STRUCTURAL ORGANIZATION OF THE SYNAPTIC CLEFT: POLYIONIC BINDING BETWEEN PRE-AND POSTSYNAPTIC MEMBRANES.

K. PFENNINGER, K. AKERT and C. SANDRI

Brain Research Institute, University of Zürich
August Forelstrasse 1, 8008 Zürich, Switzerland

The bismuth-iodide impregnation of nerve tissue (2) followed by uranyl acetate and lead hydroxyde poststaining (BIUL-method) has clearly established the presence of two electron dense layers within the synaptic cleft representing specializations of the outer fuzz coats of the two opposing membranes (Fig. 3). The affinity of the BIUL method to basic and acidic groups has been established by in vitro studies as well as by amino acetylation and carboxy methylation experiments in situ (Pfenninger, MD thesis, 1970). The two BIUL positive intracleft lines are susceptile to pronase and pepsin digestion. The effect of trypsin is of added interest because it degradates membrane components contrasted by uranyl and lead (Fig. 1 and 2), but affects BIUL treated dense lines only sligthly (Fig. 4). This observation suggests that the dense lines may contain trypsin labile peptidic components with acidic groups. The trypsin resistent membrane components which are demonstrated by the BIUL method may contain primarily basic groups. It is noteworthy that trypsin opens synaptic junctions when applied to unfixed neuropil (Fig. 4). The separation of pre-and postsynaptic elements is also accomplished by incubating neuropil in solutions of high ionic strength previous to fixation, e.g. $MgCl_2$ (0.1-2.0 M), $CaCl_2$ (0.5-1.5 M), $(NH_4)_2SO_4$ (2.0 M), LiBr (2.0 M) have been successfully used at pH 7.4 (Fig. 5 and 6). Treatment with EDTA (0.1 M), urea (3.0 M), and sucrose (3.0-5.5 M) failed to break up synaptic contacts in situ.

These experiments suggest electrostatic interactions between acidic and basic groups associated with the macromolecules at the surface of external membrane coats. The residues forming these ionic bridges failed to react with BIUL, thus sparing an electronlucent area (about 20 Å) between the two BIUL positive intra cleft lines. The splitting experiments often reveal thin filaments which cross the synaptic cleft (Fig. 6). They may represent uncoiled macromolecular chains revealing a vertical organization of cleft structures and remind of earlier observations by De Robertis (3) and Gray (1).

Supported by Swiss National Foundation of Scientific Research (Nr. 4356, 3.133.69)

References

1. Gray, E.G.: Int. Rev. Gen. exp. Zool. 2, 139 (1966).
2. Pfenninger, K., Sandri, C., Akert, K. and Eugster, C.H.: Brain Res. 12, 10 (1969).
3. Robertis De, E.: Histophysiology of synapses and neurosecretion. Pergamon Press, Oxford (1964).

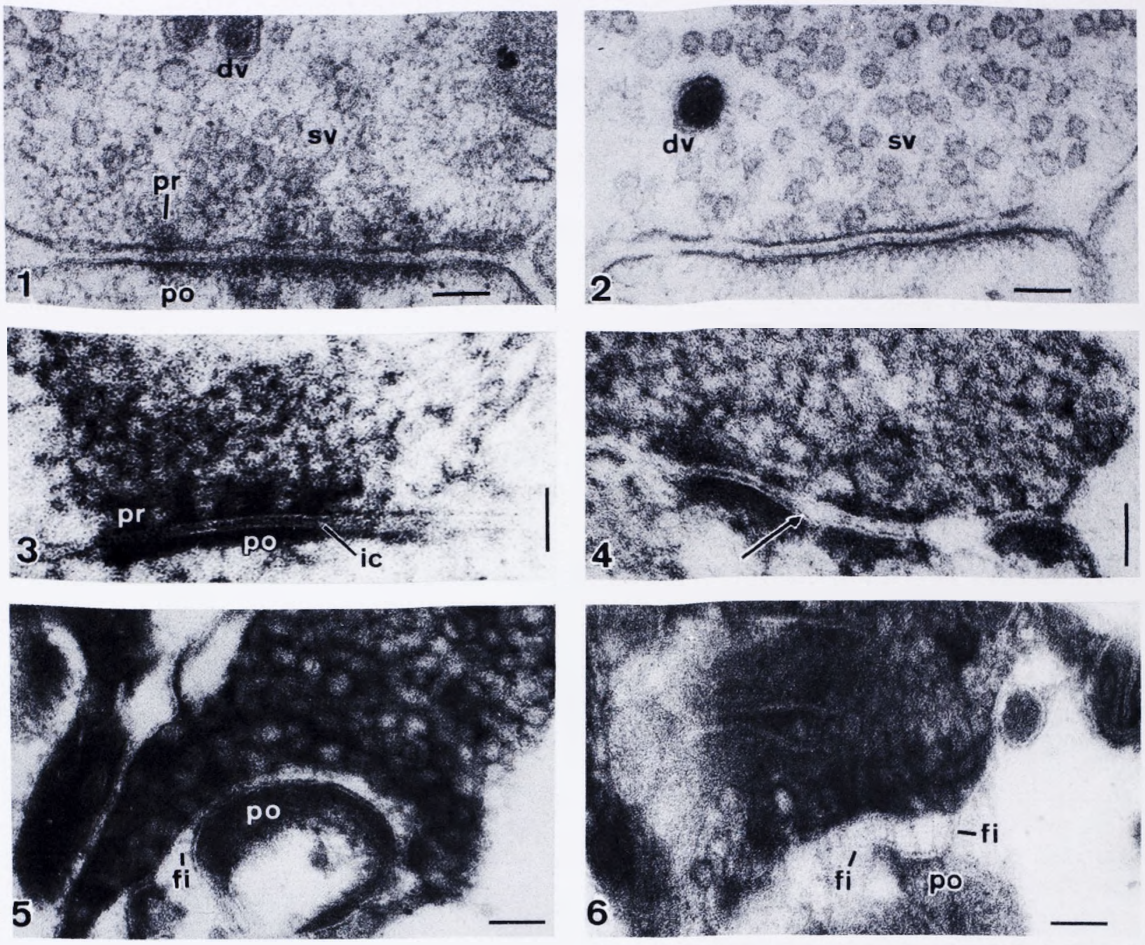

Fig. 1: Synapse in cat subfornical organ. Glutaraldehyde/OsO_4 fixation. Poststaining with uranyl and lead. Normal control. dv=dense cored vesicles; sv=synaptic vesicles; pr=presynaptic dense projections; po=postsynaptic thickening.

Fig. 2: Synapse in cat subfornical organ. Same fixation as for synapse in Fig. 1. The glutaraldehyde-fixed specimen was treated with trypsin previous to osmication. Note that the synaptic membrane complexes are drastically reduced, while plasmalemmal and vesicular membranes are preserved.

Fig. 3: Synapse in cat subfornical organ. Glutaraldehyde/Bismuth-iodide fixation. Poststaining with uranyl and lead. Note the sparing of unit membranes and the high contrast of synaptic densities and double intracleft line (ic).

Fig. 4: Synapse in cat subfornical organ. Same fixation as for synapse in Fig. 3. The unfixed specimen was treated with trypsin. Note that synaptic densities and intracleft lines are preserved. Widening of the synaptic cleft has occurred (arrow).

Fig. 5: Synapse of cat subfornical organ. Same fixation as for synapse in Fig. 3 and 4. Specimen was treated with 1.5 M $CaCl_2$ prior to fixation. Note the separation of synaptic junction.

Fig. 6: Synapse in cat subfornical organ. Fixation as for synapse in Fig. 3, 4, 5. Specimen was treated with 2.0 M $MgCl_2$. Synaptic cleft is widely opened. Filaments (fi) extend across the gap.
Bars indicate 0.1 μ in all figures.

Reprinted from
Brain Research
Elsevier Publishing Company, Amsterdam
Printed in the Netherlands

FURTHER OBSERVATIONS ON THE ENLARGEMENT OF SYNAPTIC VESICLES IN DEGENERATING OPTIC NERVE TERMINALS OF THE AVIAN TECTUM

K. AKERT, M. CUÉNOD AND H. MOOR

Brain Research Institute, University of Zurich, and Institute of General Botany, Laboratory of Electron Microscopy, Swiss Federal Institute of Technology, Zurich (Switzerland)

(Accepted July 30th, 1970)

INTRODUCTION

Cuénod *et al.*[3] have first drawn attention to the presence of large vesicles in nerve terminals as an early and reliable sign of secondary degeneration in the retinotectal system of the pigeon, and an analogous observation was recently made in degenerating cortico-caudate projections of the rat[7]. This phenomenon is not readily explained. One problem concerns the role of fixatives. The above-mentioned positive findings in our laboratory were made in aldehyde perfusion-fixed material, while Gray and Hamlyn[5] failed to point out large vesicles in the degenerated optic nerve terminals of the chicken tectum. Their material was fixed in osmium tetroxide and phosphotungstic acid (PTA). This discrepancy prompted the present investigation in which the effects of several fixation methods are compared and supplemented with those obtained from unfixed and fixed freeze-etched specimens.

MATERIAL AND METHODS

Experiments were performed in 31 pigeons (*Columba livia*). The right retina was removed under Equithesin® anesthesia by means of a fine suction pipette. The animals were sacrificed 6 h–30 days postoperatively. Optimal preservation of the tissue was obtained by perfusion with 4% paraformaldehyde (P) and postfixation with 6.5% glutaraldehyde (G) followed by 2% osmium tetroxide (OsO_4) after careful washing. The solutions used to this point had the following osmolarities: 4% paraformaldehyde in 0.05 *M* phosphate buffer, 1240 mos*M*; 6.5% glutaraldehyde in 0.1 *M* phosphate buffer, 560 mos*M*; 6.8% saccharose in 0.2 *M* phosphate buffer, 600 mos*M*.

Increased contrast was achieved by poststaining the sections with uranyl acetate and lead hydroxide (for further details see Cuénod *et al.*[3]). Additional studies with acrolein (perfusion with 5% (1200 mos*M*) and postfixation with 10%) were also performed. Similarly dissected tissue blocks of 9 animals were used for the

impregnation with zinc iodide–osmium tetroxide[1] (ZIO) according to the method of Kawana *et al.*[6] (1500 mos*M*). Osmium tetroxide fixation was carried out by removing small pieces from the superficial tectal layers *in situ* while the animal was anesthetized.

Freeze etching was performed in paraformaldehyde perfused and glutaraldehyde postfixed (4 animals) as well as in unfixed (2 animals) specimens, which were removed *in situ* during anesthesia. These were treated with 25% glycerol prior to being instantaneously frozen in liquid freon and processed according to the method of Moor and Mühlethaler[9]. Control sections for all procedures were obtained from 2 normal pigeons and from the ipsilateral tectum of the operated animals.

RESULTS

Aldehyde fixation

The presence of large vesicles in nerve terminals as described in detail by Cuénod *et al.*[3] was fully confirmed in this study. A similarly clear-cut increase in vesicular diameter was seen in specimens which were treated with acrolein (Fig. 1).

Osmium tetroxide fixation

Careful examination of optic nerve endings and 'en passant' boutons in specimens which were directly placed into the 2% buffered osmium tetroxide solution revealed the presence of vesicles having a diameter of up to 100 nm. On the 7th postoperative day, the vesicular alteration was also combined with filamentous hyperplasia and increased opacity of the cytoplasmic matrix (Fig. 1).

Zinc iodide–osmium tetroxide impregnation

Large vesicles in degenerating nerve terminals were predominantly ZIO-positive. The earliest signs of enlargement appeared 6 h after the operation. Clear-cut results were seen after 12 h. Animals with 9, 14, and 17 days of survival showed the vesicular enlargement combined with the fibrillary reaction and darkening of the cytoplasm. The largest ZIO-positive vesicles measured 100–140 nm in diameter (Figs. 2 and 3). The profiles of enlarged vesicles were either solidly black or consisted of a ZIO-positive layer subjacent to the vesicular membrane which gave a ring-like appearance. The center of these 'rings' was almost invariably filled with a ZIO-negative, finely granulated material. This argues against the possibility that the center of these vesicles was removed during the sectioning procedure.

ZIO-positive vesicles were seen as long as 17 days postoperatively (longest observation time in this series); they did not differ significantly from the appearance of earlier stages.

In 2 out of 10 cases, only the enlarged vesicles of degenerated terminals reacted with the ZIO reagent. All normal axons and endings remained mostly ZIO-negative

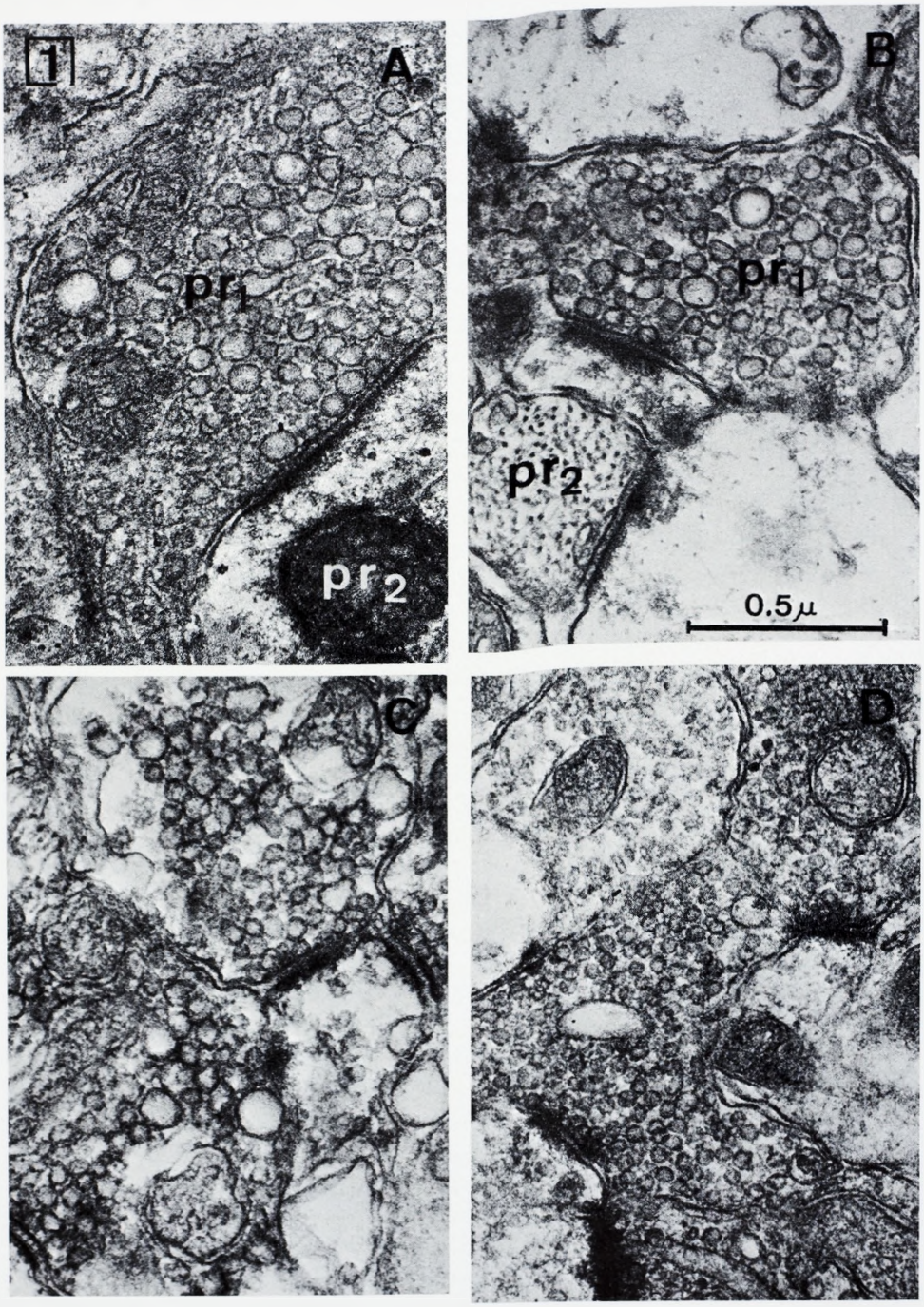

Fig. 1. Effect of aldehyde fixation. A, P, G, OsO_4 fixation, 17 days postoperatively. Large profile of degenerating optic nerve terminal (pr_1) contains many enlarged vesicles. Small profile of nerve ending (pr_2) is in an advanced stage of degeneration with dark cytoplasm. B, Acrolein, OsO_4 fixation, 11 days postoperatively. Large profile of degenerating optic nerve terminal (pr_1) contains enlarged synaptic vesicles. Small profile of degenerating nerve terminal (pr_2) is filled with neurofilaments. C, OsO_4 (veronal acetate buffer) fixation, 7 days postoperatively. Note the enlarged synaptic vesicles. D, P, G, OsO_4 fixation, normal optic nerve ending in ipsilateral tectum, 17 days postoperatively. All 4 electron micrographs are at the same magnification (primary magnification: × 40,000).

(Fig. 3). The remaining 8 cases showed about as many ZIO-positive synaptic vesicles in degenerated as in normal endings. The 2 cases with selective ZIO staining of vesicles in degenerated terminals had been treated with 5% OsO_4 (Martin *et al.*[8]), while a 2% solution had been applied to the other cases.

Freeze etching of degenerating nerve terminals

Normal nerve terminals are characterized by the presence of densely packed spheric profiles, which are approx. 30–50 nm in diameter (Moor *et al.*[10]) and have either concave or convex surfaces[2]. These spheric profiles represent synaptic vesicles. It should be emphasized that the range of vesicular diameters in normal endings is relatively narrow.

Degenerating nerve terminals in the superficial tectal layers are characterized by the occurrence of vesicles with a diameter exceeding the normal range (Fig. 4). These enlarged vesicles are grouped together and typically contain one single or very few particles (8–10 nm) on the smoothly textured inner surface (as noted in normal synaptic vesicles). Profiles of mitochondria, lysosomes and neurosecretory granules differ with respect to one or both of these criteria. Of considerable importance is the fact that fixed and unfixed specimens fail to differ markedly with respect to the vesicular alteration. Diameters of vesicles were measured in aldehyde-prefixed terminals to be 35–85 nm and in unfixed terminals to be 37.5–90 nm on the 8th–12th postoperative day. Degenerating terminals with large clusters of regularly spaced neurofibrils are occasionally seen (Fig. 4). It seems noteworthy that the cytoplasmic matrix surrounding the fibrillary bundles seems relatively homogeneous and smooth in contrast to the relatively coarse meshwork of normal axoplasm. It is suggested that the 'smooth areas' represent 'dark' cytoplasm in conventional electron micrographs of advanced terminal degeneration. In this context Gray and Hamlyn[5] stated that (at the 6–7 day stage) dense patches of cytoplasm could be seen within the groups of neurofilaments and this may be precisely what Fig. 4 illustrates.

DISCUSSION

The data obtained in this study are consistent with the report by Cuénod *et al.*[3] and confirm the existence, in degenerating nerve terminals, of vesicles having a larger diameter than the normal synaptic vesicles. Kawana *et al.*[7] have shown that vesicular enlargement is not a special feature of the pigeon retino-tectal system but occurs in the degenerating cortico-caudate system of the rat as early as 5 h postoperatively. The present study shows that aldehyde fixation of nerve tissue plays no essential role in this phenomenon since the enlargement is seen with pure OsO_4 treatment and even when no fixatives were used as in some of the freeze-etched preparations. Thus, the presence of large vesicles is more likely to be a real phenomenon rather than of artefactual origin. Gray and Hamlyn[5] studied the chicken retino-tectal system as early as 1962; they were first to describe fibrillary hyperplasia in degenerating boutons, an observation which has been confirmed by many subsequent investigators and

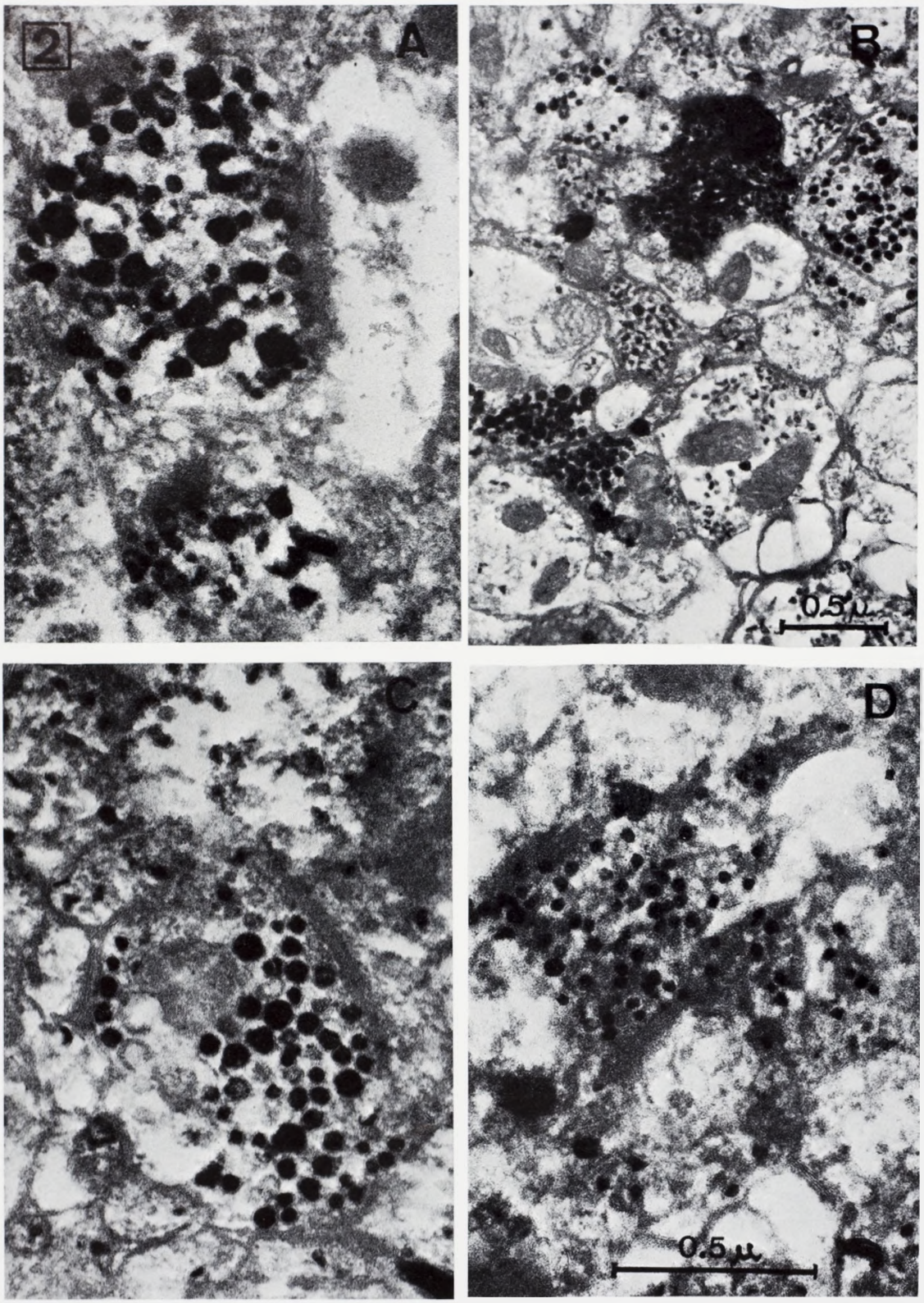

Fig. 2. Effect of zinc iodide–osmium tetroxide (ZIO) impregnation after P, G fixation. A, Degenerating optic nerve terminals, 14 days postoperatively. Two endings with enlarged synaptic vesicles with ZIO-positive reaction. B, Normal and degenerating nerve terminals in the superficial tectal layers, 14 days postoperatively. Primary magnification: × 20,000. Four endings contain enlarged ZIO-positive vesicles. The remaining boutons contain ZIO-positive vesicles of normal size (S- and F-type). One ending is in a more advanced stage of degeneration with dark cytoplasm. C, Normal and degenerating optic nerve terminals, 14 days postoperatively. Most of them are ZIO-positive. D, Normal optic nerve terminal in the ipsilateral tectum, 14 days postoperatively. Primary magnification of A, C and D: × 40,000.

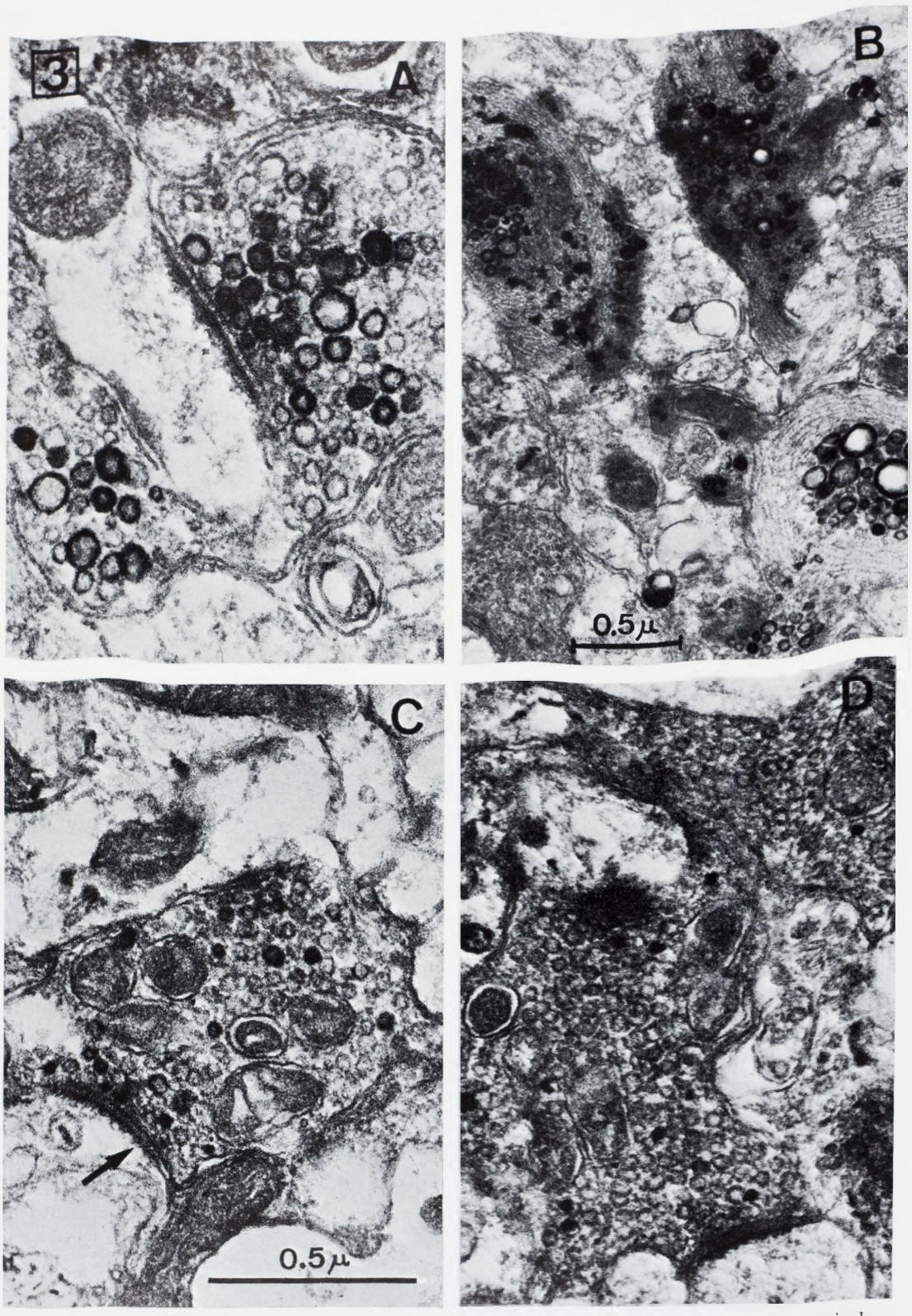

Fig. 3. Selective ZIO reaction in degenerating optic nerve terminals. A, 17 days postoperatively. Two endings contain ring-shaped, enlarged synaptic vesicles with positive ZIO reaction. Note that only about half of the vesicular profiles are ZIO-positive. Primary magnification: × 40,000. B, 17 days postoperatively degenerating nerve terminals with ZIO-positive reaction as in A. Note normal nerve terminal (lower left corner) with ZIO-negative S-type vesicles. Primary magnification: × 20,000. Endings are in different stages of degeneration showing electronopaque cytoplasm and neurofibrillary hyperplasia. C, Degenerating optic nerve terminal, 6 h postoperatively. Primary magnification: × 40,000. Note the clear-cut enlargement of synaptic vesicles with ring-like ZIO-positive precipitation. Arrow points at synapse. D, Normal control from the ipsilateral tectum, 6 h postoperatively. Primary magnification: × 40,000. Only a few synaptic vesicles have ZIO-positive reaction. The vast majority remains ZIO-negative.

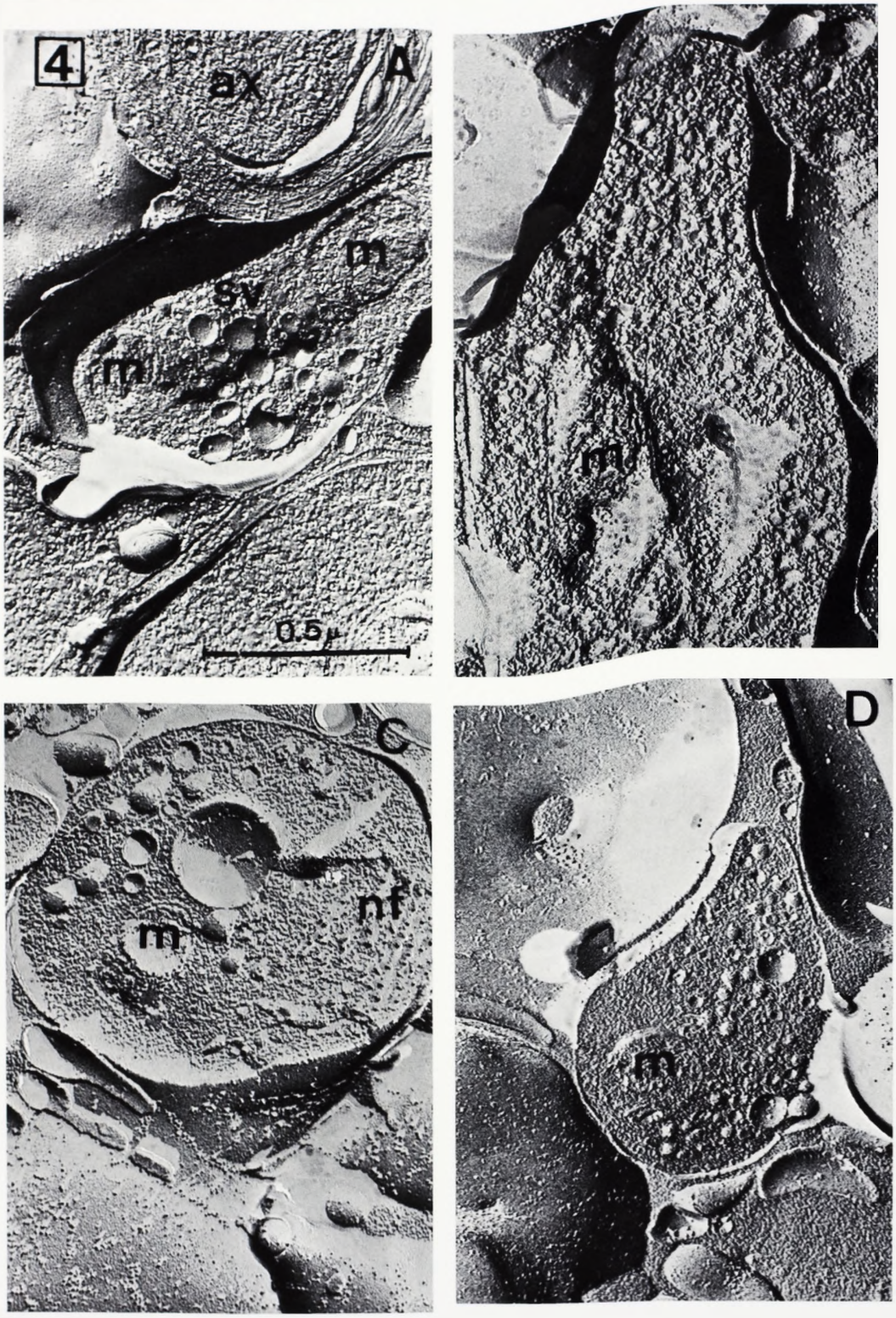

Fig. 4. Freeze-etched preparations of normal and degenerating optic nerve terminals. A, P, G fixation, 12 days postoperatively. Note the enlarged synaptic vesicles. Concave profiles with smooth surface and one particle. Ax — myelinated axon, m — mitochondrion, sv — synaptic vesicles. B, Normal control with the same fixation and magnification. C, No aldehyde fixation. Nerve terminal with neurofibrillary hyperplasia (nf) and enlarged synaptic vesicles, 8 days postoperatively. Axoplasm between fibrils is homogeneous. D, Normal control from ipsilateral tectum, 8 days postoperatively. No aldehyde fixation, same magnification as in C.

again by this study. Why did they miss the increase in vesicular size? Conceivably, the enlargement is less evident in OsO_4–PTA-treated neuropil. It is noteworthy, however, that osmium fixation alone does reveal the vesicular alteration as shown in this work.

The present data support the suggestion that the large vesicles observed during degeneration are of the same nature as the normal synaptic vesicles: not only with classical staining does their unit membrane appear identical but their characteristics in freeze-etched preparations are very similar, particularly the inner surface. Furthermore, they both react positively to ZIO. Finally, their localization frequently in direct contact with the presynaptic membrane (Figs. 1–3) is another feature which they share together. Consequently, it seems likely that the large vesicles are abnormal synaptic vesicles, although it is not possible to say which mechanism underlies this vesicular alteration: it could be related either to a malformation of new vesicles or to a transformation of already present vesicles. It remains, however, possible that they belong to a different vesicular system, like for instance, the one present in the growth cone of axons and dendrites[4].

The ZIO reaction of degenerating nerve terminals has been studied by Nickel and Waser[11] in the motor endplates of the rat diaphragm. It was shown that the vesicles may disintegrate after only a few hours and a granular ZIO-positive material remains in the terminals. This is in contrast to the present findings, where ZIO-positive vesicles remain visible for as long as 17 days postoperatively. The discrepancy may have two explanations: (a) the method of tissue preservation used by Nickel and Waser was different; (b) the time course of degeneration in motor endplates was considerably faster.

In two cases, we succeeded in staining degenerating optic nerve terminals selectively by applying a slightly modified ZIO reagent. S- and F-type vesicles in normal endings remained ZIO-negative (with few exceptions), while the enlarged vesicles of degenerating boutons showed predominantly ZIO-positive reactions. This differential behavior may reflect a higher affinity of degenerating vesicles to the ZIO compound as compared with the normal ones. More may be said about this phenomenon when the nature of the ZIO reaction is elucidated at the cytochemical level.

The significance of the present findings is 2-fold: (a) the enlargement of synaptic vesicles represents a reliable and consistent sign of Wallerian degeneration, which is not essentially dependent upon fixation methods; (b) the phenomenon may serve as an indicator of heretofore unknown biochemical changes which occur very early after separating the nerve terminals from the perikaryon. This might be useful in future investigations on the so-called 'trophic' functions of the nerve cell.

SUMMARY

The terminal degeneration of retino-tectal fibers was investigated electron microscopically in the pigeon. Continuing the research of Cuénod and coworkers, the effects of various technical procedures upon the configuration of synaptic vesicles during the initial phases of Wallerian degeneration were studied. The presence of

large vesicles in the degenerating nerve terminals was shown to be independent of the method of fixation: paraformaldehyde, glutaraldehyde and acrolein with subsequent osmication gave essentially similar results to those obtained with osmic acid fixation alone. Larger vesicles were also observed in the freeze-etched preparations of the degenerating optic nerve terminals: there was no essential difference between aldehyde-fixed and unfixed specimens with respect to the enlarged size of vesicles. Enlarged vesicles of degenerating endings turned out to be ZIO (zinc iodide–osmium tetroxide)-positive. In 2 cases, we succeeded to obtain selective vesicle staining in degenerated boutons by slightly modifying the ZIO technique.

ACKNOWLEDGEMENTS

The technical assistance of Miss C. Berger, Miss C. Sandri and Mr. A. Fäh is gratefully acknowledged.

This research was supported by Grants No. 3.133.69, 3.133.34 and 4.691.3 of the Swiss National Foundation for Scientific Research.

REFERENCES

1 AKERT, K., AND SANDRI, C., An electronmicroscopic study of zinc iodide–osmium impregnation of neurons. I. Staining of synaptic vesicles at cholinergic junctions, *Brain Research*, 7 (1968) 286–295.
2 AKERT, K., MOOR, H., PFENNINGER, K., AND SANDRI, C., Contribution of new impregnation methods and freeze etching to the problem of synaptic fine structure. In K. AKERT AND P. G. WASER (Eds.), *Mechanisms of Synaptic Transmission, Progress in Brain Research, Vol. 31*, Elsevier, Amsterdam, 1969, pp. 223–240.
3 CUÉNOD, M., SANDRI, C., AND AKERT, K., Enlarged synaptic vesicles as an early sign of secondary degeneration in the optic nerve terminals of the pigeon, *J. Cell Sci.*, 6 (1970) 605–613.
4 DEL CERRO, M. P., AND SNIDER, R. S., Studies on the developing cerebellum. Ultrastructure of the growth cones, *J. comp. Neurol.*, 133 (1968) 341–362.
5 GRAY, E. G., AND HAMLYN, L. H., Electron microscopy of experimental degeneration in the avian optic tectum, *J. Anat. (Lond.)*, 96 (1962) 309–316.
6 KAWANA, E., AKERT, K., AND SANDRI, C., Zinc iodide–osmium tetroxide impregnation of nerve terminals in the spinal cord, *Brain Research*, 16 (1969) 325–331.
7 KAWANA, E., AKERT, K., AND BRUPPACHER, H., Enlargement of synaptic vesicles as an early sign of terminal degeneration in the rat caudate nucleus, *J. comp. Neurol.*, in press.
8 MARTIN, R., BARLOW, J., AND MIRALTO, A., Application of the zinc iodide–osmium tetroxide impregnation of synaptic vesicles in cephalopod nerves, *Brain Research*, 15 (1969) 1–16.
9 MOOR, H., AND MÜHLETHALER, K., Fine structure in frozen-etched yeast cells, *J. Cell Biol.*, 17 (1963) 609–628.
10 MOOR, H., PFENNINGER, K., AND AKERT, K., Synaptic vesicles in electronmicrographs of freeze-etched nerve terminals, *Science*, 164 (1969) 1405–1407.
11 NICKEL, E., AND WASER, P. G., An electron microscopic study of degenerated motor endplates after zinc iodide–osmium impregnation, *Brain Research*, 13 (1969) 168–176.