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The efficiency of aldehyde fixation for electron microscopy: stabilization of rat brain tissue to withstand osmotic stress

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Synopsis. Rat brains were fixed either with glutaraldehyde (GA) or formaldehyde (FA). After 20 min or 24 h fixation the osmotic sensitivity of the tissue was tested by immersion in (a) distilled water (b) 0.15 M or (c) 0.3 M cacodylate buffer. GA-fixed material retained some sensitivity to osmotic stress after 20 min fixation but was entirely resistant after 24 h fixation. Ultrastructural preservation was good after only 20 min GA-fixation, provided that the subsequent treatment was with isotonic solutions. The fixation with FA was less efficient and slower. Dark neurons and other artifacts were commonly seen after the 20 min fixation with FA. Prolongation of the FA-fixation overnight gave markedly better preservation, but however, never equivalent to that with GA.

Introduction

The study of the cellular ultrastructure necessitates prompt and efficient fixation of tissues. Changes in cell membrane permeability and osmotic behaviour of the cells are important parts of a fixative's action. The importance of correct osmolarity in the fixative vehicle is well established (Schultz & Karlsson, 1965; Maunsbach, 1966; Bohman & Maunsbach, 1970; Kalimo, 1976; Collins *et al.* 1977). There is ample evidence (for example, Elbers, 1966; Bone & Denton, 1971; Bone & Ryan, 1972; Penttilä *et al.*, 1974) to show that osmium tetroxide destroys rapidly the semi-permeability of the plasma membrane and, consequently, the osmotic reactivity of the cells, but results on the effect that glutaraldehyde (GA) and formaldehyde (FA) may have on the osmotic behaviour of cells are conflicting.

Penttilä *et al.* (1974) showed that, within seconds, GA renders suspended Ehrlich ascites tumours cells resistant to osmotic stress. Carstensen *et al.* (1971) drew a similar conclusion in their dielectric study of erythrocytes. On the other hand, Bone & Denton (1971) reported that the reflecting cells on fish scales remained osmotically active after exposure to GA or FA, and Bone & Ryan (1972) showed that, even after a

long fixation with GA, the volume of crab axons changes in response to osmotic stress. Furthermore, using microconductometry, Elbers (1966) found that intracellular ions leave GA-fixed sea urchin egg cells only very slowly, which suggests that they retained semi-permeability and osmotic reactivity.

We undertook the present study to clarify the efficiency of GA- and FA-fixation in stabilizing membranes of cells *in tissue* instead of *suspended cells*. We report light and electron microscopic observations on the resistance of rat brain cells to osmotic stresses of varying severity after fixation by intravascular perfusion with GA or FA.

Materials and methods

Adult male Wistar rats weighing 250–300 g were used as experimental animals. They were anaesthetized with ether and killed by perfusion fixation via the ascending aorta. The compositions of the fixatives were:

(1) 3% GA in 0.15 M cacodylate buffer, with 8 mM $CaCl_2$. Osmolarity of the buffer 295 mosmol/l, total osmolarity 640 mosmol/l, pH 7.4.
(2) The same as (1) with 2% polyvinylpyrrolidine (PVP; mol. wt. 40 000; May & Baker Ltd) added. Total osmolarity 650 mosmol/l.
(3) 4% FA in 0.15 M cacodylate buffer, with 8 mM $CaCl_2$. Osmolarity of the buffer 295 mosmol/l, total osmolarity 1800 mosmol/l, pH 7.4.

Six animals were perfused with fixative (1), four with (2) and three with (3).

GA was purified according to Anderson (1967) by Leiras Oy, Turku, Finland. FA was prepared from paraformaldehyde. The fixative solutions were prepared immediately before use. Osmolarities were measured cryoscopically with a Knauer M or Ollituote OM osmometer.

The fixatives were introduced via the ascending aorta according to a modification of the method of Schultz & Karlsson (1965) as presented earlier (Kalimo, 1976). Thoracotomy and cannulation could be carried out in 1–1.5 min thus minimizing the effects of ischemia (Kalimo *et al.*, 1977). The vascular bed was prewashed before fixation by filling the tubing with 0.9% saline (about 25 ml). The saline, as well as the fixatives, were warmed to 38 °C. For each animal 300 ml of fixative was perfused, which took about 4 min (3.5–5 min) at a pressure of 17.6 kPa (180 cm H_2O). The skull was quickly opened and samples of frontal cortex were removed and kept in fixative until sectioned into 230 μm slices using a Sorvall TC-2 tissue sectioner either after 20 min or 24 h from the start of the fixation. The sectioned slices were immersed in distilled water, 0.15 or 0.3 M cacodylate buffer (osmolarities 0, 300 and 600 mosmol/l, respectively), in which the samples were stored either for 30 min or overnight at 4 °C. They were post-fixed for 2 h in 1% osmium tetroxide buffered with the same solution in which the tissues were kept hitherto. Post-fixation with osmium tetroxide in distilled water usually produced a fine precipitate throughout the samples (Friend & Murray 1965). After staining *en bloc* in buffered 0.5% uranyl acetate, the tissues were dehydrated in a graded series of ethanol and embedded in Epon. Semithin sections were cut with a Sorvall MT-1 ultramicrotome and stained for light microscopy with Toluidine Blue. Thin sections were cut with a Sorvall MT-2 ultramicrotome and double-stained with uranyl acetate and lead citrate. Thin sections were examined in a JEM-100 C electron microscope.

Results

Macroscopic appearance

The brains were firm and uniformly fixed after perfusion with either aldehyde. Those fixed with FA appeared somewhat shrunken, and they were slightly paler in colour that the GA-fixed ones.

Light microscopic observations

A good preservation of the tissue at the light microscopic level was obtained with GA after a fixation period as short as 20 min and regardless of the post-fixation treatment, yet the results were slightly better with isotonic than with the hypo- or hypertonic rinsing solutions (Figs. 1*a* & *b*). After overnight fixation with GA the tissue was resistant to any osmotic stress.

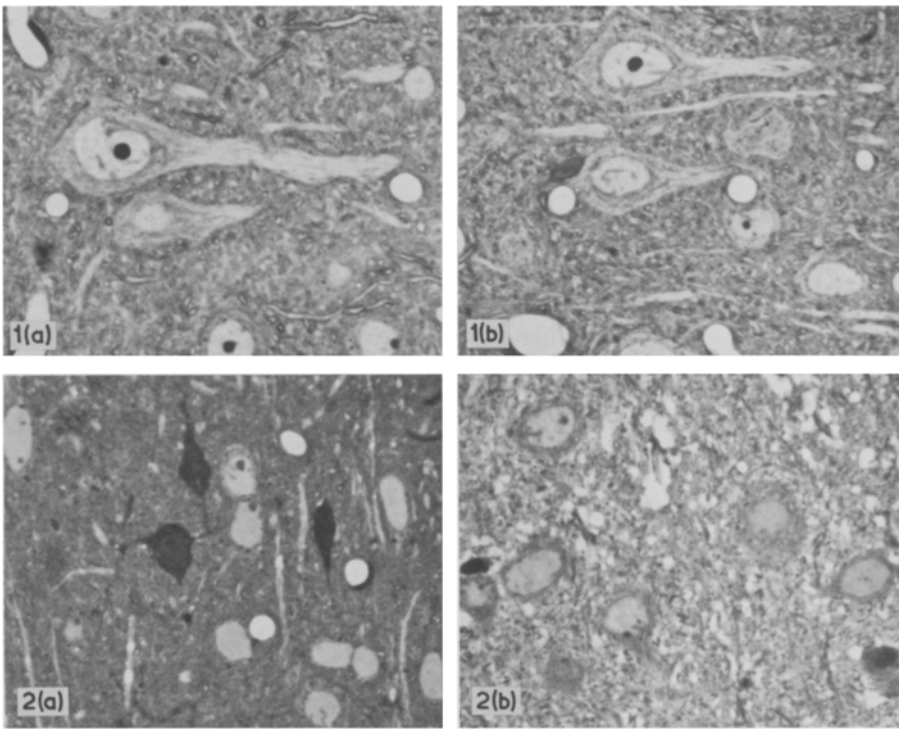

Figure 1. Light micrograph of an Epon section from a brain fixed with GA for 20 min followed by an overnight rinse in 0.15 M buffer. The preservation of the tissue is good. Toluidine Blue. x 360. (*b*) Tissue fixed as in (*a*) but followed by an overnight rinse in water. No essential difference in the cellular structures is seen between Figs. 1*a* & *b*. Toluidine Blue. x 360

Figure 2. (*a*) Epon section from a brain fixed with FA for 20 min and rinsed overnight in water. The structure is severely damaged with dark neurons and vacuolated neuropil. Toluidine Blue. x 360. (*b*) Tissue fixed as in Fig. 2*a* but rinsed in 0.15 M buffer. The structure is somewhat better than in Fig. 2*a*, but dark neurons are still relatively common indicating that FA does not stabilize the tissue in 20 min as GA does (compare with Fig. 1*b*). Toluidine Blue. x 360

Tissue samples fixed with FA for 20 min were markedly damaged by the hypo-osmotic treatment: the neuropil appeared vacuolated and myelin sheaths in the subcortical white matter were extensively damaged. Dark, condensed neurons were frequent (Fig. 2*a*). With iso- or hypertonic buffers markedly better preservation for the light microscopy was obtained, although some dark neurons were present (Fig. 2*b*). Prolonging FA-fixation overnight rendered the tissue considerably more stable; the vacuolation was clearly less pronounced and dark neurons were fewer in number, but FA-fixation was always less efficient than that with GA.

Electron microscopic observations

Fixation with GA for 20 min gave good results, whenever post-fixation treatment was kept isotonic (Fig. 3). Only a few dendrites and astrocytic processes appeared electron lucent and very few cytoplasmic artefactual vacuoles were seen. Such changes appeared to be slightly less frequent when PVP was added to the fixative. Rinsing in hypertonic buffer after 20 min GA-fixation produced a few more adverse structural changes, condensed glial cells and neurons, but no significant damage to the organelles was seen. The extracellular spaces were slightly widened and some cytoplasmic

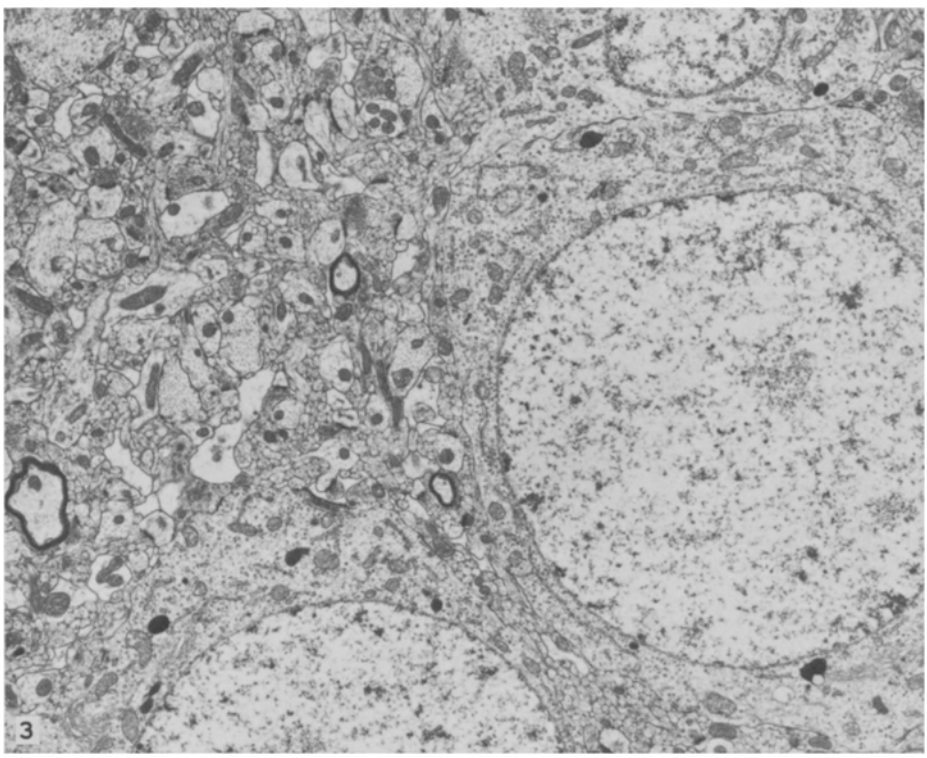

Figure 3. Fixation with GA for 20 min followed by overnight rinse in 0.15 M buffer. The ultrastructure of the rat cerebral cortex is well preserved with evenly narrow extracellular space, intact cell membranes, finely dispersed nuclear chromatin and regularly shaped organelles. x 6000

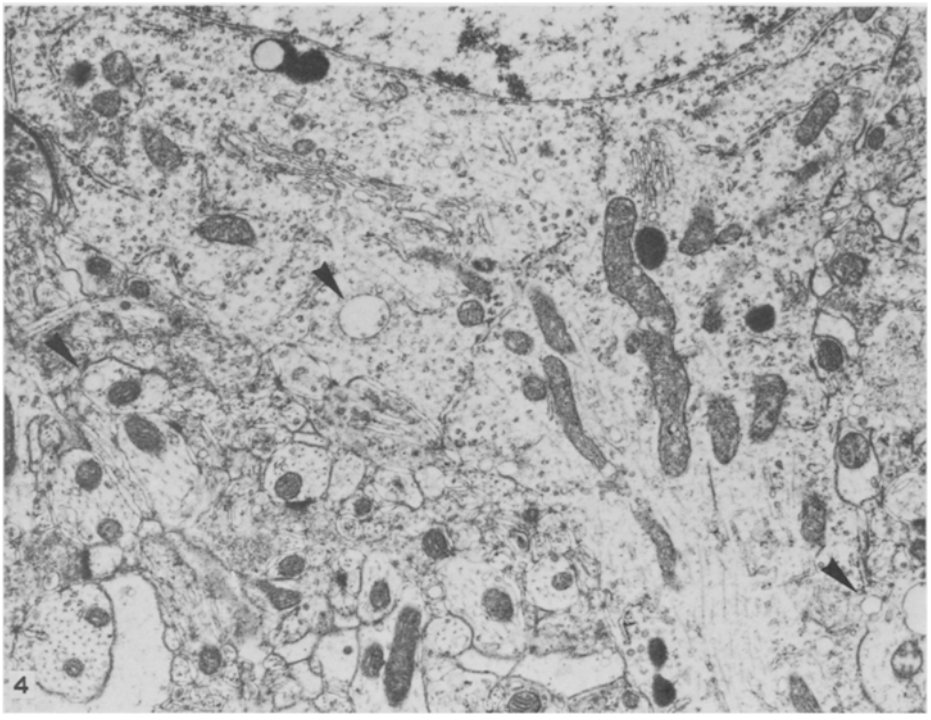

Figure 4. Rat cerebral cortex after fixation with GA for 20 min followed by immersion in water overnight. The extracellular space is somewhat irregular; the neuronal cytoplasm, some dendrites and astrocytic processes display slightly increased electron lucency. A few cytoplasmic vacuoles (arrowheads) of possible artefactual character are seen. x 12 250

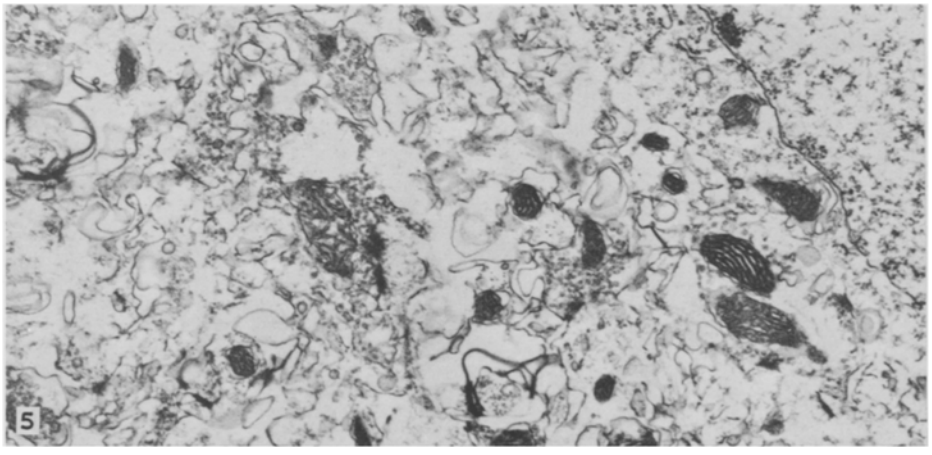

Figure 5. Fixation in FA for 20 min, post-fixation treatment with water overnight. The cellular structures are severely destroyed with disrupted cell membranes and extensive vacuolization of the tissue. x 13 000

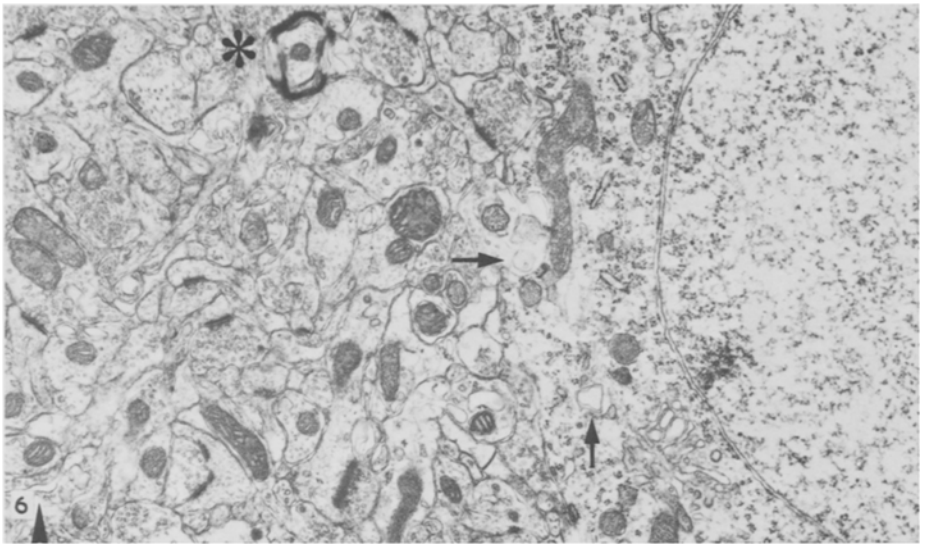

Figure 6. Fixation with FA overnight followed by rinse in 0.15 M buffer. The preservation is satisfactory with somewhat irregular extracellular space (arrowhead), a few artefactual cytoplasmic vacuoles (arrows), slightly increased electron lucency of the cytoplasm in perikarya and cell processes, and loosened myelin (asterisk). x 13 600

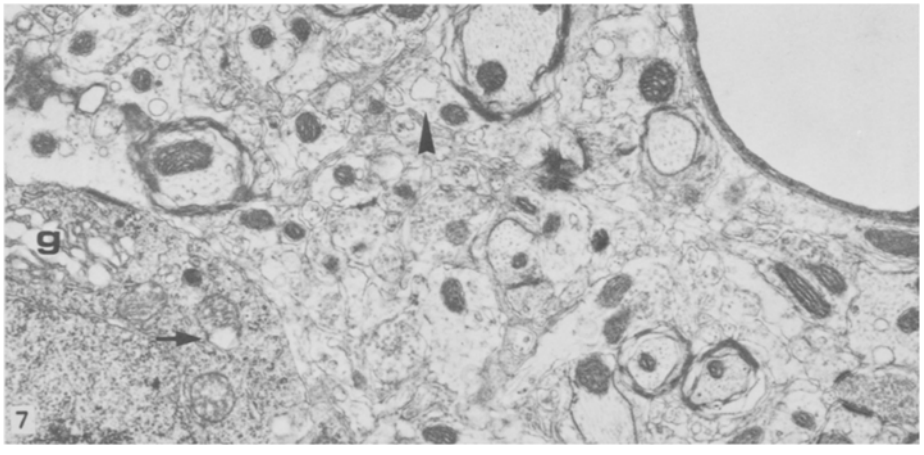

Figure 7. Fixation with FA overnight followed by rinse in water. There is condensation of the cytoplasm and karyoplasm with widening of the Golgi cisternae (g) and mitochondrial vacuolation (arrow) in the neuron at the lower left corner. The cellular membranes are often fuzzy, the myelin is loosened and cytoplasmic vacuoles (arrowhead) are common. x 12 250

Table 1. A summary of the microscopic findings.

Fixative	*Distilled water (0 mosm/l)*	*0.15 M buffer (300 mosm/l)*	*0.3 M buffer (600 mosm/l)*
3% GA for 20 min (with or without PVP added)	LM: Good EM: Satisfactory – irregular extracellular space, increased electron lucency of dendrites and astrocytic processes, cytoplasmic vacuoles	LM: Good EM: Good – minimal alterations similar in quality as for distilled water	LM: Good EM: Satisfactory, extracellular space slightly widened and some cytoplasmic vacuoles. No marked damage of organelles
3% GA overnight	LM: Good EM: Good – osmium precipitate	LM: Good EM: Good	LM: Good EM: Good
4% FA for 20 min	LM: Severe damage, dark neurons EM: Extremely poor – widespread disruption of cellular membranes, washed out appearance of cells	LM: Poor – satisfactory, dark neurons EM: Poor – large cytoplasmic vacuoles, dark neurons or electron lucent cells	LM: Poor – satisfactory, dark neurons EM: As with isotonic buffer
4% FA overnight	LM: Moderate damage EM: Poor – satisfactory, changes like after 20 min GA followed by distilled water	LM: Satisfactory – good EM: Satisfactory – good, minor changes similar to those after 20 min fixation with GA followed by distilled water rinse	LM: Satisfactory – good EM: Satisfactory – good as after isotonic buffer

vacuoles were detected. Immersion in distilled water of similarly fixed samples resulted in minor irregularity of the extracellular space, slightly increased electron lucency of the neuronal perikarya, dendrites and astrocytic processes and somewhat enhanced number of cytoplasmic vacuoles (Fig. 4). After prolonged overnight GA-fixation the above mentioned alterations were prevented and the ultrastructural preservation was considered good.

After fixation with FA for 20 min, the tissue was still very unstable to osmotic challenges. Even rinsing with an isotonic buffer produced numerous large cytoplasmic vacuoles, some dark neurons and increased electron lucency of the dendrites and astrocytes which were interpreted as signs of osmotic reactivity due to an inadequate fixation. Hypotonic treatment in distilled water further accentuated these changes resulting in a wide-spread disruption of the plasma membranes and a washed-out appearance of the cells (Fig. 5). Though brain tissue fixed with FA was osmotically reactive to hypotonic treatment, it appeared to be relatively more resistant to the hypertonic rinse; structural preservation in the latter situation was comparable to that obtained with isotonic buffer.

Prolongation of the fixation in FA overnight markedly improved the fixation giving satisfactory preservation of the ultrastructure after isotonic buffer rinse. Likewise, after treatment with a hypertonic buffer the results were satisfactory, occasional cytoplasmic vacuoles and widening of the extracellular space were the most noticeable adverse effects (Fig. 6). The deleterious effects of treatment with distilled water were considerably less pronounced after the prolonged fixation, but the preservation of the fine structure in tissues rinsed in distilled water still remained unsatisfactory (Fig. 7).

A summary of the microscopic findings is given in Table1.

Discussion

As was expected, GA fixes brain tissue more efficiently than FA as judged by the resistance of the fixed tissue to osmotic shock. GA either rapidly destroys the semi-permeability of the cell membranes or it makes the cell structure rigid through cross-linking of the proteins or it does both (Hopwood, 1962). The rapidity and irreversibility of GA-action seems to overweigh its slower penetration, as compared to FA, when fixing tissue that has such a dense capillary network as it exists in the cerebral cortex. A fixative composed of both GA and FA (Karnovsky, 1967) has been widely used for perfusion fixation on the premise that smaller FA molecules penetrate the tissue more rapidly to 'prestabilize' cells until the GA molecules arrive. Our results indicating that within 20 min fixation GA renders cells more resistant to osmotic stress than FA makes us question the role of FA in these combination fixatives unless these two fixatives potentiate each other. Such a effect, however, has not been proved. One reason for combining FA with GA might be the need for a more rapidly diffusing agent in tissues with very sparse capillary network such as the white matter of the brain.

FA alone was not very efficient with the short fixation time, but using longer fixation and post-fixation solutions of appropriate tonicity, that is, slightly hypertonic, satisfactory results can be obtained. This may be relevant in electron microscopic enzyme histochemistry, where a compromise must be made between the

efficiency of fixation and the preservation of enzyme activity. Our results point out the importance of controlling the tonicity of post-fixation solutions (for example, incubation solutions for enzymes) in order to obtain optimal preservation of structure. However, it should be mentioned that the less efficient fixation with FA does not necessarily mean lesser inactivation of the enzyme activity as reported for example, for horseradish peroxidase (Malmgren & Olsson, 1978).

After completing the perfusion it is a common practice to leave the brain *in situ* for a couple of hours or overnight for the purpose of letting the brain tissues stabilize to withstand subsequent manipulation better. Condensed dark neurons are easily produced by the mechanical damage to the brain before adequate fixation has taken place (Cammermeyer, 1972). The nearly total absence of dark neurons in the GA-fixed material and their presence in FA-fixed tissue after only 20 min total fixation implies that GA rapidly also renders the tissue insensitive to mechanical manipulation (including chopping with TC-2 tissue sectioner, which must create considerable pressures in the tissue). Leaving the brain *in situ* after the perfusion and placing samples for additional immersion fixation certainly improve the structural stabilization, but as pointed out above, histochemical studies may necessitate fixation of the shortest possible duration.

Comparing our results with those of Penttilä *et al.* (1974), they may be considered concordant in the respect that GA obviously makes cell membranes lose their semi-permeability fairly rapidly and consequently become resistant to osmotic stress. Likewise GA is also very efficient in making the cells resistant to mechanical stress. It is understandable that a longer fixation time is needed for equivalent efficiency in tissue samples as in suspensions of free-floating cells, since GA arrives at cells in tissue more slowly and at lower concentrations. Comparison of our data with the results of Bone & Denton (1971) and Bone & Ryan (1972), on the other hand, is difficult, as mammalian nervous tissue may not be adequately compared with fish scale cells, neither can axonal swelling in brain occur in the same way as it can in isolated crab axons. The fixation time needed before the tissue will be entirely resistant to osmotic and mechanical stresses obviously depends on several factors, for example, the quality of the tissue, the concentration and purity of the aldehyde in the fixative, the duration of the perfusion versus immersion, and on the perfusion pressure, which affects the extent of fixative penetration into the tissue (Kalimo, 1976). Because of all these variables, exact time intervals cannot be given here. However, GA evidently makes cells become resistant even to the severe osmotic stress of water immersion after a total fixation time of just a few hours whereas such an efficiency will not be reached by FA even within a day.

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