J. exp. Biol. (1980), **89**, 19–29
With 5 figures
Printed in Great Britain

USE OF ALDEHYDE FIXATIVES TO DETERMINE THE RATE OF SYNAPTIC TRANSMITTER RELEASE

BY J. E. SMITH AND T. S. REESE

Section on Functional Neuroanatomy, National Institute of Neurological and Communicative Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20205, *U.S.A.*

SUMMARY

Aldehyde fixation continues to be useful to prepare synapses for freeze-fracture, but it may increase the rate of transmitter release. The effects of different aldehyde fixatives on spontaneous quantal release (m.e.p.p.s), and on the corresponding synaptic vesicle exocytosis at frog nerve-muscle synapses were investigated with the hope of finding a way to minimize side effects of fixation. Increases in m.e.p.p.s of up to 50 s^{-1} occurred during fixation, despite the species of aldehyde used in the fixative, and this fixative effect decreased only slightly as aldehyde concentration was increased. Increases in m.e.p.p. frequency were not blocked by tetrodotoxin, by lowering external calcium and raising external magnesium concentration, or by lowering the total osmotic strength of the fixative. The smallest increase in m.e.p.p. frequency was in 3% glutaraldehyde and corresponded to the lowest level of synaptic vesicle exocytosis seen by freeze-fracture, 0·15 per μm of active zone. The effects of aldehyde fixation on m.e.p.p. frequency and synaptic vesicle exocytosis could not be avoided, but this study suggests how its effect on morphological changes in synapses might be minimized.

INTRODUCTION

Interactions of synaptic vesicles with the surfaces of nerve terminals are readily demonstrated with the freeze-fracture technique (Akert *et al.* 1972; Streit *et al.* 1972; and Dreyer *et al.* 1973; Heuser, Reese & Landis, 1974). A particular contribution of this earlier work was to show that these interactions are limited to discrete areas of the presynaptic membrane, the *active zones*, where it faces the postsynaptic cell. However, durable conclusions about the exact nature of these interactions and their relationship to the release of transmitter are severely limited by uncertainties about the time course and side effects of the chemical fixatives conventionally used to prepare tissues for freezing.

The limitations of chemical fixation were avoided when techniques were developed to rapid freeze tissues directly for freeze-fracture by pressing them against a metal surface cooled by liquid helium (Van Harreveld & Crowell, 1964; Heuser, Reese & Landis, 1976; Heuser *et al.* 1979). Unfortunately, only the layer of tissue, 15–50 μm thick, next to the metal surface is frozen rapidly enough, in 1–2 ms, to avoid distortion by ice crystals (Heuser *et al.* 1979). This requirement places severe limits

on the types of synaptic preparation that can be rapid frozen, so it seemed useful to explore further the use of chemical fixatives to prepare synaptic preparations for quantitative study of transmitter release. We chose the frog neuromuscular junction for this purpose because previous results with rapid freezing provided a background against which to evaluate the physiological and anatomical effects of the aldehyde fixatives which are conventionally used to prepare tissues for freeze-fracture (Heuser *et al.* 1979).

METHODS

Cutaneous pectoris muscles from small grass frogs were dissected in Ringer containing: NaCl (111 mM); KCl (2 mM); $CaCl_2$ (1·8 mM) and Hepes (5 mM, pH 7·2). 'Low Ca' Ringer lacked added calcium, EDTA, and magnesium. Tetrodotoxin (10^{-6} g/l) was always applied in low Ca Ringer for 1 h prior to a fixation experiment in order to prevent muscle twitching.

After recording the resting rate of miniature end-plate potentials (m.e.p.p.s), the Ringer was removed and replaced by Ringer containing glutaraldehyde, formaldehyde or crotonaldehyde. Replacement was done in a manner which permitted continuous recording from the same muscle fibre. M.e.p.p.s were then recorded for 15 min on a paper tape unless the resting potential fell below 40 mV; this much loss of resting potential occurred in only a few muscles in the highest concentrations of aldehydes.

Muscles prepared for freeze-fracture were fixed for 15 min in either 0·8 or 3·0% glutaraldehyde in normal Ringer (with the pH adjusted back to 7·2–7·3), followed by fixation for 45 min in 3·0% glutaraldehyde. A few were also fixed for 5 min in 3% formaldehyde followed by 55 min in 3% glutaraldehyde (Pumplin & Reese, 1977), or in some of the media used for the physiological experiments as described below.

RESULTS

(*a*) *Physiology*. Frequencies of spontaneous miniature end-plate potentials (m.e.p.p.s) were sampled one or more times during each of the 15 min following application of different concentrations of aldehyde fixatives in frog Ringer. The experimental variables are summarized in Table 1 and the results presented in Table 1 and Figs. 1–3.

Every concentration of each aldehyde produced an increase in m.e.p.p. frequency and fasiculations in some, but not all muscles. The fasiculations were not blocked by curare and therefore were probably not related to the increase in transmitter release. Glutaraldehyde fixation resulted in the smallest increases during the 15 min interval of observation (Figs. 1–2). Increasing the concentration of glutaraldehyde from 0·75 to 3·0% resulted in an earlier peak m.e.p.p. frequency, which then fell to near zero more rapidly than in the dilute glutaraldehyde. After further dilution to 0·08%, this effect was even more pronounced; the average period of increased m.e.p.p. frequency lasted for 6 rather than 3 min, independently of whether tetrodotoxin was added to the Ringer. When calcium was omitted from the Ringer, in the presence of tetrodotoxin and 0·08% glutaraldehyde, m.e.p.p. frequency increased to 3 s^{-1} in one muscle, but this small increase lasted almost as long as the increase

Table 1. *Summary of physiological experiments*

Fixative (%)	Calcium (mM)	Peak (m.e.p.p.s sec^{-1})	Time to 1st peak (min)	Total m.e.p.p.s × 1000 ± S.D.	No. of experiments (number with TTX)	No. active after 15 min
G* (0·08)	0·0	3·0*	4·0*	0·41 ± 0·4	6 (6)	0
G (0·08)	1·8	3·3	2·5	0·95 ± 0·3	8 (6)	0
G (0·75)	1·8	5·7	1·0	0·36 ± 0·2	5	0
G (3.00)	1·8	7·0	0·5	0·23 ± 0·1	4	0
C (0·08)	1·8	47·0	15·0	18·00 ± 5·0	3 (3)	3
F (0·08)	0·0	51·0	7·0	25·00 ± 9·0	4 (4)	4
F (0·08)	1·8	33·0	2·8	17·00 ± 7·0	5 (3)	5
F (3·00)	1·8	23·0	3·3	5·20 ± 3·0	7	3

G = glutaraldehyde; C = crotanaldehyde; F = formaldehyde.
* From a single muscle which showed a clear peak and total m.e.p.p.s of 1220.

3–4 s^{-1} in normal Ringer. Therefore, the absence of calcium may result in only a small decrease in the frequency of m.e.p.p.s provoked by glutaraldehyde fixation.

Formaldehyde fixation was accompanied by an order of magnitude more m.e.p.p.s and greater m.e.p.p. frequency than was glutaraldehyde fixation (Figs. 3*a–c*; Table 1). Increasing the concentration of formaldehyde resulted in fewer total m.e.p.p.s during the 15 min period of fixation that we observed; this parallels the effect of increasing glutaraldehyde concentration. Peak frequencies were reached later in formaldehyde and increasing its concentration also had the effect of making the peak earlier. Removing calcium (in tetrodotoxin) had the opposite effect from the parallel experiment with glutaraldehyde; the m.e.p.p. frequency rose to a higher level and stayed high during the whole 15 min of fixation. The results with formaldehyde were far more variable than with glutaraldehyde with respect to both the peak m.e.p.p. rate and the onset and duration of the peak. In two out of ten experiments with 3% formaldehyde, the peak m.e.p.p. frequency was less than 10^{-1} s, whereas in two others it exceeded 50 s^{-1}. With 0·75 and 3% formaldehyde, there were fluctuations in m.e.p.p. frequencies, often resulting in two frequency peaks several minutes apart (Fig. 1*b*). Four experiments in which 3% glutaraldehyde was mixed with 2% formaldehyde resulted in an early rise in m.e.p.p. frequency similar to that in glutaraldehyde which, however, reached a frequency more typical of formaldehyde. The m.e.p.p.s then disappeared within 3–4 min.

Crotonaldehyde (C_4H_6O) produced the largest increases in m.e.p.p. frequency, but only the lowest concentration (0·08%) was tolerable to use in an open laboratory. The onset of the m.e.p.p. increase was also more delayed than in glutaraldehyde. Intolerance to the fumes prevented completion of the experiments with acrolein (C_3H_4O).

The decreases in m.e.p.p. frequency which followed fixation-induced increases could not be attributed to decreases in the resting potentials of the muscle fibres. The maximum decrease in resting potential which accompanied the first minute of decrease in m.e.p.p. frequency was 10 mV in 3% glutaraldehyde or formaldehyde. However, the decrease in m.e.p.p. amplitude was somewhat greater than predicted from these small changes in membrane potential. For example, in one experiment in

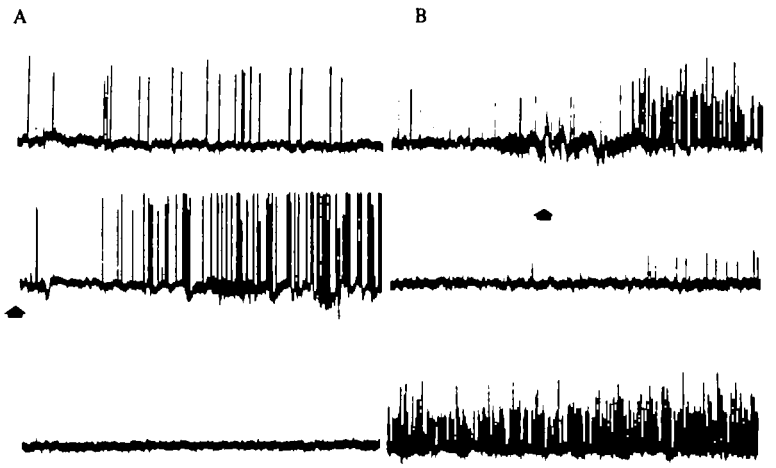

Fig. 1. Spontaneous miniature endplate potentials (m.e.p.p.s) before and after application (arrow) of 3 % glutaraldehyde (A) or formaldehyde (B) in normal Ringer. A second burst of m.e.p.p.s (lower trace in B) was characteristic of the response to high concentrations of formaldehyde. Each trace represents 17 s. The lower trace in A begins 1 min after application of glutaraldehyde, and the two lower traces in B begin 2 min and 5 min after application of formaldehyde. Amplitude of first m.e.p.p. in A is 1·5 mV.

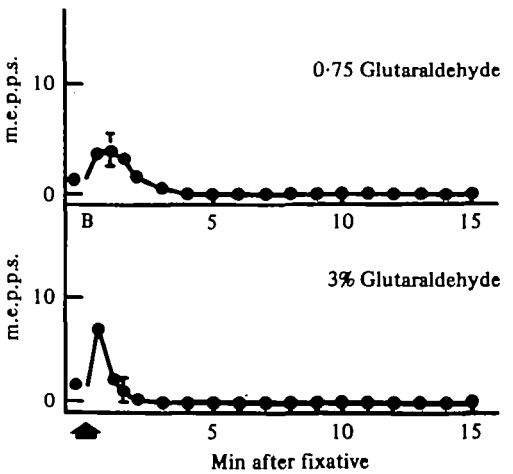

Fig. 2. M.e.p.p.s sampled at intervals of 1 min or less for 15 min after application of glutaraldehyde (arrow). The points from the experiment with 0·75 glutaraldehyde are the mean from 5 muscles; those with 3 % glutaraldehyde are the means of four muscles. One standard deviation is bracketed.

3 % formaldehyde, the mean m.e.p.p. amplitude decreased from 1·0 to 0·5 mV, even while the resting potential decreased by only 2 mV. These disproportionate decreases are presumably caused by a direct action of the aldehyde on the post synaptic receptors

Substitution of magnesium (10 mM) for calcium did not inhibit the fixation-induced increase in m.e.p.p. frequency (Birks, 1973). Nine muscles exposed to 3 % formaldehyde with magnesium substituted for calcium showed an increase in m.e.p.p. frequency ranging between 5 and 50 s^{-1}, which was not significantly different from the fixation effect in normal Ringer.

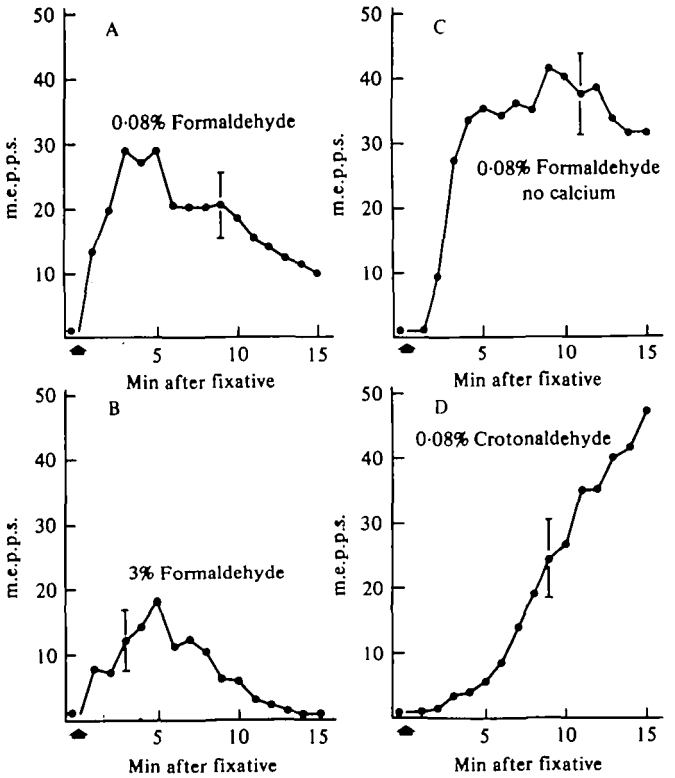

Fig. 3. M.e.p.p.s sampled at intervals of 1 min or less after application (arrows) of crotonaldehyde or formaldehyde. Concentrations of aldehyde and other significant components of the Ringer are given on each graph. Each point is the mean from several muscles: A from 5; B from 11; C from 4; and D from 4. Most of these experiments were done in TTX to prevent muscle twitches.

We also attempted to test the possibility that the increases in m.e.p.p. frequency were caused by a momentary osmotic effect of the aldehyde-Ringer mixture by exploring the effects of formaldehyde (127 mM) in Ringer diluted to half strength (4 experiments) or to three quarter strength Ringer (4 experiments). Typically, the micropipette was ejected from the muscle fibre when the fixative was added, but repenetration showed that m.e.p.p.s increased up to 50 s^{-1}. No increases were found in muscles exposed to the dilute Ringer in the absence of formaldehyde, nor was the increase induced by formaldehyde blocked by pretreating muscles with tetrodotoxin. We wish to stress, however, the preliminary nature of these osmolarity experiments and that none of our solutions were calibrated with an osmometer.

To determine whether the morphological results could be correlated with the physiological results, recordings were made from four muscles during 15 min of treatment in 0·8% glutaraldehyde followed by 15 min of treatment in 3% glutaraldehyde. The m.e.p.p. rate did not show a secondary increase when the higher concentration of glutaraldehyde was introduced, though the muscle resting potential fell rapidly.

(*b*) *Anatomy*. Several nerve terminals, each with 5–50 contiguous active zones

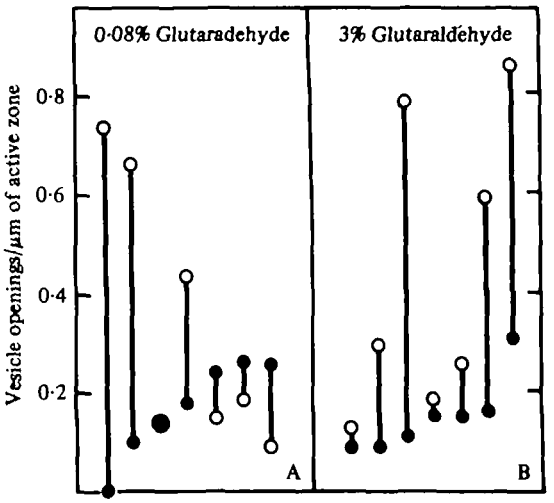

Fig. 5. Frequency of vesicle openings per μm of active zone at 14 terminals, each with 11–50 active zones. The bars connect the mean value for vesicle openings at the active zone (●) to the mean value for vesicle openings outside of the active zone (○) at the same nerve terminal.

typically were found in each series of aldehyde fixed muscles. A few impocketings of the nerve terminal plasmalemma were found consistently after fixation in either 0·8 or 3·0% glutaraldehyde in Ringer (Figs. 4–5). Roughly one third of these impocketings were at active zones adjacent to the attachment of the presynaptic dense bar where synaptic vesicles open during transmitter release (Heuser *et al.* 1977, 1979). Thus, their appearance and location at active zones suggests that they are openings of synaptic vesicles. Other impocketings of the plasmalemma were spread over the adjacent regions of the nerve terminal plasmalemma where it is not covered by Schwann cells. Their identity is not certain, though their appearance also suggests they could be either synaptic vesicle openings or sites of coated vesicle formation (Pumplin & Reese, 1978). Fig. 5 summarizes the range and variability in the frequency of vesicle openings at and away from the active zone from 14 representative nerve terminals These data indicate that there is approximately one vesicle opening at every 5–6 active zones.

Brief prefixation in 3% formaldehyde followed after 5 min by fixation in glutaraldehyde also increased the number of vesicle openings, particularly at the active zone. This result was more variable than with glutaraldehyde in that an occasional segment of a nerve terminal had many vesicle openings (Fig. 4) whereas many other segments exposed by freeze-fracture had few or none. Similar results have been seen previously following fixation in formaldehyde alone (Miller & Heuser, personal communication). In one successful but preliminary experiment, vesicle openings at active zones were still seen after fixation in 3% formaldehyde when 110 mM-Mg^{2+} was substituted for Na^{+} and Ca^{2+} (Birks, 1973; McKinlay & Usherwood, 1978). Vesicle openings were also frequent at active zones in another muscle fixed in the half-strength Ringer containing 0·38% (127 mM) formaldehyde.

The mean diameter of the vesicle openings at active zones seemed to increase

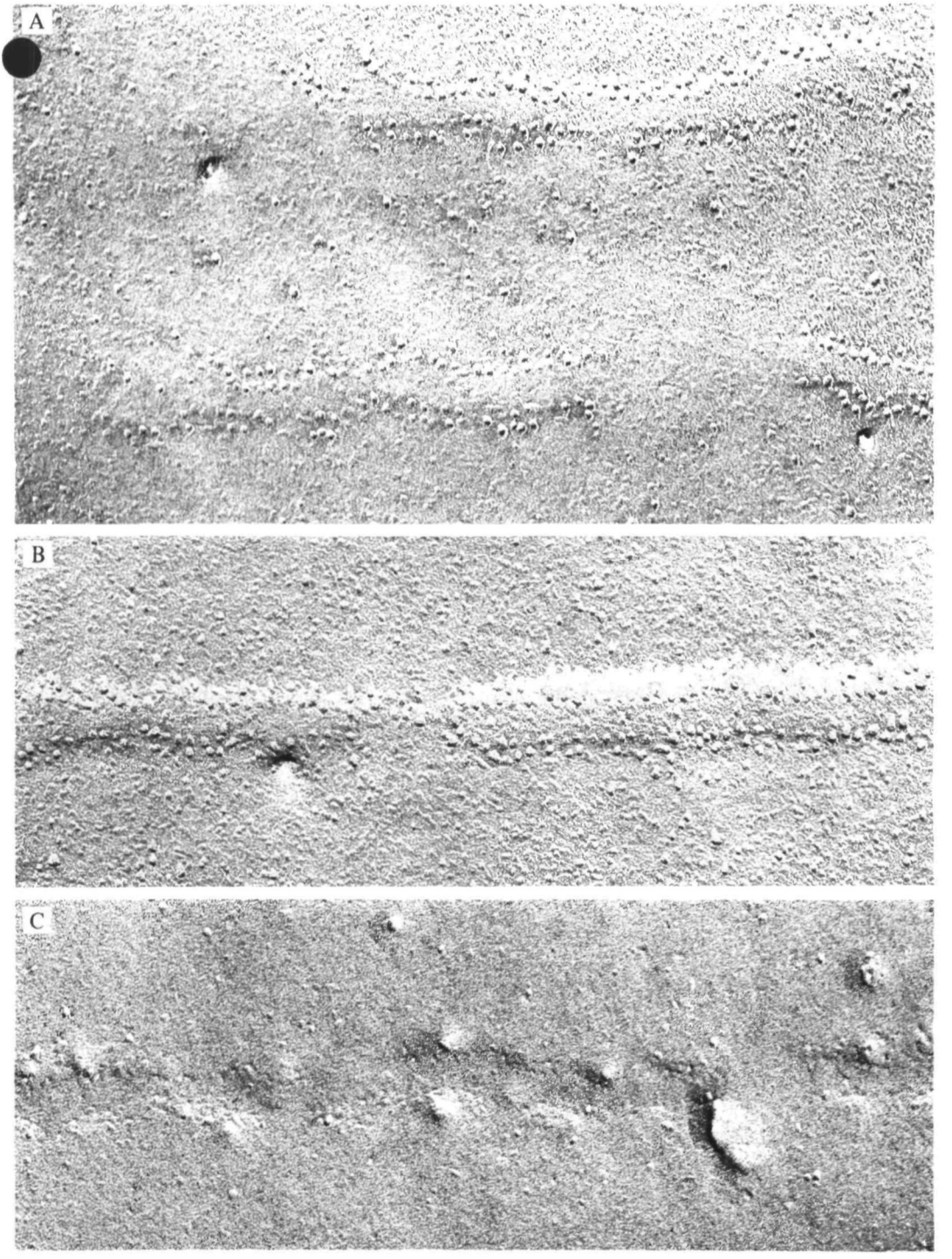

Fig. 4. Active zones from muscles fixed in 0·08 % glutaraldehyde (A), 3 % glutaraldehyde (B), or 3 % formaldehyde (C) in normal Ringer. Most plasmalemmal dimples represent openings of synaptic vesicles and occur at active zones within 50 nm of the particle rows. The identity of those outside of active zones is not certain. The terminal shown in C had the highest concentration of active zone vesicle openings we found, 12 μm^{-1} of active zone. The terminals in A and B show cytoplasmic (P) leaflets while the one in C has its external (E) leaflet exposed. × 100000.

slightly in the higher concentration of glutaraldehyde (from 32·7 to 38 nm), though this difference was not significant at the 99% level. These diameters are comparable to those found in rapid frozen nerves, though the latter may show a greater variety of shapes, representing all the stages in exocytosis, than are captured with glutaraldehyde (Heuser & Reese, 1980). After formaldehyde fixation, many of the vesicle openings appear to be smaller and have a different shape, though this point has not yet been examined with careful measurements. These differences could represent the selective capture of a stage in exocytosis not commonly seen with other methods, a distortion of the exocytotic process during or after fixation, or an extraneous artifact unrelated to exocytosis.

DISCUSSION

Every concentration and species of aldehyde in every variant of Ringer that we tried increased the frequency of miniature endplate potentials (m.e.p.p.s). These experiments therefore eliminate or narrow the possible interpretations of this aldehyde effect. Clearly, the effect does not depend on a particular aldehyde or its impurities. A similar aldehyde effect is also found at insect nerve muscle junctions (McKinley & Usherwood, 1978).

The aldehyde effect is also unlikely to depend on any depolarization of the nerve that is propagated or mediated by the normal sodium channels because no differences were found in doses of tetrodotoxin sufficient to block nerve action potentials. The lack of any effect from increasing the concentration of external magnesium with respect to the concentration of calcium suggests that the quantal release evoked by aldehyde fixative is not mediated by an influx of external calcium, either caused by the direct effects of fixative or by indirect effects such as depolarization of the nerve. In fact, the membrane potential of the muscle changed little during the interval of increased m.e.p.p. frequency evoked by glutaraldehyde.

The aldehyde fixatives must therefore be added to the list of agents known to increase quantal transmitter release independently of normal concentrations of external calcium (Quastel, 1974). External application of hypertonic medium also belongs on this list (Kita & Van der Kloot, 1977), and aldehyde fixatives as ordinarily used are quite hypertonic. A 3% solution of glutaraldehyde, for instance, is 300 mM in addition to the Ringer in which it is dissolved. However, our preliminary results showing the persistence of a fixative effect, even when the aldehyde Ringer solutions are mixed to an isomotic level (calculated), suggests that the aldehyde effect shares properties with, but is fundamentally different from the osmotic effect. This interpretation requires that aldehyde fixatives penetrate cells so rapidly that their hypertonic effect is absent or short-lived. This rapid penetration may account for the fact that the concentration of aldehyde is almost universally ignored in setting the osmolarity of fixatives without resultant gross shrinkage of cells (Hayat, 1970).

The similarity of the fixative effect to that of other agents which are independent of normal external calcium suggests an approach to suppressing it that was not tried in the present study. It is now known that this kind of quantal release, at least at the frog neuromuscular junction, is dependent on the availability of small amounts of external divalent cation (Ornberg, 1977). It is therefore conceivable that strong che-

lators might suppress the effects of aldehyde fixatives on spontaneous quantal release. It should also be stressed that the present experiments and our interpretations of them do not rule out the possibility that the same result could be achieved by lowering external sodium or by fixation in one of the buffers often used in anatomy instead of in Ringer.

All the variants of gluaraldehyde and formaldehyde fixation tried initially for physiological experiments also resulted in plasmalemmal impocketings at active zones. It has recently become clear that each plasmalemma pocket is the opening of a synaptic vesicle undergoing exocytosis and that it corresponds to the release of one quantum of transmitter (Heuser *et al.* 1979). If the nerves were slowly immobilized during quantal release induced by aldehyde, vesicle openings which started during fixation should be captured. However, the exact number that would be captured during aldehyde-induced release is difficult to predict because the exact time and the interval during which the fixative immobilized the presynaptic membrane is unknown. It is even conceivable that immobilization would have occurred only after quantal release stopped, or that immobilization would have proceeded so rapidly once it began that the low level of release in the aldehyde fixatives would have produced only a few vesicle openings in a whole neuromuscular junction. Evidently, neither of these possibilities is applicable to the results with any of the aldehyde–Ringer combinations tried by us, or by Miller & Heuser (personal communication).

It is also possible to ask the converse question, how rapidly the immobilization progresses from the time it begins to capture vesicle openings until vesicle opening is stopped, under the assumption that vesicle opening stops at the same moment that quantal release stops. For instance, the fixation effect in 3% glutaraldehyde was typically 100–500 quanta released during 1·5–2 min. Yet, the anatomical data shows that only 50–125 vesicle openings are captured under these conditions, which could be interpreted to mean that fixation in 3% glutaraldehyde has captured the anatomical substrate of up to a minute of quantal release (Heuser *et al.* 1974). By this interpretation, dilution of the glutaraldehyde, or substitution of formaldehyde results in even longer fixation and therefore capturing times. An additional assumption which underlies this calculation of the time course of the fixative effect on anatomical events is that vesicle openings are resolved uniformly and rapidly under normal circumstances. Indeed, recent results with the rapid freezing technique have shown that they normally disappear within msecs after they form (Heuser & Reese, 1980). However, during very high levels of release, or after sustained low levels of release in hypertonic sucrose, they last much longer (Heuser & Reese, 1980). If the sustained release produced by aldehyde fixatives has a similar effect of prolonging the life times of vesicle openings, then the time course of the immobilization phase of fixation would be shorter than that calculated above.

Aldehyde fixation also produced an increase in vesicle openings outside the active zone which, in some instances, exceeded those at the active zone. Endocytosis of coated vesicles is known to occur in these regions of synaptic terminals (Heuser & Reese, 1973; Landis & Reese, 1974), and forming coated vesicles are often distinguished from exocytotic sites in rapid frozen tissue by their larger diameter and content of large plasmalemmal particles. However, these differences are not easily seen in aldehyde fixed tissues, presumably because of distortion by the fixative (Ceccarelli *et al.*

1979*a*, *b*). Distributions of vesicle openings outside of active zones similar to those reported here are found after treatment with brown widow spider venom, and it has been suggested that these may represent synaptic vesicle exocytosis, particularly if the effects of spider venom depend on a general increase in the level of cytoplasmic calcium (Pumplin & Reese, 1977). However, the nature of vesicle openings found outside the active zone in aldehyde fixed nerve terminals treated with spider venom remains unresolved and factors producing them remain difficult to sort out from co-operative effects with the fixatives used to perform these experiments or even from effects provoked by aldehyde fixation itself.

Our results therefore raise serious questions about quantitative interpretations of data from experiments on synaptic activity which depend on aldehyde fixation. In particular, the intervals over which vesicle openings are captured and the effects of level of activity, whether induced by the aldehyde fixative or the experimental conditions, on the lengths of these intervals are unknown. These factors remain undefined even at the frog neuromuscular junction where the results from direct rapid freezing are available for comparison.

Nevertheless, important new information about activities in different components of synaptic circuits has been derived from experiments on the brain which have depended on aldehyde fixatives (Pfenninger & Rovainen, 1974; Landis & Reese, 1974; Gulley, 1978). The use of aldehyde fixatives to capture large numbers of vesicle openings has also been helpful for recognizing active zones and understanding their distribution in the brain (Akert, *et al.* 1972). However, the increases in structural activity relative to unstimulated synapses has tended to be low, usually from 3–5 times (Streit *et al.* 1972; Heuser *et al.* 1974; Pfenniger *et al.* 1974; Gulley, 1978; Rheuben & Reese, 1978). If these synapses function like the frog neuromuscular junction, where the increase of activity during evoked quantal release is known to be much greater (Heuser *et al.* 1979), then these results cannot be accepted uncritically as reflecting the actual activities at these synapses.

An alternative to observing vesicle openings directly is to look at their aftermath, the appearance of large intramembrane particles 120 Å in diameter, around the active zone (Heuser & Reese, 1975; Venzin *et al.* 1977; Heuser & Reese, 1979*a*, *b*); Heuser & Reese, 1980). Since these particles are found in synaptic vesicle membrane (an average of 3 per vesicle); are larger than those which define the edge of the active zone; appear at the active zone immediately after exocytotic synaptic vesicle openings disappear, and subsequently spread over the rest of the presynaptic membrane, they are considered to be components of synaptic vesicle membrane which are added to the presynaptic surface during exocytosis (Heuser & Reese, 1980). They are ultimately recovered by endocytosis, so their concentration on the presynaptic surface should reflect the ***net*** effect of the relative rates of these two processes prior to fixation.

Increases in the numbers of large particles have also been seen at nerve–nerve synapses and at synapses in the brain which, unlike the nerve muscle synapses, are almost certainly not cholinergic (Venzin *et al.* 1977; Heuser & Reese, 1979*a*). It has been suggested that measuring these increases may be an alternative method for evaluating the activities of synapses prior to fixation (Heuser & Reese, 1979*a*, *b*), though different investigators have differed in the details of their interpetations of these increases and it is not known yet whether they occur at all types of synapses. Since

particle increases would reflect a period of activity *prior* to fixation, and the point at which vesicle openings are captured by fixation would presumably be the *end* of the period of large particle addition, the numbers of large particles might not be so heavily influenced by the fixative effects as the numbers of vesicle openings. In, fact, the resting levels of large particles are similar regardless of whether frog neuromuscular junctions are fixed in glutaraldehyde or rapid frozen (Heuser & Reese, 1979*a*, *b*; Heuser & Reese, 1980). Nevertheless, a minimum period of aldehyde-induced quantal release is desirable when counts of large particles are to be applied to quantitative evaluation of synaptic activity. If other systems behave like the frog neuromuscular junction, then fixation for particle counts should be in a high concentration of glutaraldehyde alone.

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