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Significance of the maillet method (ZIO) for cytochemical studies of subcellular structures

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Summary. The present knowledge on the methodology, specificity, localization, cytochemical significance and experimental variations of the zinc iodide-osmium tetroxide (ZIO) staining are reviewed. Some new findings supporting the concept that ZIO reacts with -SH groups in some structures under specific conditions are also communicated. In this respect the effect of in vitro pretreatment with -SH reagents on ZIO staining, already described in different kinds of synaptic vesicles, was studied in rod photoreceptor outer segments. It was observed that the intra- and extradiskal electron opaque deposits, present in untreated rod outer segments directly stained with ZIO at 4 °C for 2 h, were enhanced by previous incubation with dithioerythrytol (DTE), which protects -SH groups and reduce -S-S-bridges; when N-ethyl-maleimide (NEM), a -SH blocking agent was used directly or after DTE, the electron opaque deposits were considerably reduced or abolished. Furthermore the influence of previous incubation with cysteine on ZIO staining was studied in the rat pineal gland. In the controls ZIO made at 4 °C for 2 h, reacts strongly with the matrix of the small granulated synaptic vesicles in the pineal nerves; no electron opaque deposits are seen in the membranes of the vacuolar system. Previous incubation with cysteine 5 m*M* in phosphate buffer makes negative ZIO reaction in the matrix of the synaptic vesicles while with a concentration of 50 m*M* cysteine, a partial recovery is observed. In both concentrations the membranes of the vacuolar system appeared covered by patchy electron opaque deposits.

In 1959 MAILLET[1] introduced the zinc iodide-osmium tetroxide (ZIO) mixture as a stain for the demonstration of autonomic nerves at the light microscopic level. Many structures were also found to be stained by the mixture. The interest in studying the mechanism involved in the staining reaction received a great impulse following its introduction to electron microscopy[2], specially after the work of AKERT and SANDRI[3] demonstrating the staining of cholinergic synaptic vesicles with ZIO. This confirmed that ZIO, apart from staining membranous structures as conventional fixatives do, also results in electron opaque precipitates which were restricted to some cells and organelles. These initial observations prompted a thorough study on the nature of the possible substances reacting with ZIO, the influences exerted by the conditions in which the staining was carried out and the modifications produced by pharmacological and experimental manipulations. Thus, in recent years much informations has accumulated on these problems and it is the purpose of this review to summarize the present knowledge on the methodological aspects of the ZIO procedure as well as on the localization, significance and experimental modifications of the material stained.

Remarks on methodology

ZIO mixture is a modification introduced by MAILLET to the CHAMPY reagent[4] originally prepared with osmium tetroxide and potassium iodide. MAILLET[5] found that the use of zinc iodide increased the sensitivity and reproducibility of the staining in com-

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parison with potassium, sodium, calcium and cadmiun iodides. Furthermore he observed that trivalent iodides (arsenic, tin, antimony iodides) were inadequate for staining, because they form an unstable complex which precipitates giving osmium dioxide. On the other hand, the mixture of osmium tetroxide with bromides, chlorides and fluorides gives similar results to diluted osmium tetroxide, indicating that the presence of the iodine ion is required for obtaining the specific staining.

ZIO mixture has proved to be useful for revealing many subcellular structures. These structures do not show the same sensitivity toward ZIO at a given schedule of fixation. Important parameters involved in this differential sensitivity are: temperature[5–8], pH[9–12], duration of impregnation [5,8,12], the presence of certain ions as Ca, Mg[10] and phosphates[13], the previous fixation with aldehydes or other fixatives[14–17], the use of the mixture diluted[10,12,14,18] or not[5,3,19] in different buffers.

Experimental conditions such as light[20,21] or the use of anesthesia[12] also influence the reaction. According to different authors[12,14] the impregnation of tissue blocks represents a handicap, because the superficial layer is bleached when the reaction products are extracted by alcohol during dehydration, while the deeper layers are almost unaffected.

These facts and the circumstance that each author follows a different procedure when applying ZIO staining, makes it difficult to compare strictly the results published by different laboratories. However some general conclusions can be formulated.

When ZIO mixture is used directly, tissue preservation is not as good as with other varieties of the staining procedure, although the general structure of the tissues and organs can be recognized. Some structures like synaptic boutons and outer segments of photoreceptor cells are relatively more resistent than other structures to the deleterious effect of the fixative. Prefixation in aldehydes and the use of hyperosmolar buffer solutions improve the preservation of the tissues[12,14,18]. However, the different results obtained in monoaminergic vesicles using the mixture directly[19] or after prefixation in aldehydes[22] and other findings, like the different effects of -SH reagents on ZIO reaction in both conditions[23], indicate that these procedures are not identical and may have different applications. Furthermore, prefixation in glutaraldehyde reduces the affinity of the ZIO stain for some structures and increases its affinity for others[12,16,17,24]. We have observed that the duration of washing after aldehyde prefixation modifies the ZIO affinity for some structures (unpublished). When the impregnation is made at 4 °C[14,19] artifactual precipitates, commonly present when the impregnation is made a room temperature as described by MAILLET[5], are not observed; on the other hand, the use of higher temperatures (20, 40, 60 °C) led to the stain of a greater number of structures[5–8]. There is agreement in that the pH of the reagent is of critical importance, but the optimal pH has been differently estimated by the authors[9,10–12]; however they applied different varieties of the technique to diverse structures. The optimal time of impregnation has also been differently appretiated[5,12,14,24,25], but perhaps, as pH, it is related to the other parameters of the staining procedure and the structure studied.

ZIO mixture can be prepared using the zinc iodide salt or preparing the zinc iodide with zinc powder and resublimated iodine[26]. The solution can be preserved at 4 °C in the dark over a period of several months[12]. According to our experience, the pH of the solution increases with time when kept at room temperature.

Specificity of the ZIO staining

a) Chemical reacting with ZIO. Test-tube assays show that many different substances react with ZIO giving a black precipitate[5,20,27]. Amongst the more reactive substances were catechol and indoleamines, their precursors and structurally related substances, amino acids with -SH, -S-S- or dicarboxylic groups. Reduced gluthation reacts considerably faster than oxidized gluthation. Retinol but not retinal reacts intensively with the ZIO mixture. Similar but slower reactions were observed with the mixture of potassium iodide-osmium tetroxide. It is interesting that cysteine, methionine and aspartic acids, as well as reduced and oxidized gluthation, produce a colored reaction with

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osmium tetroxide alone but do not give a precipitate as observed with the CHAMPY-MAILLET mixtures[20]. Also aldehydes reduce ZIO mixture without affecting osmium tetroxide alone[5]. As is well known, catechol and indoleamines give an intensely black precipitate with osmium tetroxide. It can be mentioned in this context that phospholipids isolated from rat pineal gland and retina by thin layer chromatography strongly reduce ZIO in silica gel plates, while cholesterol does not react[28].

b) Structures stained by ZIO. The structures stained by ZIO as observed with the light microscope have been enumerated by MAILLET[5]. One of the main applications of this technique in light microscopy was the demonstration of peripheral nerve fibres and endings which appear totally stained, in a similar way to that obtained with methylene blue[5,26,29–32]. Although some histological observations and in vitro reactions suggested initially that adrenergic autonomic nerve fibres and endings could be specifically revealed by this mixture, it was soon demonstrated that cholinergic preganglionic fibres[26] and sensitive nerve structures (see[5,33]) were also stained. ZIO impregnates the plexiform layers of the retina and granular structures in the neurons of the superior cervical ganglion cells[5]. Furthermore, many other cells not related to nervous tissue were partially or totally stained by ZIO, including epithelial, glandular and connective cells. Chromaffin and intestinal cells were also reactive. MAILLET has mentioned other reactive loci to ZIO like phenolic inclusions in some plants and different inclusions in the venon glands of the toad.

Electron microscopic studies not only confirmed that many tissues react with ZIO but defined to a greater extent the cytological localization of the specific electron opaque deposits. Although non-artifactual granular deposits may be seen in the cytoplasm of some structures[6,34], in most cases they are localized in organelles like synaptic vesicles[3,10,12–16,19,34–41], the vacuolar system[7,10,11,22,42], lysosomes[19], mitochondria[10,15,16,42], multivesicular bodies[13], coated vesicles[10], cytoplasmic vesicles[10], synaptic bar vesicles[10,20], synaptoid vesicles in the rat neurohypophysis[43] and in the several types of nerve endings of the median eminence of different species[44] or connected with membranes like in the myelin sheath[10], in the outer segments of the photoreceptor cells[7,15,17], in the lamellar bodies of type II pneumonocytes[45] and in the central periodic lamellas of the Langerhans cell granules[46].

In summary, different subcellular structures may be stained by ZIO and various substances or reactive groups could be responsible for the presence of the selectively localized precipitates. These concepts, which were already outlined in the publications of MAILLET, have been confirmed and extended by other authors using different methodological modifications.

Staining of the synaptic region

According to AKERT and SANDRI[12], the affinity for ZIO shown by synaptic vesicles, makes this method 'the most prominent approach to the development of a specific synaptic stain when a proper methodology is applied', taking into consideration that synaptic vesicles are the most characteristic component of nerve endings (Figures 1–4). The impregnation of different kinds of synaptic vesicles has been demonstrated by many authors[3,10,12–16,34–41] (Figures 3 and 4). Negative vesicles have also been described. The problem of ZIO positive and ZIO negative synaptic vesicles has been extensively reviewed by AKERT et al.[12,22], and its significance is not yet clearly understood. It has been demonstrated that in many cases the variations in the stainability depend on the methodological conditions[6,8,47]. This variability, which could be specific for each type of synaptic vesicle, may be related to the chemical organization or to the functional state of the vesicles. It has also been suggested that more than one substance may be responsible of ZIO staining in synaptic vesicles[48].

Studies made in our laboratory[19,27] have demonstrated that in small monoaminergic synaptic vesicles (Figure 4), ZIO applied directly reveals two compartments: the core and the matrix more intensively stained than the core and comprizing the space between the core and the vesicle membrane. The correlation of ZIO reaction in the pineal nerves with the noradrenaline and serotonin content of the pineal gland after different pharmacological treatments[27,49] indicates that neither noradrenaline nor serotonin are

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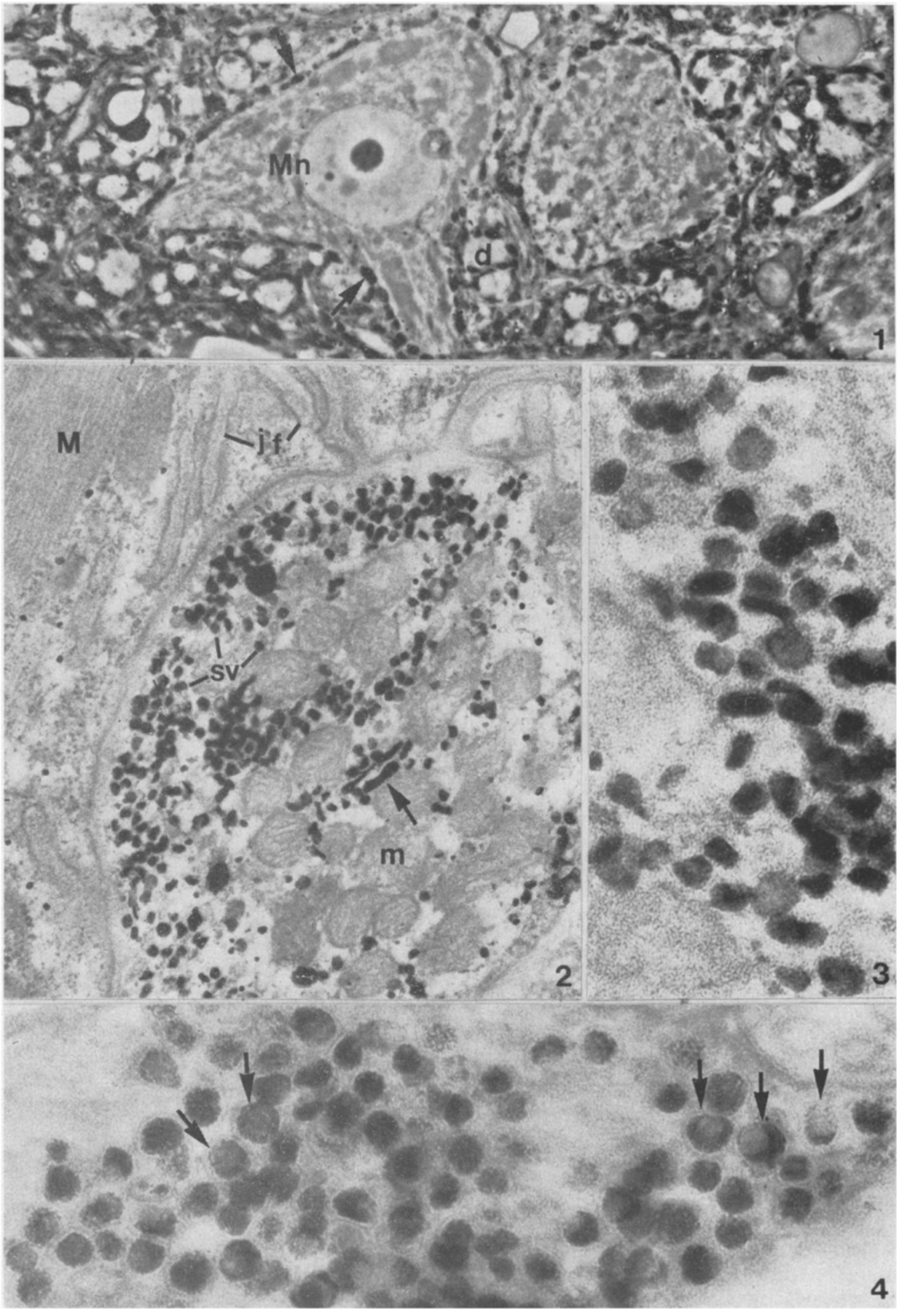
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responsible for ZIO reaction in the matrix of these vesicles, although many drugs interfering with storage, uptake, release and synthesis of monoamines may affect ZIO staining in this compartment[19,27,48,50–54]. Likewise ZIO may contribute to the definition of two compartments in the granulated vesicles of the adrenal medulla[8]. These findings indicate that ZIO staining can be useful to investigate the function of a non-aminergic compartment in synaptic vesicles containing biogenic amines. A similar compartmentation has not been described in other ZIO reactive synaptic vesicles.

A lack of correlation between ZIO positivity of cholinergic terminals and the cholinergic transmission in the sympathetic ganglia of the cat has been reported by PÁRDUCZ et al.[55]. The authors found that hemicholinium pretreatment, with or without preganglionic stimulation, did not cause any significant changes in ZIO positivity of cholinergic nerve terminals after aldehyde perfusion. This result could indicate that also in cholinergic vesicles ZIO reaction does not reveal the neurotransmitter but other vesicular component. According to AKERT and SANDRI[12], if the two compartments exist in cholinergic vesicles as well, they may not be readily differentiated by means of ZIO method because of the well known affinity of acetylcholine for iodine compounds[56].

An interesting difference has been described by HALÁZ et al.[47] in excitatory and inhibitory synapses in the olfactory bulb and the cerebellum prefixed in glutaraldehyde. These authors found that most of the ovoid vesicles of the inhibitory synapses were ZIO positive whereas the spherical vesicles of the excitatory synapses were predominantly ZIO negative.

A negative ZIO reaction was noted consistently in dense cored synaptic vesicles of 1200–2000 Å present in monoaminergic and cholinergic nerve endings[3,19,22,34–36,57–58]. However, in monoaminergic nerve endings, ZIO positive vesicles of about 1000 Å can be found when the impregnation is made at 20°C for 15 h.

The mixture of sodium iodide[59] and potassium and cadmium iodides[9,52] have also been applied in electron microscopy to stain synaptic vesicles. Some of these studies[9,52] suggest that different iodides are not identical in their staining properties.

In conclusion, it is well demonstrated that ZIO stains different types of synaptic vesicles, although the nature of the reactive substances has not been identified. At least in vesicles containing monoamines, it seems well established that the neurotransmitter is not involved in the reaction and there is some evidence indicating that this might be also the case in cholinergic vesicles. Moreover, vesicles present in nerve terminals, probably containing other neurotransmitters, also react with ZIO but in this case there is no information concerning the possible participation of the transmitter in the reaction. However, since ZIO reacts in vitro with many different substances, the nature of the component or components stained in the synaptic vesicles must be carefully investigated in each particular situation. Other components of the synaptic region may be stained with the ZIO mixture. Tubular elements which may represent the continuation of axonal smooth endoplasmic reticulum and could have a role in the origin of synaptic vesicles, are likewise impregnated[9,17,22,38]. Furthermore ZIO stain has helped considerably in establishing the concept of a presynaptic grid[60–62] as a marker of the vesicleplasmalemma relationships and the 'recycling' of synaptic vesicles[12].

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Fig. 1. Motoneurons, rat spinal cord. Perikarya and dendritic profiles are surronded by synaptic boutons (arrows) darkly stained by ZIO. Mn, motoneuron; d, dendrite. Primary magnification approximately × 900 (Courtesy of K. AKERT and C. SANDRI).

Fig. 2. Cholinergic synaptic vesicles (sv) stained by ZIO (motor end plate, rat diaphragm). ZIO positive tubular profiles may be identified as smooth endoplasmic reticulum (arrow). m, mitochondria; jf, junctional folds; M, muscle fibre. × 20,000. (Courtesy of K. AKERT and C. SANDRI.)

Fig. 3. The same as Figure 2 at a larger magnification (× 120,000). The staining of the vesicles is homogeneous (compare with Figure 4). (Courtesy of K. AKERT and C. SANDRI.) × 120,000.

Fig. 4. Monoaminergic synaptic vesicles stained by ZIO (rat pineal nerves). In many vesicles a pale core, in contact with the vesicle membrane, is seen (arrows). The matrix comprizing the space between the core and the vesicle membrane appears as a crescent densely stained. Compare with Figure 3. × 120,000.

Staining of the vacuolar system

The staining of the Golgi complex with ZIO was well established by light microsco pists[5]. The reactivity of the other components of the vacuolar system has been described with electron microscopy[6,10,22,63]. This technique has been applied to the study of the relatioships between synaptic vesicles, Golgi apparatus and smooth endoplasmic reticulum[38].

The ZIO reactivity of the vacuolar system is variable in different cell types and in some cases may be absent. In ZIO positive cells, ZIO reaction is temperature- and time-dependent as shown in photoreceptor cells[7]. Similar observations have been made in our laboratory in pineal cells (unpublished).

ZIO electron opaque deposits are located inside the cisterns, attached to the membranes, giving them a scalloped profile. In frontal view, they appear as patches of a variable diameter which can be isolated or fused together. MADARÁSZ and HÁMORI[64] have described ZIO positive globular subunits of 300–350 Å in the rough endoplasmic of neurons, which rapidly lost their affinity after denervation. ZIO positivity of the Golgi apparatus and synaptic vesicles has been correlated with the observation that both these structures contain thiamine pyrophosphatase[24].

Rod outer segments

Electron opaque deposits are present all along the rod photoreceptor cells in different species[7,15,20], although they are mainly concentrated in the outer segment, where they are located in the intra- and interdiskal spaces as well as between the plasma and disk membranes. These deposits are unevenly distributed along the outer segments, being more concentrated at their tip, as well as between the outer segments of different cells. As in rods, in cone photoreceptor cells electron opaque deposits were observed in synaptic vesicles and in the inner segments; in the outer segments, only the membranes were revealed in most of the cases[7,20]. However, a small reaction could be observed in some intradiskal spaces in certain conditions. Positive vacuoles were seen in rods and cones at the base or at the border of outer segments[65].

It has been shown that ZIO reactive material of rod outer segments is influenced by light. It is more abundant in adult rats exposed to light for 96 h than after 96 h of darkness[20]. Briefer periods of light after dark adaptation also produced a greater reaction than dark adaptation in rod outer segments of *Bufo arenarum* Hensel[21]. In both cases, extradiskal deposits predominate in rod outer segments exposed to light. These changes are better observed when the reaction is done at 4°C than at 20°C or at 60°C. A positive reaction has also been reported in the photoreceptive cells of the pike pineal organ[16].

Applications in experimental pathology

The ZIO method has been applied to the study of wallerian degeneration. In the central nervous system, AKERT et al.[12,66–68] found that many of the enlarged synaptic vesicles, which may represent an initial sign of synaptic degeneration[69,70], are peripherally reactive the center being filled with a ZIO negative, finely granulated material in nerve endings while synaptic

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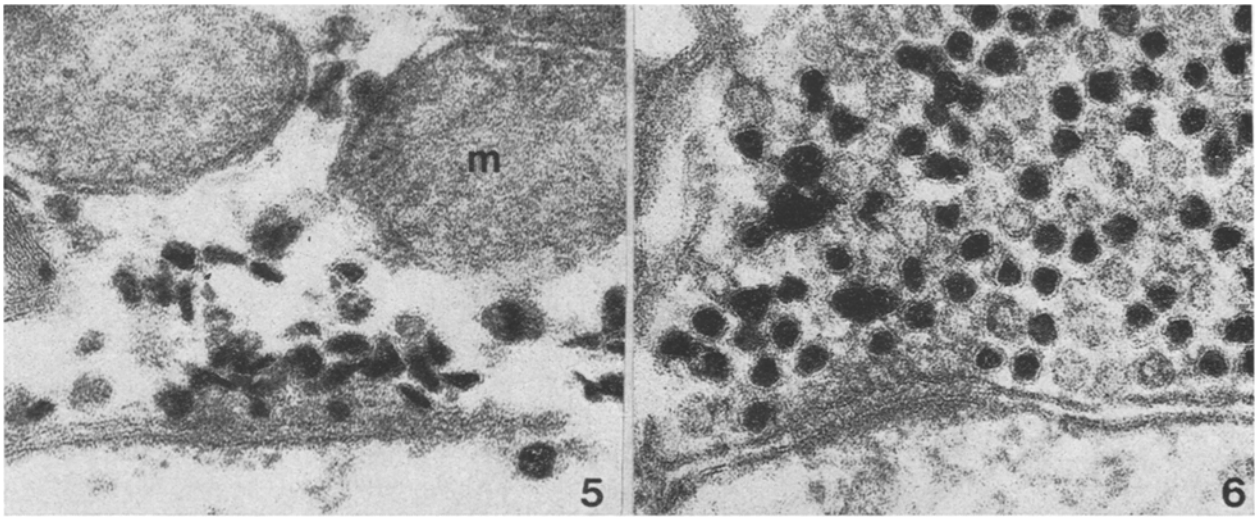

Figs. 5 and 6. Rat spinal cord ventral gray. Relationship between synaptic vesicles and presynaptic membrane in anesthetized (nembutal 40 mg/kg) (Figure 5) and unanesthetized (Figure 6) animals. Note the absence of ZIO positive vesicles from the immediate vicinity of the presynaptic membrane in the waking state (× 120,000). (Courtesy of K. AKERT and C. SANDRI.)

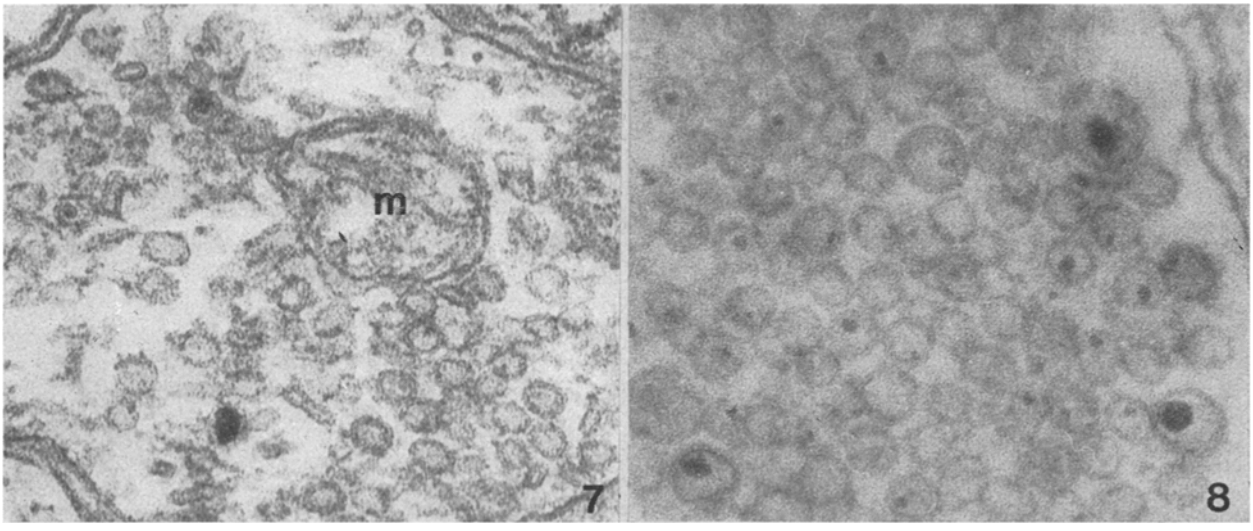

Fig. 7. Rat pineal nerves 2.5 h after the administration of reserpine (5 mg/kg, i.p.). ZIO staining made at 4°C is negative in most of the vesicles. Compare with Figure 4. × 120,000.

Fig. 8. Rat pineal nerves after 10 doses of tyramine (50 mg/kg, i.p.) at 15 min intervals. ZIO was made at 4°C. The matrix of the synaptic vesicles is ZIO negative. A small pale core is seen in many vesicles and a larger and denser one appears in a few of them. × 120,000.

vesicles normally appear clear or totally stained. SNYDER[71] has made similar attempts in this direction. His results seem to indicate that it will be possible selectively to impregnate degenerating boutons by changing the pH of the mixture. In denervated motor end plates, NICKEL and WASSER[72] observed a dense staining of the whole nerve internal structure 7 h after the axotomy only leaving the membranes of the synaptic vesicles and mitochondria free. In normal end plates, the staining is restricted to the synaptic vesicles[3].

After continuous illumination of the retina of adult and immature rats[73–75], rod disks are transformed into tubular and vesicular structures. Intra- and extradiskal spaces are ZIO reactive at 4, 20 and 60°C in controls maintained in rhythmic variations of darkness and light[7,15,20]. Following transformation into tubular and vesicular structures, both spaces are ZIO negative at 4°C. At 20 and 60°C, ZIO reaction is only positive inside the tubular and vesicular structures[76]. Thus ZIO shows a difference between both compartments in rod outer segments after continuous illumination which is not revealed by current fixation methods.

Pharmacological studies

ZIO reaction in synaptic vesicles is influenced by different drugs used in clinical or experimental pharmacology.

In the synaptic vesicles of the spinal cord ventral gray, a differential ZIO affinity of the synaptic vesicles was found between the rats anesthetized with sodium pentobarbital and those unanesthetized. The majority of the vesicles trapped within the presynaptic grid are ZIO positive in the former (Figure 5) and ZIO negative in the latter[12] (Figure 6).

In the monoaminergic vesicles of the rat pineal gland, the effect of the administration of drugs which act on the biogenic amine stores, has been explored with ZIO reaction at different temperatures. It was observed that the administration of reserpine led to a negative ZIO reaction (Figure 7)[19,27,48]. Tyramine hinders ZIO reaction at 4°C[53] but has little effect on ZIO made at 20 and 60°C[49]. Similar results has been obtained with *p*-chlorophenylalanine[48,52] a drug which inhibits tryptophan hydroxylase activity and the uptake of 5-hydroxytryptophan[77]. When disulfiram, an inhibitor of dopamine-*β*-hydroxylase, or *α*-methyl-*p*-tyrosine, which inhibits tyrosine hydroxylase, are administered, little or not effect is observed on ZIO reaction at 4, 20 and 60°C. However, if tyramine is given simultaneously, ZIO reaction is almost totally negative at the three temperatures studies[50,54]. *α*-methyl DOPA, which inhibits DOPA decarboxylase, completely abolishes ZIO reaction made at 4°C whereas the effect is partial at 20°C and negative at 60°C. When tyramine is added to the treatment, ZIO is totally negative at 20°C and partially affected at 60°C[51]. Oxipertine also abolishes ZIO reaction at 4°C[27]. It is interesting to correlate the influence of these drugs on ZIO electron opaque deposits in the vesicles with the fact that the deposits are not determined by

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the presence of catechol or indoleamines (see staining of the synaptic region, and cytochemical significance of the ZIO reaction).

Cytochemical significance of the ZIO reaction

The distribution of Zn, Os and I in stained zones of histological preparations has been investigated by electron probe mycroanalysis to determine their participation in the electron opaque deposits. There is general agreement with respect to the role played by osmium and iodine in the reaction but there is discrepancy on the significance of zinc. The study of lamellar bodies of type II pneumonocytes after ZIO impregnation showed an intensity distribution of X-ray irradiation with the two distinct peaks of α-particle activity, which are characteristic of osmium and zinc. No evidence of iodine could be detected[45]. The electron dense precipitates contained osmium and zinc atoms in a ratio NOs/NZn which varied from 3/2 to 4/1[78]. GILLOTEAUX et al.[79,80] found in stained zones of histological preparations of the muscular byssus complex innervation of *Mytilus edulis* L. that the distribution of osmium and zinc was remarkably similar and located in the same histological structures. A relatively high concentration of zinc in the photoreceptor cells has been observed by LIBANATI (see [15]). OSBORNE and THORNHILL[13] found in ZIO stained sectioned mitochondria that ZIO staining is composed mainly of osmium and possibly with a trace amount of zinc. Iodine is not shown to be present.

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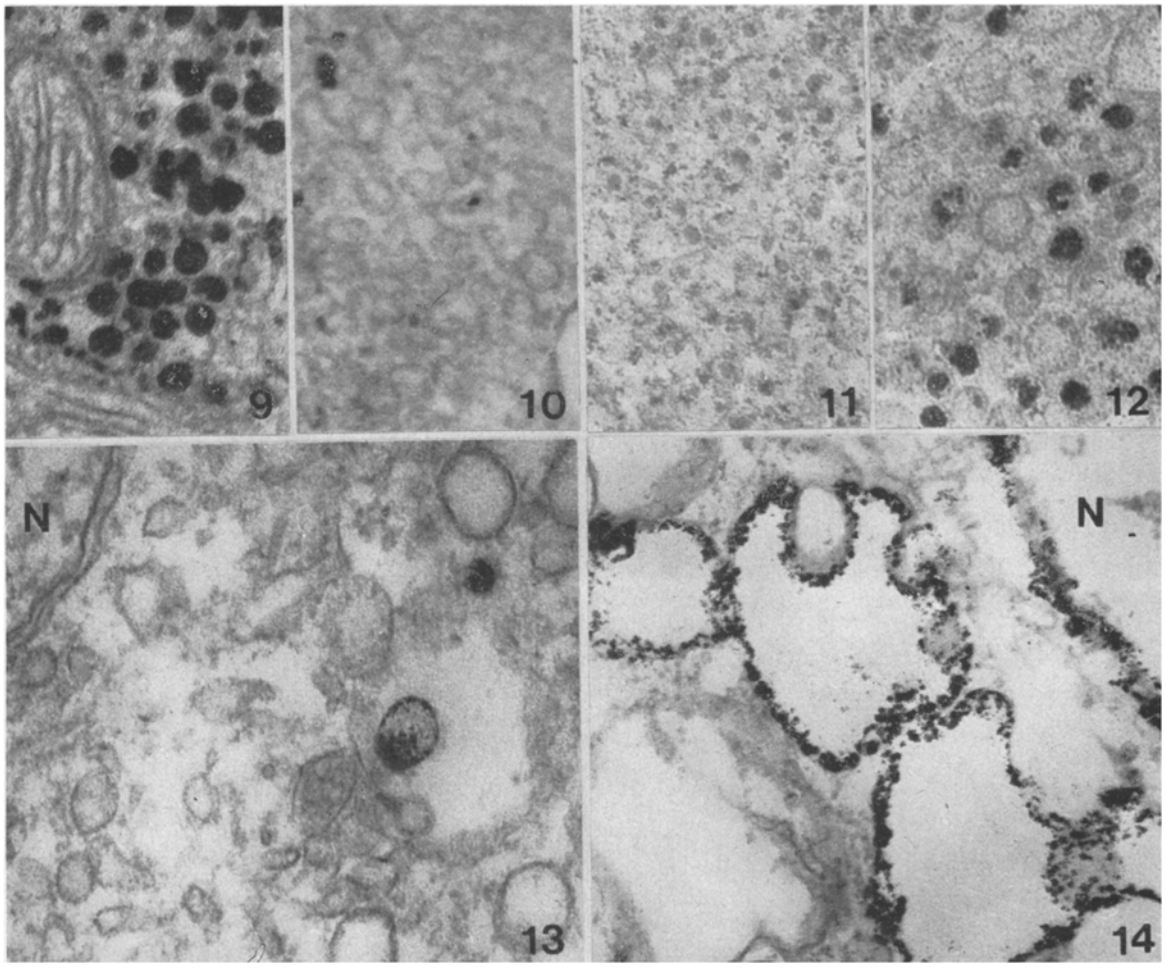

Figs. 9–12. Monoaminergic synaptic vesicles from rat pineal nerves stained with ZIO at 4 °C. Before staining, pineal glands were incubated for 30 min at room temperature in 0.1 *M* phosphate buffer pH 6.9 (Figure 9), 0.1 *M* N-ethyl-maleimide in phosphate buffer (Figure 10), 5 m*M* cysteine in phosphate buffer (Figure 11) and 50 m*M* cysteine in phosphate buffer (Figure 12). Note the disappearance of ZIO reaction in the synaptic vesicles after incubation with N-ethyl-maleimide and 5 m*M* cysteine and its recovery after incubation in 50 m*M* cysteine. × 120,000. Figs. 13 and 14. Endoplasmic reticulum from rat pinealocytes stained with ZIO at 4 °C. Pineal glands were incubated before staining for 30 min at room temperature in 0.1 *M* phosphate buffer pH 6.9 (Figure 13) and in 5 m*M* cysteine in the phosphate buffer (Figure 14). Note in the gland incubated with cysteine attached to the membranes of the nuclear and cytoplasmic cisternae patchy electron opaque deposits. × 60,000.

OSBORNE and THORNHILL[13] think that the ZIO reaction results in a deposition of metallic osmium or possibly a lower oxide than the tetroxide at the positive sites, presumably dependent upon some type of reduction reaction. MAILLET[5] explains the staining by the fixation of an osmical iodate complex at the sites of several cellular structures, iodine being essential for the development of the stain. Thus bromides, chlorides and fluorides cannot be substituted for iodides in the staining mixtures of CHAMPY. GILLOTEAUX et al[80] consider that the reducing process involves complex cations Os-Zn^{++} to oxidize double bonds from organic reducing substrates in the cells. GARRETT[59] has suggested that the deposition of sodium iodide-osmium stain within cell organelles depends upon their redox potential. The indispensable components of the staining mixture would be Os and I catalizing the latter deposition of osmium. BIMES has postulated a catalizing role for the potassium iodide in the CHAMPY mixture (see [5]). It may be mentioned in this context, taking into account the influence of the pH in the redox potential, that the mixture of potassium iodide-osmium tetroxide[9] stains the matrix of many synaptic vesicles of the pineal nerves similarly to ZIO when the pH of the mixture is 5.5 being negative when the final pH is 7.2.

MAILLET[81] has postulated that ZIO uncouples lipid moieties from lipoprotein complexes and that newly exposed groups of the lipid would then be available for increased deposition of metal. Other authors[19,25,46] have also suggested that ZIO reacts with lipoproteins or more simply with a lipid component, based on the fact that the application of lipid solvents to tissues makes the reaction negative. This will be in agreement with the fact that ZIO reacts with phospholipids isolated by thin layer chromatography[28]. However, it must be considered that lipid solvents applied to tissues have a complex action on them. As a matter of fact, they may remove non-lipid components and to cause conformational changes in protein molecules.

ELIAS et al.[25] have demonstrated that the incubation of hydras with hyaluronydase and neuraminidase inactivates ZIO reaction in some places suggesting that ZIO could stain mucosaccharides.

Studies made in our laboratory, e.g. test-tube assays[20,27], the enhancement of ZIO reaction by light in rod outer segments[20,21] and considerations about the role of -SH groups in monoaminergic vesicles[82–84] and visual excitation processes[85] led us to think that -SH groups could be responsible for ZIO reaction in

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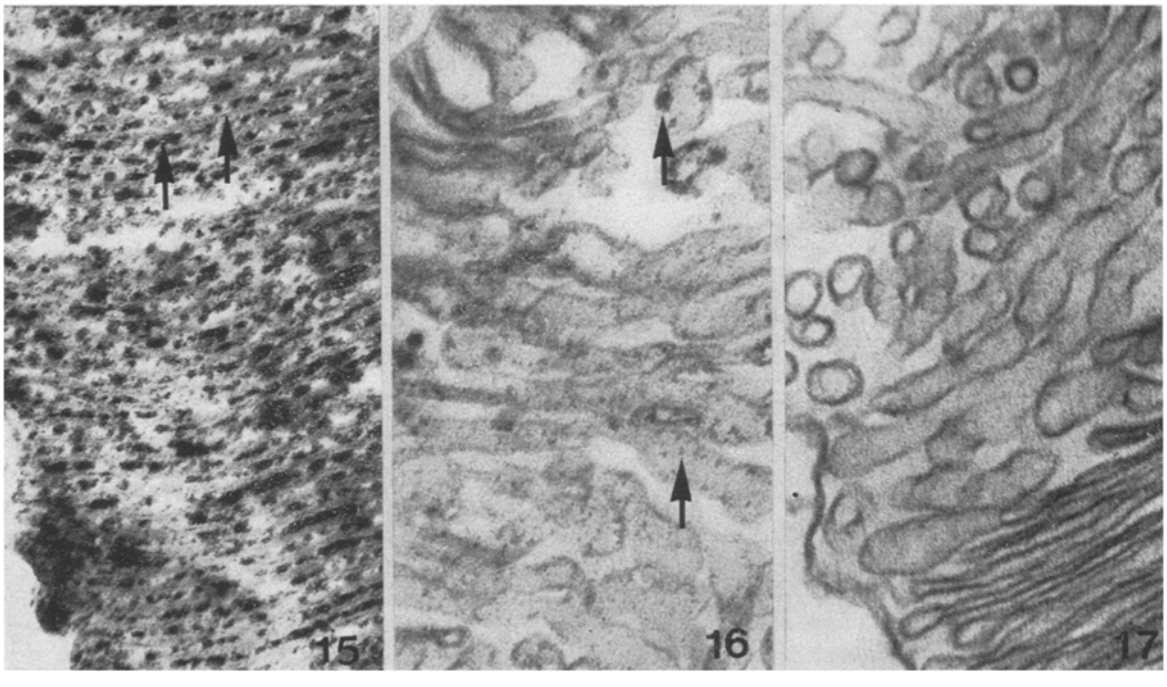

Figs. 15–17. Rod outer segments of rat photoreceptor cells treated with –SH reagents before staining with ZIO at 4°C. Figure 15: control incubated in 0.1 *M* phosphate buffer, pH 6.9 for 30 min at room temperature. There are ZIO electrom opaque deposits in the intra- and extradiskal spaces. The intradiskal deposits are very small. Fig. 16: after incubation for 30 min in 0.1 *M* N-ethyl-maleimide in phosphate buffer. Only small granular opaque deposits are seen inside tubular structures replacing the disks. The extradiskal space is ZIO negative. × 120,000. Figure 17: after incubation for 30 min in 5 m*M* dithioerythritol in phosphate buffer., followed by incubation for 30 min in 0.1 *M* N-ethyl-maleimide in phosphate buffer. ZIO reaction is totally negative both in the intra- and extradiskal spaces. × 120,000.

many tissues. To test this hypothesis, retinas and pineal glands were treated with -SH reagents [86–88] before staining. It was observed that dithioerythritol, which protects -SH groups and reduces -S-S-groups[89] enhances the reaction, whereas N-ethyl-maleimide, a -SH blocking agent[90], renders ZIO staining negative, either used directly or after dithioerythritol (Figures 9, 10, 15–17). We also observed[91] that previous incubation of rat pineal glands in cysteine in weak concentrations in phosphate buffer makes negative ZIO reaction made at 4°C in the granulated vesicles of rat pineal nerves (Figure 11); if the concentration of cysteine is increased the staining of the vesicles reappear (Figure 12). Many of the membranes of the vacuolar system, which are ZIO negative at 4°C (Figure 13) appear covered by a patchy electron opaque precipitate after incubation with cysteine (Figure 14), giving a similar image to that obtained with ZIO at 60°C. In all these experiments ZIO was applied without previous treatment with other fixatives. These observations can be correlated with the varying results obtained with this technique according to the different schedules adopted for the staining procedure, as mentioned in a precedent section. The negative reaction after previous fixation in osmium tetroxide[15] could be explained by the oxidation of available -SH groups; the enhancement of the reaction[8] by the increase of the temperature could be due to the rupture of -S-S-bridges. The critical influence of the pH can also be explained in this context. The differential reactivity of -SH and -S-S-groups has been extensively studied, especially in correlation with their role in the tertiary protein structure. Their availability could depend on diverse conformational changes and marked differences in susceptibility to denaturing agents according to their location in diverse parts of the protein molecules (for a review see [92]). Furthermore the amino acids cystine and cysteine form a redox system.

It has been reported[42] that ZIO reactivity is inhibited by N-ethyl-maleimide in mitochondria, but it is not altered in the vacuolar system in human blood and bone marrow cells prefixed in glutaraldehyde. We have observed that, in photoreceptor cells prefixed in glutaraldehyde, N-ethyl-maleimide blocks and dithierythritol enhances ZIO reaction in mitochondria but have little effect on the other reactive sites[23]. The fact that formaldehyde and glutaraldehyde are denaturing protein agents and that aldehydes may react with -SH groups must be taken into consideration to criticize the results obtained with ZIO after glutar or formaldehyde fixation. The influence of such prefixation on subsequent ZIO staining should be further analyzed to determine its cytochemical significance.

The circumstance that ZIO reacts in vitro with many chemicals, and the possibility that the appearance of electron opaque deposits could be dependent on the redox potential of tissues, makes a special study of each particular case necessary to determine the active groups or chemical components involved in the reaction.

Concluding remarks

The ZIO method was introduced as a general staining procedure for light microscopy with special application to the study of the peripheral nervous system. In this respect, its value has been intensively investigated and fully recognized. Now it can be compared with the best techniques available for the demonstration of nerve fibres and endings, not only in the peripheral nervous system but in the central nervous system as well. At the electron microscopic level, ZIO stains selectively many subcellular structures, especially synaptic vesicles and other components of the synaptic region.

Trying to improve the resolution of the staining procedure, many modifications were introduced to the original method of MAILLET. They led to a better knowledge of the parameters influencing ZIO impregnation in each structure. Although at first sight the variability of the technique can appear as a drawback, the study of the conditions determining this variability may contribute to a better understanding of its possible histochemical significance. Histochemistry does not deal with isolated substances but with reactive groups and molecules organized in complex and variable arrays, in structures submitted to different physiological and pathological conditions. Then their availability for chemical reactions can display a great variability. From the beginning, MAILLET was interested in defining the specificity and the possible histochemical significance of ZIO impregnation and this interest was shared by the authors who used the technique thereafter. It is now well established that ZIO stains different structures and reacts in vitro with many chemicals. However, the findings here reviewed indicate that, when used in an adequate context, the reaction can reach a more defined histochemical significance. They also point to its usefulness in physiological, pharmacological and pathological studies.

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