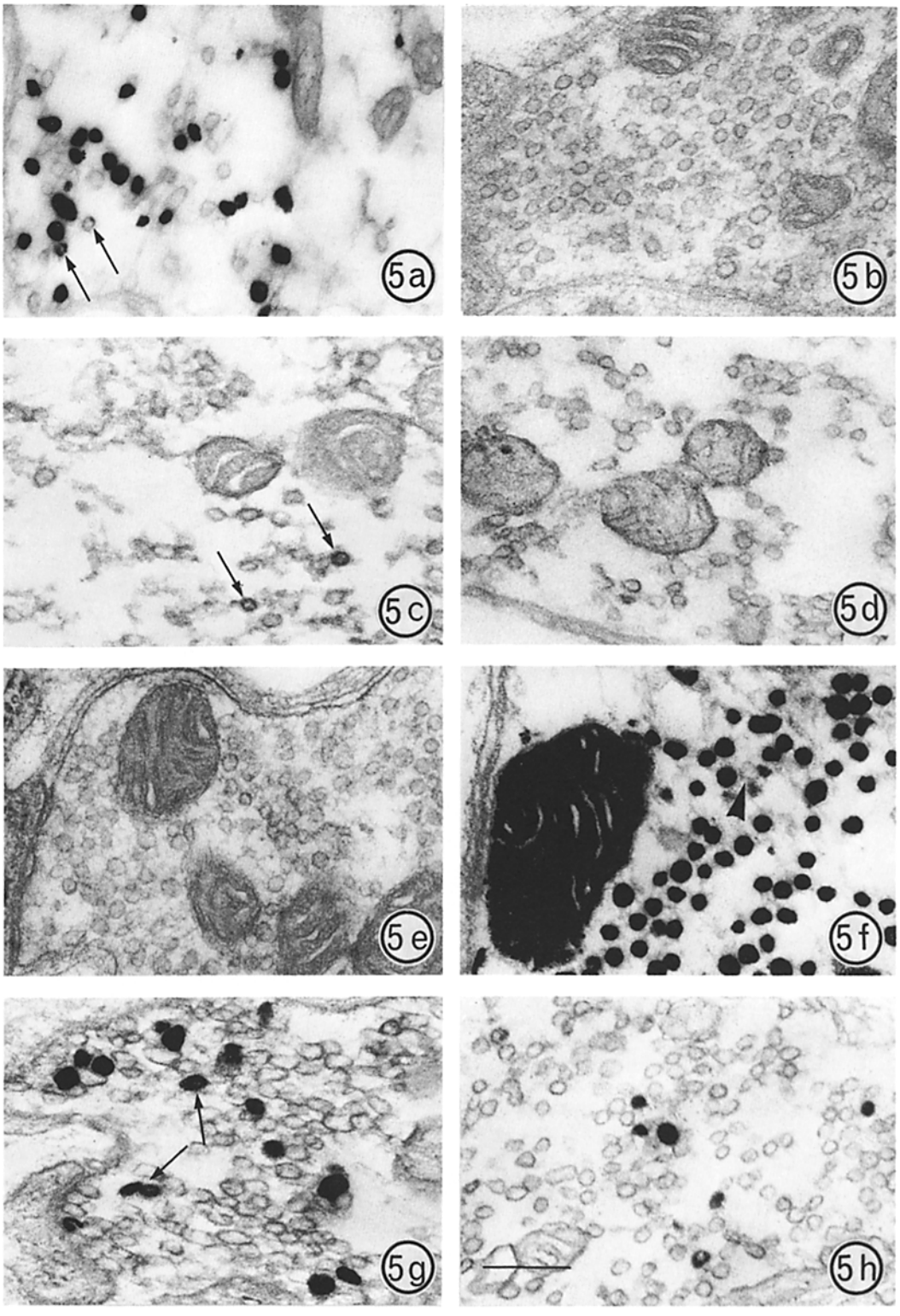
Effect of Pretreatment with Various Agents on the Percentage of ZIO-Positive Vesicles (Completely Plus Partially Stained Ones)

	Percent of ZIO-positive vesicles (% ± SEM)	Number of synapses
Control	56.75 ± 2.89	51
5% TCA	2.49 ± 0.70	14
0.1 mM *p*-CMPS	3.66 ± 0.63	23
1 mM NEM	2.07 ± 0.43	22
1 mM diamide	1.47 ± 0.51	17
1 mM DTT	86.25 ± 1.46	28
0.1 mM *p*-CMPS + 1 mM DTT	48.71 ± 4.25	15
1 mM DTT + 0.1 mM *p*-CMPS	9.32 ± 1.80	16

THIOL-OXIDIZING AGENT: Diamide oxidizes SH-groups, especially of glutathione (29). After incubation with this substance the ultrastructure is preserved rather well (Fig. 5*e*). Beadlike arrangements of synaptic vesicles (see first section of Results) are observed more frequently than in control preparations. Again, the proportion of ZIO-positive synaptic vesicles is drastically reduced, to <2%, and these are only partially stained.

SH-PROTECTING AGENT: DTT (9) which reduces disulfide bridges and prevents SH-groups from oxidation hardly affects the general structure of the tissue (Fig. 5*f*). It leads to an increase in the number of the ZIO-positive vesicles to nearly

FIGURE 5 Portions of nerve terminals from preparations treated with various agents before carrying out ZIO reaction at pH 5.0 (see also Table I). Incubation was performed for 30 min after fixation in glutaraldehyde. (*a*) Control. Among completely ZIO-positive vesicles, some partially stained ones (arrows) occur. (*b*) 5% TCA. ZIO-positive vesicles are absent. (*c*) 0.1 mM *p*-CMPS. Only some partially ZIO-positive vesicles (arrows) occur. (*d*) 1 mM NEM. Only ZIO-negative vesicles are present. (*e*) 1 mM diamide. ZIO-positive vesicles are absent. (*f*) 1 mM DTT. The matrix of the mitochondrion is stained. No partially ZIO-positive and only few ZIO-negative vesicles (arrowhead) are present. (*g*) 0.1 mM *p*-CMPS followed by 1 mM DTT. Both completely and partially (arrows) ZIO-positive vesicles occur. (*h*) 1 mM DTT followed by 0.1 mM *p*-CMPS. Few completely ZIO-positive vesicles are present. Bar, 0.2 μm. × 78,000.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90% and these are all completely stained. Many of the mitochondria present in the nerve terminals and the glial or muscle cells show a positive reaction of the matrices. Like synaptic vesicles, they stain either completely or not at all. Profiles of SER are stained whereas the neurotubules are clearly ZIO-negative as was found under other conditions except for low pH (see third section of Results). A great deal of the T system and the sarcoplasmic reticulum is filled with black deposit.

CROSSREACTION OF P-CMPS VS. DTT: If DTT is applied after *p*-CMPS or vice versa the preservation of the tissue is rather bad, but ZIO-positive and -negative vesicles can still be distinguished (Fig. 5*g* and *h*). The percentage of positive vesicles is considerably higher if DTT is given after rather than before *p*-CMPS (Table I), which clearly indicates some reversibility of both reactions. In both cases the majority of the ZIO-positive vesicles is completely filled with black deposit. From these results, it is unlikely that loss of ZIO-reactivity after incubation with *p*-CMPS is due to loss of the substrate caused, for example, by the vesicular membrane becoming leaky.

Number and Size of ZIO-Positive and ZIO-Negative Vesicles and the Effect of Nerve Stimulation

The results reported so far refer to preparations in the unstimulated, resting condition. To assess the physiological significance of the differential ZIO reactivity, the number and size of ZIO-positive and ZIO-negative vesicles were compared in preparations fixed without stimulation, immediately after, 2 min after, and 1 h after stimulation of the motor nerve at 20 Hz for 7 min. The results of these morphometric comparisons were subjected to the *t* test and the outcomes are listed for convenience in Tables II and III.

ELECTRICAL RESPONSE: The pattern of stimulation, i.e., 20 Hz for 7 min, was chosen because it had been shown previously, in the same muscle in the locust *S. gregaria,* to cause considerable fatigue of neuromuscular transmission and to lead to a decrease in the average vesicle volume (37). In a resting preparation, nerve stimulation evokes at all fibres a large, often overshooting depolarization composed of an excitatory junction potential (ejp) of 20–40 mV and a graded electrically excited response (Fig. 6*a*).

With 20-Hz stimulation, the combined response declines continuously (Fig. 6*a–d*). Usually after 7 min the ejps, now without electrically excited components, have amplitudes of 5–10 mV. The stimulation may be continued up to 30 min without completely fatiguing transmission, but the decline of the response is more pronounced in the fibres of one bundle than in those of the other, which is consistent with the situation in *Schistocerca* (37, 64). Within the first 30 s after a 7-min stimulation, the response recovers to a level some 10–20 mV below the initial value and then slowly approaches that value within the following 60 min.

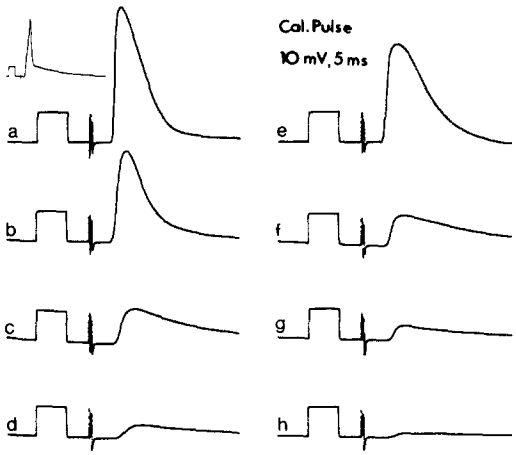

FIGURE 6 Averaged electrical responses of a muscle fibre during various periods of tetanic stimulation and rest. (*a*) Before the start of stimulation. (*b, c, d*) At the end of the 1st, 4th, and 7th min, respectively, of continuous stimulation at 20 Hz for 7 min. (*e*) 2 min after the end of this stimulation. (*f, g, h*) At the end of the first 30 s, the 1st and 2nd min of another 20-Hz stimulation which had started 2 min after the end of the first. The resting potential initially increased from 45 mV (Fig. 6*a*) to 51 mV (Fig. 6*d*) and then remained at this level. An average of 16 responses is presented in Fig. 6*a–d* and *f–g* and an average of 4 in Fig. 6*e*. Inset at Fig. 6*a* shows a single recording from another fibre before stimulation; the start of the electrically excited membrane response arising from the ejp is clearly to be seen here but is smoothened out by the averaging procedure on records *a*, *b*, and *e*.

If a second 20-Hz stimulation is started 2 min after the first, the response is reduced to <10 mV usually within 1 min (Fig. 6*e–h*). Even if 1 h of rest is given before a second 20-Hz stimulation, the decline of the response is quicker than on the first stimulation.

NUMBER OF SYNAPTIC VESICLES: Stimulation of the motor nerve with 20 Hz for 7 min leads

to depletion of vesicles (Fig. 7*b*). There is a quick recovery, however, since the total number of vesicles per unit of cross-sectional nerve terminal area (from which the mitochondrial area has been subtracted) increases from 48 to at least 100% within 2 min after the end of stimulation and then is maintained at about this level for 1 h (Figs. 7 and 8). A more detailed morphometric analysis (to be published elsewhere) shows that the cross-sectional area (both with and without mitochondrial area) of the nerve terminals stays almost the same with stimulation and subsequent rest. Thus, our

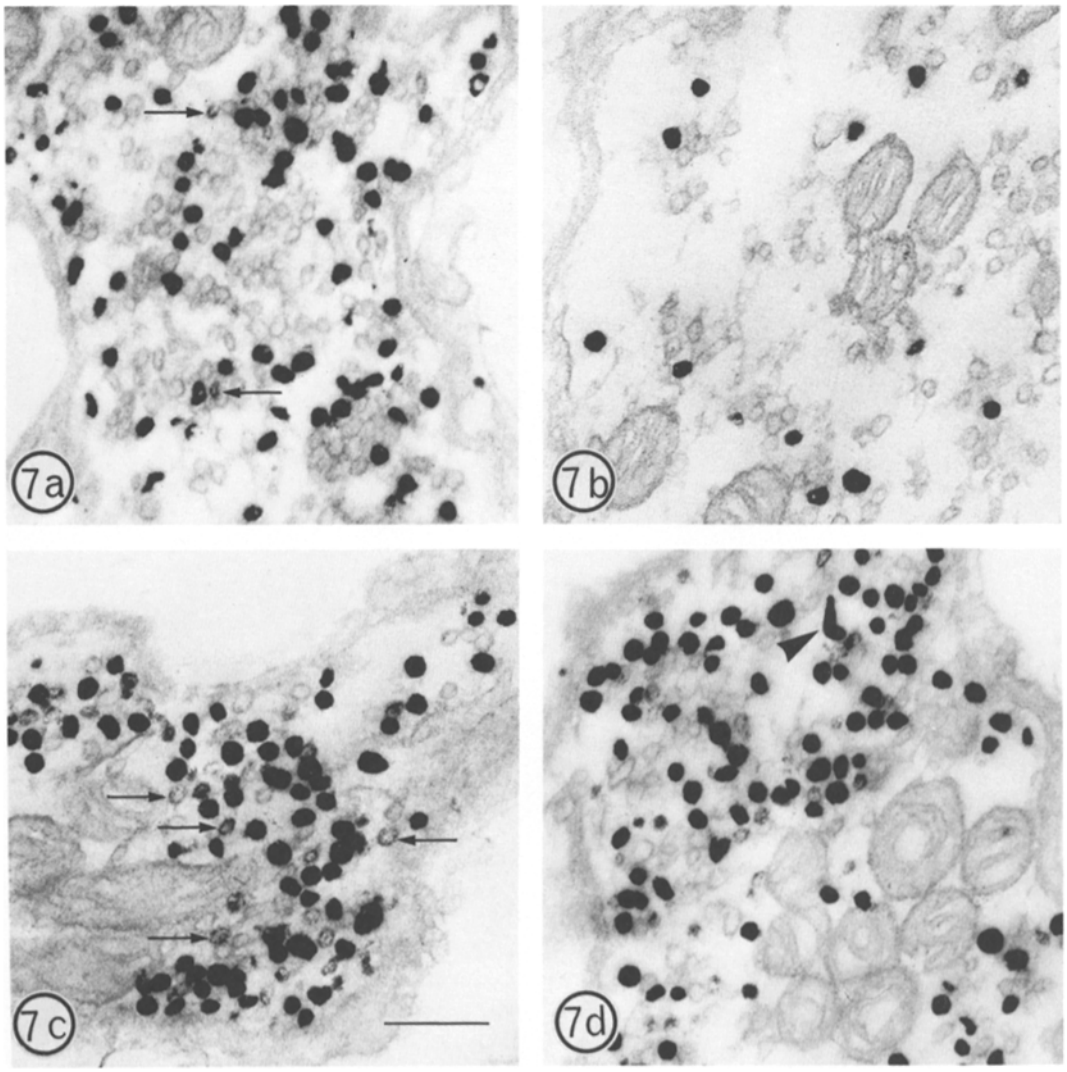

FIGURE 7 Portions of cross-sectioned nerve terminals fixed without stimulation and at various times after stimulation of the motor nerve at 20 Hz for 7 min, ZIO reaction at pH 5.0. (*a*) Unstimulated preparation. About half of the synaptic vesicles are ZIO-positive; a few partially stained ones (arrows) occur. (*b*) Immediately after stimulation. The terminal is rather depleted of vesicles, and of the remainder only a few are ZIO-positive. (*c*) 2 min after stimulation. Completely ZIO-positive vesicles are about as numerous as in Fig. 7*a*. Note that the partially ZIO-positive vesicles (arrows) are considerably more common than in Fig. 7*a*. (*d*) 1 h after stimulation. ZIO-positive and ZIO-negative vesicles are about as frequent as in Fig. 7*a*. The short ZIO-positive profiles (arrowheads) possibly represent SER. Bar, 0.2 μm. × 78,000.

figures in fact reflect differences in the vesicle content of the nerve terminals.

From Fig. 8, it is apparent that the changes in the total number of vesicles are largely accounted for by changes in the number of the ZIO-positive vesicles. The number of ZIO-positive vesicles is decreased immediately after the stimulation period by 85% compared with the number before stimulation, whereas the number of the ZIO-negative vesicles is reduced by only 16%. The partially ZIO-positive vesicles, by contrast, are not decreased in number immediately after stimulation and, after the subsequent 2 min of rest, are three times more frequent than before stimulation ($P < 0.00001$). At this time, also, more short ZIO-positive profiles of SER are observed than in resting preparations and, in addition, occasionally small ZIO-positive areas occur in the matrices of mitochondria. With 1 h of rest, the number of partially ZIO-positive vesicles is close to the initial value or perhaps somewhat higher (cf. Table II).

VESICLE SIZE: The differential effect of stimulation on the number of ZIO-positive and -negative vesicles leads to the question whether we are visualizing different types of vesicles or vesicles of the same kind at different "developmental" stages. With this in mind, we have looked for possible differences in the size of the ZIO-positive and -negative vesicles under various physiological conditions. Because the vesicles are more or less elliptical in shape, their volumes (V) without the contribution of the membrane were calculated, assuming oblate spheroids, from measurements of the longest ($2a$) and shortest ($2b$) inner diameters of the vesicles, according to $V = {}^4/_3\pi a^2 b$.

It could be that the ZIO treatment by itself causes some change in the volume of those vesicles that give a positive reaction. To test this, the volumes of vesicles were compared for unstimulated preparations which had been incubated either in a normal ZIO mixture or in a solution where the zinc iodide had been replaced by sucrose (cf. Materials and Methods). Inasmuch as both the shapes of the distributions (Fig. 9) and

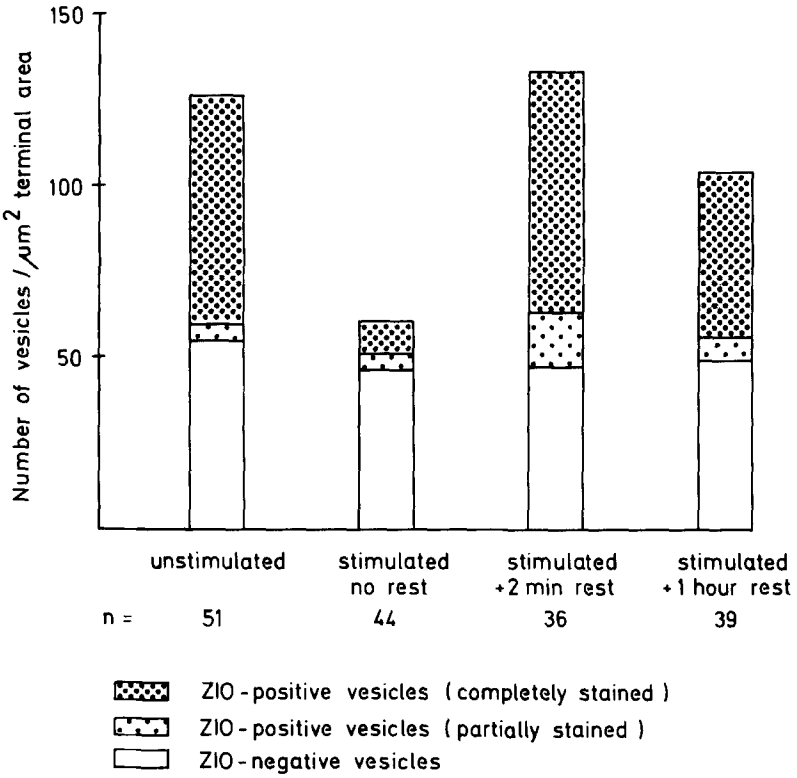

FIGURE 8 Effect of nerve stimulation and subsequent rest on the number of vesicles in the nerve terminal. Stimulation was performed at 20 Hz for 7 min. Note considerable increase in the number of partially ZIO-positive vesicles 2 min after the end of stimulation. See also Table II.

TABLE II

Comparison of the Average Numbers (±SEM) of Synaptic Vesicles/μm^2 Terminal Area under Four Different Experimental Conditions

	Unstimulated	Stimulated	Stimulated + 2 min rest	Stimulated + 1 h rest
Completely ZIO-positive vesicles:				
Number*	66.55 ± 3.41	9.88 ± 0.94	70.12 ± 2.83	48.43 ± 3.38
Unstimulated		$P < 0.00001$	n.s.‡	n.s.
Stimulated			$P < 0.00001$	$P < 0.00001$
Stimulated + 2 min rest				n.s.
Partially ZIO-positive vesicles				
Number*	4.97 ± 0.64	4.17 ± 0.48	15.75 ± 1.11	6.14 ± 1.51
Unstimulated		n.s.	$P < 0.00001$	n.s.
Stimulated			$P < 0.0001$	$P < 0.05$
Stimulated + 2 min rest				$P < 0.001$
ZIO-negative vesicles				
Number*	55.37 ± 3.63	46.48 ± 1.16	47.19 ± 3.10	51.19 ± 3.41
Unstimulated		$P < 0.05$	n.s.	n.s.
Stimulated			n.s.	n.s.
Stimulated + 2 min rest				n.s.

t-tests were carried out separately for (*a*) completely ZIO-positive vesicles, (*b*) partially ZIO-positive vesicles, and (*c*) ZIO-negative vesicles. Stimulation was performed at 20 Hz for 7 min. The numbers of synapses evaluated were 51, 44, 36, and 39 for unstimulated, stimulated, stimulated + 2 min rest, and stimulated + 1 h rest, respectively.

* Number of vesicles/μm^2 terminal area.

‡ not significant.

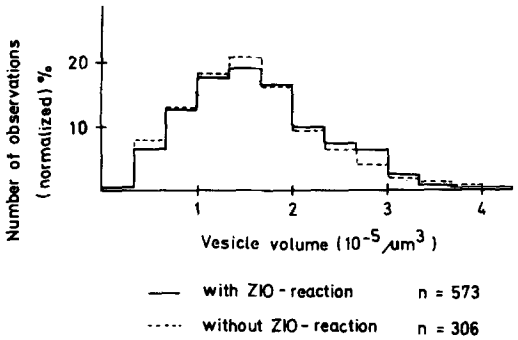

FIGURE 9 Comparison between vesicle volumes from preparations incubated either in a ZIO- or in a sucrose-OsO_4 solution. Both solutions had the same osmolarity and were applied after prefixation in glutaraldehyde. Vesicle volumes were calculated from inner diameters, assuming oblate spheroids. Mean values 1.61 ± 0.04 with ZIO and 1.54 ± 0.04 with sucrose-OsO_4. The difference is statistically not significant ($P > 0.1$).

the mean values (ZIO: 1.61 ± 0.04; sucrose-OsO_4:1.54 ± 0.04, $P > 0.1$) agree closely, there is little reason to believe that the black deposits themselves affect the vesicle volume.

A comparison of the volumes of ZIO-positive and ZIO-negative vesicles is shown in Fig. 10. The completely ZIO-positive vesicles are bigger than the ZIO-negative ones in the unstimulated condition as well as at various times after stimulation ($P < 0.00001$ under all conditions). Their average volume is slightly decreased immediately after stimulation and subsequently returns to the initial value. This effect of stimulation is much more pronounced with the ZIO-negative vesicles. Here there is a 25% decrease in the average volume immediately after stimulation, and 1 h after stimulation the volume is still smaller than before stimulation (cf. Table III). It should be pointed out that the number per square micrometer of terminal area (without mitochondrial area) of the smallest ZIO-negative vesicles, i.e., which have volumes $<1.0 \times 10^{-5}$ μm^3, is increased by ~40% immediately and 2 min after stimulation. After 1 h, it is still somewhat higher than in the unstimulated condition (Figs. 8 and 10).

The average volume of the partially ZIO-posi-

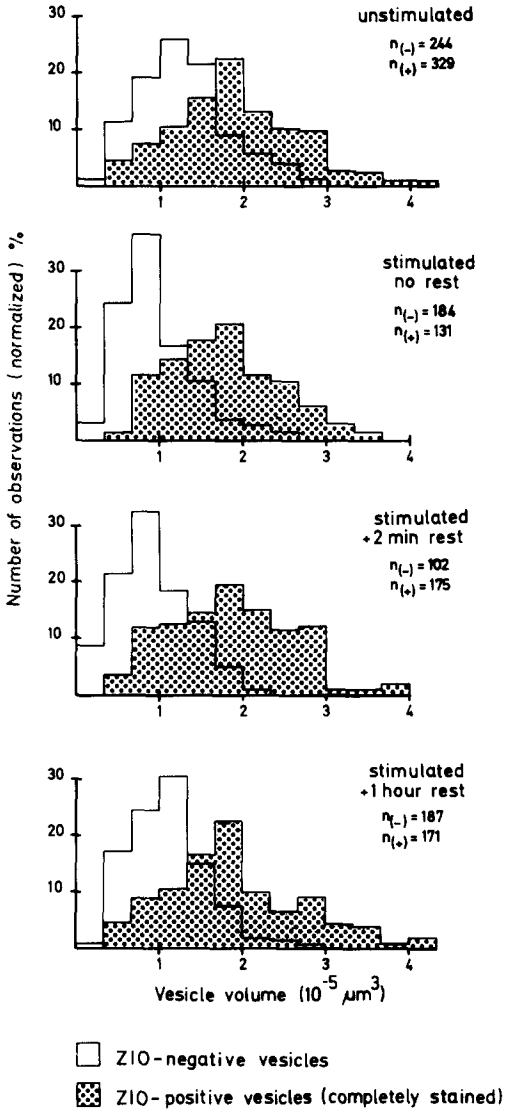

FIGURE 10 Comparison between the volumes of ZIO-negative and completely ZIO-positive vesicles under four experimental conditions. Stimulation was performed at 20 Hz for 7 min. Vesicle volumes were calculated from inner diameters, assuming oblate spheroids. Whereas the distribution of the volumes of completely ZIO-positive vesicles is nearly unaffected by stimulation or subsequent rest, the peak of the distribution of ZIO-negative vesicles is shifted to lower values immediately and 2 min after the end of stimulation. See also Table III.

tive vesicles was found to be intermediate between that of the ZIO-negative and that of the completely ZIO-positive vesicles, yet more close to that of the latter. The mean values (1.66 ± 0.08, $n = 52$; 1.68 ± 0.13, $n = 30$; 1.62 ± 0.09, $n = 51$; 1.67 ± 0.13, $n = 28$; without stimulation, immediately after, 2 min, and 1 h after stimulation, respectively; differences statistically not significant) differ from those of the ZIO-negative vesicles statistically highly significantly ($P < 0.00001$). The difference between the mean values of the partially and the completely ZIO-positive vesicles was significant only in the unstimulated condition ($P < 0.01$).

DISCUSSION

Chemical Basis for the ZIO Reaction

There are conflicting views about the chemical basis of the ZIO-reaction. From a recent review on the subject (46) it is rather unlikely that one single substrate is responsible for the cytochemical reaction. In vitro molecules as different as certain amino acids, catecholamines, aldehydes, and phospholipids can yield black precipitations with ZIO (46, 48). In the locust neuromuscular preparation, our results from preincubation of the tissue with *p*-CMPS, NEM, DTT, and diamide all lead to the conclusion that ZIO reacts mainly with free sulfhydryl groups of proteins. Our findings agree with those of Pellegrino de Iraldi (45, 46) who demonstrated, in rod outer segments, enhancement or negative outcome of the ZIO reaction after incubation in dithioerythritol and NEM, respectively. Because we applied the reagents in a concentration 100 times less than she did in order to minimize reaction with substrates other than SH-groups (5, 52, 57), our conditions were even more likely to warrant specificity for SH-groups.

The specific involvement of SH-groups suggests that proteins may be predominantly the substrate for the ZIO reaction. Intravesicular proteins have been demonstrated, for example, in various hormone-secreting cells: neurophysine in the neurohypophysis (49), chromogranins and dopamine β-hydroxylase in adrenal medulla (68), or the protein associated with heparin in mast cells (65).

It has been shown that cysteine, free or integrated in protein, but not, for example, glutathione, after forming a mercaptide with silver can complex additional silver (4, 27). Such additional complexing with mercaptide was also found with mercury (7) and it is possible that this also happens with zinc and/or osmium. This could lead to an intensification of the ZIO reaction at SH-groups of proteins, so that a few SH-groups per molecule might suffice to give a visible reaction product.

TABLE III

Comparison of the Average Volumes (±SEM) of Synaptic Vesicles under Four Different Experimental Conditions

	Unstimulated	Stimulated	Stimulated + 2 min rest	Stimulated + 1 hr rest
Completely ZIO-positive vesicles				
Vesicle volume	1.86 ± 0.04	1.73 ± 0.06	1.76 ± 0.06	1.84 ± 0.06
(10^{-5} μm^3)	n = 329	n = 131	n = 175	n = 171
Unstimulated		$P < 0.05$	n.s.‡	n.s.
Stimulated			n.s.	n.s.
Stimulated + 2 min rest				n.s.
ZIO-negative vesicles				
Vesicle volume	1.26 ± 0.04	0.94 ± 0.03	0.96 ± 0.04	1.10 ± 0.03
(10^{-5} μm^3)	n = 244	n = 184	n = 102	n = 187
Unstimulated		$P < 0.00001$	$P < 0.00001$	$P < 0.01$
Stimulated			n.s.	$P < 0.0005$
Stimulated + 2 min rest				$P < 0.02$

t-tests were carried out separately for (*a*) completely ZIO-positive vesicles and (*b*) ZIO-negative vesicles. The difference between the average volumes of the completely ZIO-positive and the ZIO-negative vesicles is highly significant ($P < 0.00001$) under all experimental conditions. Stimulation was performed at 20 Hz for 7 min.
‡ not significant.

The original idea, put forward by Maillet (34), that lipid moieties uncoupled from lipoprotein complexes account for the reaction is rather unlikely in our preparation, for the following reasons: The reactivity of the synaptic vesicles with ZIO was almost totally prevented by TCA, and the same was found after heating (80°C). Both treatments should have affected proteins rather than lipids. Evidence in favor of lipoproteins or simply lipids, put forward later on (14, 16, 39), mainly relies on the failure of the ZIO reaction after extraction of tissues with lipid solvents, e.g., methanol-chloroform (1:1) or 70% methanol. These solvents may, however, also remove nonlipid tissue components and lead to conformational changes in proteins (43).

Structural Basis of the ZIO Reaction

If ZIO predominantly reacts with protein, then the question arises why does the reaction not take place throughout the whole tissue. Obviously, it would have to distinguish between different kinds of proteins. Thus, for example, the mitochondrial matrix and the interior of an additional 30% of the synaptic vesicles stain only after pretreatment with DTT. This would imply that the proteins involved lack free SH-groups under the conditions used for the stimulation experiments (see section entitled Number and Size of ZIO-Positive and ZIO-Negative Vesicles and the Effect of Nerve Stimulation). Furthermore, cysteine residues are more accessible in some kinds of protein than in others and can be entirely hidden in an apolar core. The massive overall reaction found at low pH (<3.5) could be explained by assuming some degree of denaturation of protein, leading to free access to additional SH-groups (cf. similar considerations by Pellegrino de Iraldi [46]).

The reactivity of potentially ZIO-positive material might also be modified by the presence or absence of small molecules, e.g., transmitter (in our preparation, presumably glutamic acid [63]) or ions, including protons. In aminergic nerve endings, there is some indication that a reserpine-induced loss of transmitter is accompanied by a reduction of ZIO reactivity within granular synaptic vesicles (47, 48) though the evidence is conflicting (36, 41). In both aminergic and cholinergic vesicles, in addition to transmitter, high concentrations of ATP and Ca^{++} have been demonstrated (10, 54, 68, 69). With chromaffin granules, a low internal pH due to a proton pump has been found (25). It is conceivable that intravesicular proteins undergo changes in conformation and aggregation (19) as the concentration of transmitter, ATP, Ca^{++}, and possibly protons builds up during maturation of vesicles, perhaps leading to formation of complexes.

Physiological Aspects

The value of ~57% ZIO-positive vesicles (completely plus partially stained ones) which was found in resting nerve terminals compares well with the figure of ~50% previously reported for rat spinal cord (24) under almost identical experimental conditions, including pH. With pretreatment by DTT, after which partially stained vesicles do not occur any more, this percentage is increased to nearly 90%. Preliminary experiments show that in preparations fixed immediately after stimulation DTT also causes an increase in the number of ZIO-positive vesicles. However, the number of those vesicles which become ZIO-positive only by the DTT treatment is only about half that in unstimulated preparations. As a working hypothesis, we suggest that the total number of ZIO-positive vesicles found after DTT treatment represents the subpopulation of vesicles containing a certain kind of protein (see above).

In the following discussion the different results concerning ZIO-reactivity and the number and size of the synaptic vesicles are interpreted on the basis of a hypothetical scheme for their turnover and biogenesis (Fig. 11). Most features incorporated into it have been demonstrated for a variety of synaptic and glandular systems (for a recent review, see reference 22). According to this scheme, vesicles would be derived from SER and, after some maturational process, be used via exocytosis for transmitter release. Vesicle membrane would be retrieved via endocytosis of vesicles which, at least in part, fuse with SER. This fits in with current views of the situation in adrenal chromaffin cells (68), although in those cells recycling vesicles are suggested to fuse with dictyosomes.

The ZIO-negative vesicles would in this model be regarded as those not ready for exocytosis. On the average, they are smaller than the ZIO-positive ones and they are further reduced in size but not in number after stimulation. In part, they seem to be of endocytotic origin for the following reasons: Endocytosis, which in preliminary experiments we have demonstrated in this preparation with horseradish peroxidase, is known to be enhanced by nervous activity (e.g., references 6, 20, 23). Recently, in *Torpedo* electric tissue it has been shown that stimulation-dependent uptake of dextran preferentially occurs in a class of particularly small vesicles (70, 71). In our preparation, the smallest ZIO-negative vesicles are clearly more numerous after nerve stimulation (Figs. 8 and 10).

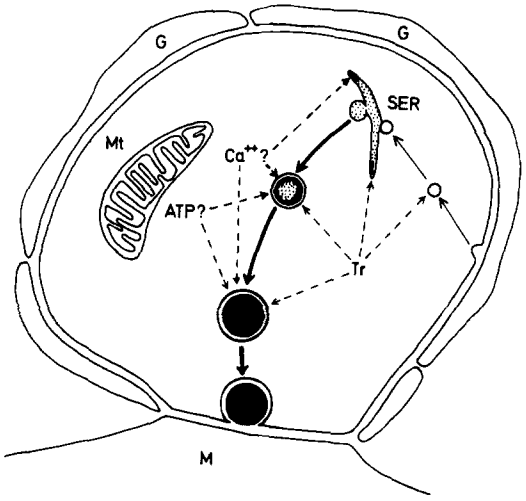

FIGURE 11 Hypothetical scheme for the biogenesis of synaptic vesicles within the nerve terminal. Thick arrows show course of development of a vesicle which starts by budding from SER. Stippling indicates presence of protein which becomes progressively reactive with ZIO (shown as blackening) as transmitter and/or perhaps Ca^{++} and ATP are taken up from cytoplasm (broken arrows). Thin arrows indicate retrieval of vesicular membrane via endocytotic vesicles which may fuse with SER. The ZIO reactivity in portions of SER which is found more often after nerve stimulation might be accounted for by uptake of Ca^{++} and/or transmitter. (*G*, glia; *M*, muscle; *Mt*, mitochondrion; *Tr*, transmitter [in the preparation used for this study, presumably glutamic acid]).

The completely ZIO-positive vesicles would represent the mature ones, i.e., those ready for exocytosis. Whereas their average volume under the different conditions stays about the same, their number immediately after massive tetanic stimulation is drastically reduced. Results similar to the latter have been reported for synaptosomes (18). The surprising finding that already within 2 min after the end of stimulation the number of vesicles has returned to the initial level is mainly due to the increase in number of completely ZIO-positive ones. This indicates a highly activated transmitter supply mechanism which requires formation of new ZIO-positive vesicles. Transport of vesicles from stores at some distance from the junctions would account for only a minor part of the supply since synaptic vesicles are rarely found on axonal cross-sections ($4.7 \pm 1.0/\mu m^2$; $n = 21$) between junctions, and at most some 40% of these are ZIO-positive.

The partially ZIO-positive vesicles, which are infrequent both in the unstimulated and in the stimulated condition but which are tripled in number 2 min after stimulation, would be considered as an intermediate stage in the formation of new ZIO-positive vesicles. Their average volume, lying between that of ZIO-negative ones and that of completely ZIO-positive ones, is consistent with this, suggesting that maturation of the vesicles is accompanied by their growth.

If the substrate of the ZIO-reaction is in fact an intravesicular protein which is released during exocytosis, such as for example, dopamine β-hydroxylase (67), a continuous supply of this protein would be required even if part of it is recovered by endocytosis. SER has been suggested to transport proteins from the perikaryon to nerve endings (11, 12, 55, 58). Transfer of protein into synaptic vesicles would thus be conceivable if vesicles can bud off from SER, as has been reported in a variety of synapses (13, 21, 22, 59, 62) including insect neuromuscular synapses (40). In neuronal perikarya, formation of vesicles from SER (and dictyosomes) was also suggested on the basis of ZIO studies (31, 56). The converse, i.e., fusion of endocytotic vesicles with SER, might be indicated by the fact that exogenous peroxidase is found not only in vesicles but also within cisternae and tubular structures at nerve terminals (20, 32, 53, 60, 61, 62; unpublished results of these authors).

In the locust, the SER in nerve terminals and axons is in part ZIO-positive (see second section of Results; cf. also reference 31) as reported also for vertebrate central nervous system (1, 56). After stimulation, more ZIO-positive profiles are seen in our preparation. This accords with an increase in the number of SER cisternae or tubules after nerve stimulation observed in vertebrate central and peripheral synapses (28, 38).

A positive or negative reaction of the synaptic vesicles with ZIO should not simply be equated with the presence or absence of the hypothetical protein(s) (cf. above). Rather, for the reasons given in the second part of this discussion, uptake of transmitter, Ca^{++}, and ATP has been incorporated into the scheme (Fig. 11), assuming that this takes place mainly after separation from the SER. It could be this latter process which is reflected in the increasing ZIO-positivity of the vesicular matrix.

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