MICROTUBULE LOSS WITH ACROLEIN AND BICARBONATE-CONTAINING FIXATIVES

ROBERT L. SCHULTZ and NORMAN M. CASE. From the Department of Anatomy, Loma Linda University, Loma Linda, California 92354

In the process of evaluating various fixatives for perfusion fixation of the central nervous system we have found that the neuronal microtubules are singularly sensitive to the perfusate used. In our experience the use of bicarbonate buffer results in a disintegration of neuronal microtubules; surprisingly, acrolein fixation alone uncombined with any other aldehyde also causes a similar loss. This finding is particularly noteworthy because other morphological features can remain apparently unchanged and well preserved.

MATERIALS AND METHODS

The major part of the material reported on here consists of visual cerebral cortex obtained from adult rats; however, optic nerve, corpus callosum, superior colliculus, cerebellum, and lateral geniculate have also been observed. The technique of perfusion used has been described by Karlsson and Schultz (1) and consists of perfusion of an aldehyde, or aldehydes, for about 15 min into a live Nembutal-anesthetized animal. The pH of all solutions, both perfusates and postfixatives, was maintained at 7.2–7.5. The osmolality was determined with a Mechrolab 301A vapor pressure osmometer F & M Scientific Division of Hewlett-Packard Co., Avondale, Pa. and an Advanced Instruments, Inc. (Newton Highland, Mass.) 31LAS freezing-point depression osmometer. The buffer (or buffer with sodium chloride in the case of *s*-collidine) was kept close to isotonic (300–320 milliosmols). After perfusion, blocks of tissue were dissected out and postfixed in 1% osmium tetroxide buffered with the same buffer used for the perfusate. Embedding was done in Vestopal, and sections were stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 1A.

RESULTS AND DISCUSSION

When a 0.2 M bicarbonate buffer was used with 3% glutaraldehyde, 2% glutaraldehyde–1% acrolein mixture, or 37% formaldehyde, the results were the same, as far as the neuronal microtubules were concerned. The tubular elements normally seen (Fig. 1) in neuronal processes were replaced by a dispersion of flocculent-appearing material and a few fine, much smaller, solid-appearing filaments (similar to Fig. 2). Combining equal amounts of bicarbonate and phosphate buffer (total of both components about 320 milliosmols) and using this combination with 3% glutaraldehyde did not help to preserve the microtubules.

The results with bicarbonate buffer contrast the results obtained by using a 320 milliosmol phosphate buffer with 3% glutaraldehyde (Fig. 1), 2% glutaraldehyde–1% acrolein, or formaldehyde (4 or 37%). With phosphate buffer, the microtubules are routinely well preserved. Using cacodylate buffer (0.17 M) produced a result similar to that obtained with phosphate. While the over-all picture was different when *s*-collidine buffer was used, there was no noticeable loss of microtubules.

Bicarbonate-buffered 37% formaldehyde has been recommended by Gonzalez-Aguilar as a fixative for the central nervous system (2). We attempted to duplicate the procedure used by Gonzalez-Aguilar, but we found that, although fine preservation of morphological detail was obtained, there was the peculiar loss of microtubules already mentioned. Incidentally there was some gross, over-all shrinkage of the brain even though the perfusion was done for only 2 min.

Using mainly liver as a test tissue, Wood and Luft (3) tried bicarbonate as a buffer with osmium tetroxide. Their results were favorable, and they stated that bicarbonate buffer appeared to provide good fixation with a wide variety of tissue types. It must be noted, though, that they were not using perfusion technique or aldehyde primary fixation followed by osmium tetroxide postfixation; they used only osmium tetroxide fixation.

The reason for the loss of neuronal microtubules in the presence of bicarbonate buffer is obscure. In an attempt to determine whether the aldehydes caused a loss of buffering action, we have made titration curves for bicarbonate buffer both with and without aldehydes added. As would be expected, the curves show that bicarbonate is an excellent buffer and that aldehydes only slightly affect this buffering capacity. One is forced to conclude that the effect of the bicarbonate ion is very specific. Perhaps it causes a disaggregation or dissolution of the microtubules. A general sensitivity

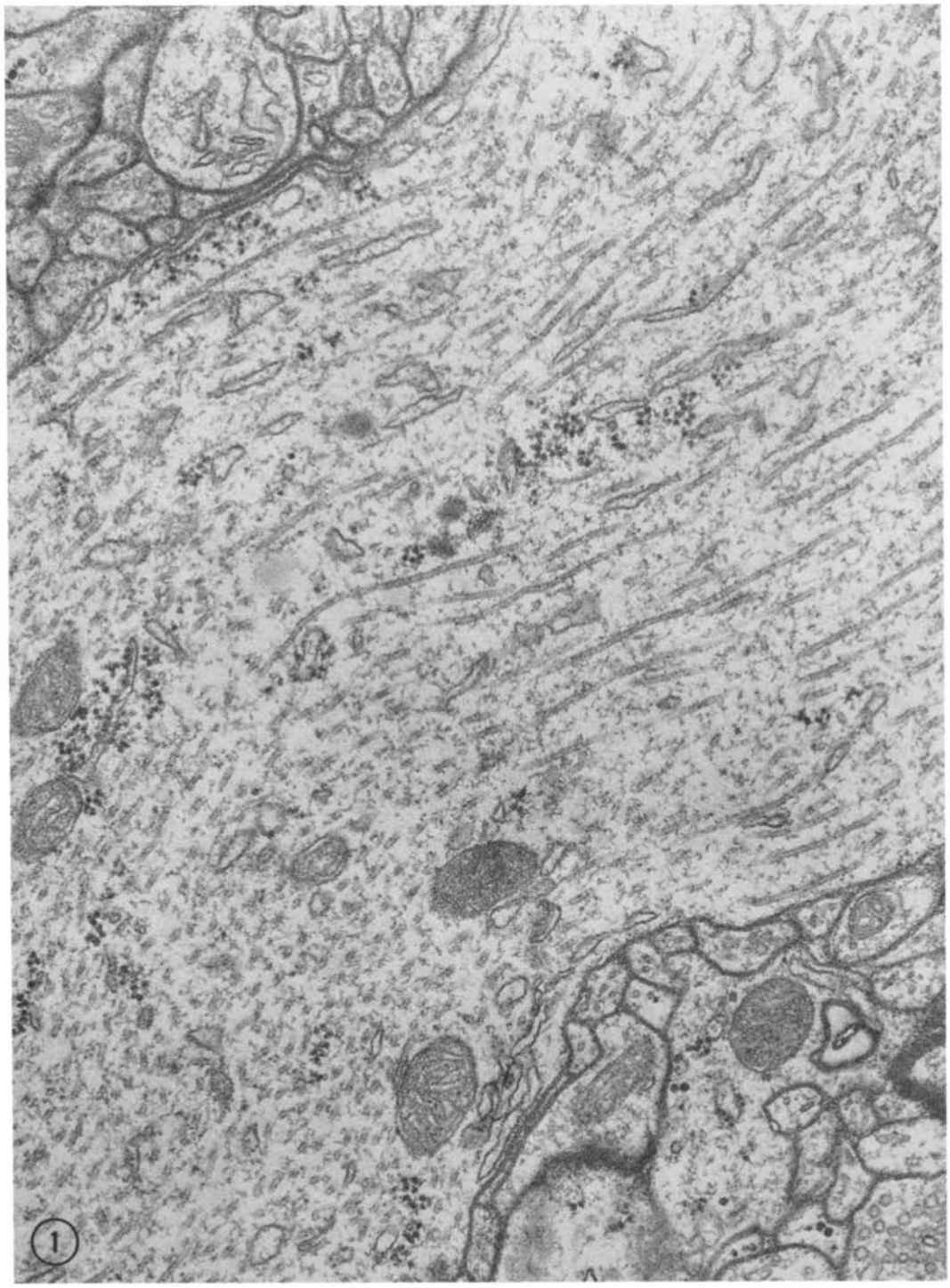

FIGURE 1 Part of a large dendrite, from visual cerebral cortex perfused with 3% glutaraldehyde in isotonic phosphate buffer, extends across the micrograph from lower left to upper right. Microtubules in the dendrite are oriented longitudinally near the center of this print, while those at the lower left are tangential and cross-sectioned. × 57,000.

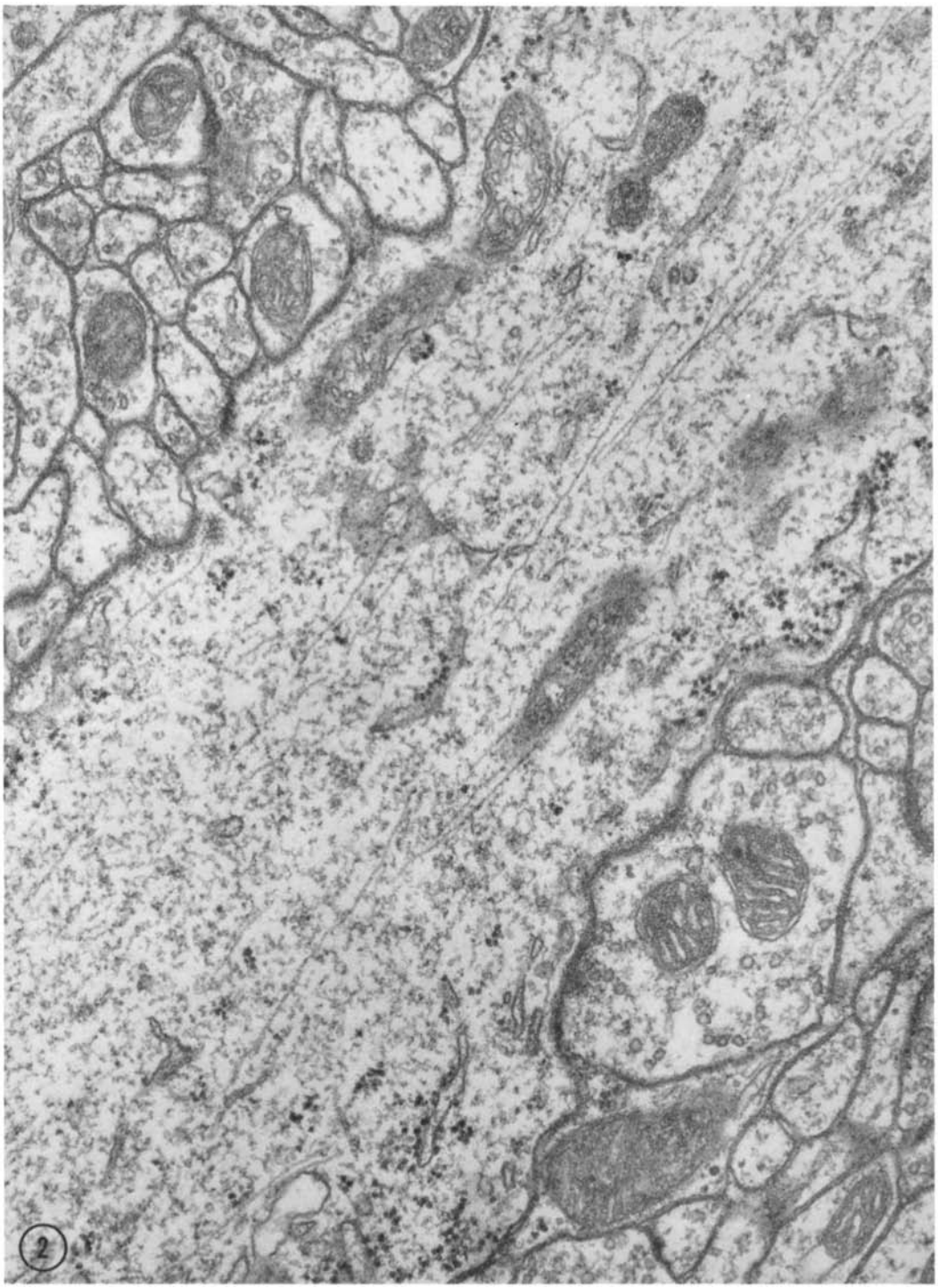

FIGURE 2 This dendrite is oriented as in the previous figure. The perfusate used was 3% acrolein in an isotonic phosphate buffer. Microtubules are not seen, but only fine filaments and a flocculent background. Other morphological features appear essentially the same as with glutaraldehyde and phosphate-buffer perfusion. × 57,000.

of neurons of the central nervous system to carbon dioxide, an increased level of which has a stimulating effect, has been suggested by Hornbein (5). One can speculate that this sensitivity is related to the microtubules. Such a hypothesis has already been suggested by Schmitt (4) who proposed that, under conditions prevailing in the normal axon, changes in ionic environment may cause either aggregation or disaggregation of fibrous axonal protein. However, he did not limit the fibrous axonal protein to only the axon filaments but stated that it may also be deposited upon the limiting membrane of the axon, the axolemma.

Acrolein was given a rating equivalent to glutaraldehyde by Sabatini et al. (6), as concerns fixation of morphological detail. Sandborn (7) stated that a mixture of glutaraldehyde and acrolein in phosphate buffer gave better preservation of cytoplasmic microtubules than was obtainable with either fixaive alone. One would assume, from those results, that neuronal microtubules should be just about as well preserved with phosphate-buffered acrolein alone as with phosphate-buffered glutaraldehyde alone. Such was not the case. We found that phosphate-buffered 3% acrolein caused a nearly total loss of microtubules (Fig. 2). The acrolein apparently did not preserve the tubules even though it gave excellent preservation of membranes, mitochondria, synaptic vesicles, and other features. If glutaraldehyde, a mixture of glutaraldehyde and acrolein, or formaldehyde was used in place of acrolein by itself, the microtubules were preserved. Unlike Sandborn, we could not observe better preservation of microtubules with glutaraldehyde-acrolein than with glutaraldehyde alone. Formaldehyde did not preserve microtubules quite so well as glutaraldehyde or glutaraldehyde-acrolein, but this was difficult to detect.

We made an attempt to determine how quickly the microtubules were lost. In one experiment acrolein (phosphate buffered) was perfused for about 30 sec, and then glutaraldehyde (also phosphate buffered) was perfused for about 15 min. With this procedure there was no apparent loss of tubules. In another experiment acrolein was perfused for 5 min, and then glutaraldehyde was perfused for 10 min. This procedure resulted in a partial loss of tubules. It is interesting to note that Karlsson and Schultz (8) found that delayed perfusion after exsanguination caused little change in cytoplasmic microtubules. From that result, one would conclude that the tubules are not very sensitive metabolically.

Some emphasis has been placed on the loss of microtubules when bicarbonate and acrolein are used because it is possible to achieve with these agents a rather excellent preservation of other morphological features. However, other procedures have been tried which result in inferior fixation and often a loss of microtubules. Simply cutting out a piece of cerebral cortex and placing it into fixative, or dripping fixative onto the exposed surface, will result in a varying loss of microtubules. A number of aldehydes which also possess no fixative ability or, at best, fix poorly do not preserve microtubules well. The aldehydes which have been tried and which fit this category are crotonaldehyde, methacrolein, propynal, hydroxyadipaldehyde, glyoxal, acetaldehyde, pyruvic aldehyde, and malondialdehyde.

One might logically question what happened to the microtubules in the glial cells under the various conditions of fixation. From the evidence we have, the glial microtubules seem to react like the neuronal microtubules, but to observe glial microtubules is not so simple and easy as to observe neuronal microtubules, and further explanation of this situation is needed. In this context, reference to the comments of Sandborn (7) concerning glial cells and microtubules is appropriate because of his emphasis upon the better preservation obtained with phosphate-buffered glutaraldehyde-acrolein. Using a similar fixative, we indeed see microtubules in glial cells, but we are unwilling to state, as Sandborn has, that in astrocytes these microtubules are so abundant as to form the fibers of the fibrous astrocytes described from light microscopy. Where the preservation of microtubules in our material is good, we observe a considerable size differential between microtubules and astrocytic fibrils, the fibril diameter being much smaller. Both structures appear hollow on close inspection, but the microtubules are obviously so. We find that in astrocytes the larger microtubules are sparsely distributed in contrast to the very common fibrils. Cells which we choose to identify as oligodendrocytes show variable accumulations of microtubules, but since the identification of oligodendrocytes and microglia and even their status as separate cell categories are presently questioned by a number of investigators, it is not judicious to make definitive statements concerning the microtubules in these glial cells.

As an example of a study which could have arrived at erroneous conclusions in regard to microtubules because of fixation, we would cite the observations of Peters and Vaughn (9) concerning the changes in the population of microtubules and filaments within maturing axons and astrocytes. Fortunately, those authors used phosphate-buffered 4% formaldehyde and 0.5% glutaraldehyde in their perfusion. As has been stated, we agree with their finding that filaments are abundant in mature astrocytes and that microtubules are rare. Peters and Vaughn suggest that the filaments seen in astrocytes and axons are formed by the breakdown of microtubules.

Our observations of the effects of bicarbonate buffer and acrolein fixative on neuronal microtubules show these microtubules to be singularly sensitive. Future studies involving microtubules might profit from an awareness of this sensitivity.

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