The Effect of Acetic Acid on Cytoplasmic Inclusions

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With one plate (fig. 1)

SUMMARY

Acetic acid at 5% is not necessarily destructive of cytoplasmic inclusions. Hermann's fluid gives excellent mitochondrial preparations if tissues are postosmicated for several days at 34° C. The mitochondria are blackened by this treatment. Mann's fluid with the addition of 5% of acetic acid gives, on postosmication, very good preparations of the 'Golgi apparatus' of certain cells.

INTRODUCTION

IT was remarked by von Brunn in 1884 that the granules grouped round the axial filament of the mammalian spermatid 'show the reaction of protoplasmic granules on treatment with acetic acid; that is to say, they first become pale, then indistinct, and finally disappear altogether, while the axial filament remains still clearly recognizable'.

It was this finding of von Brunn's that led Benda (1902) to reduce greatly the amount of acetic acid when making up Flemming's strong fluid for studies of mitochondria, and this example was followed by others. Acetic acid was omitted from Flemming's fluid by Lewitsky (1911) in his studies of mitochondria. No acetic acid was included by Golgi (1898) in the fixative he used to demonstrate his 'apparatus'. It has always been omitted or used at low concentration by his successors.

As a result of the example of these and other authorities, the general practice is to omit acetic acid from fixatives used in studies of cytoplasmic inclusions, or to reduce its concentration far below 5%. In general histology and studies of chromosomes, on the contrary, it is usual to include acetic acid at 5% v/v or thereabouts, because its swelling effect counteracts the shrinkage caused by other constituents of fixative mixtures.

Two questions are raised by these familiar practices. Is it possible that acetic acid at about 5% does not in fact necessarily destroy mitochondria? And if it does destroy them, *how* does it do so?

With regard to the first question, those who have placed living cells in solutions of acetic acid have not always observed a sudden destruction of mitochondria (Lewis and Lewis, 1915; Strangeways and Canti, 1927), though filamentous ones are often transformed into faint rows of granules. Chromium trioxide, used alone, is much more destructive than acetic acid; yet it is included in several valuable fixative mixtures used in studies of mitochondria. These organelles (or the remains of them) have been seen from time to time in microscopical preparations made from tissues fixed in solutions containing quite high concentrations of acetic acid (Champy, 1911; Romeis, 1913;

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Nicholson, 1916; Young, 1928; Baker, 1932; Marquez, 1934). Nevertheless, there can be no doubt that as a general rule one does not obtain a clear view of them if acetic acid is used at the concentration that is usual in routine histology.

With regard to the second question, it is pointed out that mitochondria and certain other cytoplasmic inclusions are composed partly of lipids, and that acetic acid is a lipid-solvent. This, however, is irrelevant; for although glacial acetic acid does indeed dissolve certain lipids, the aqueous solution at 5% does not. The hydronium ion cannot be responsible, for, as Casselman and Jordan (1954) showed, one can actually fix mitochondria in 0·1 N hydrochloric acid. There is no obvious reason why the acetate ion or the undissociated acid, in aqueous solution, should disperse lipids.

The known facts, taken together, lead to the following conclusions:

(1) Acetic acid, used by itself at 5%, is not necessarily very destructive of mitochondria.

(2) It is not, however, a fixative for mitochondria: that is to say, it does not protect them against dehydration and other processes of routine microtechnique.

(3) There is no proof that acetic acid at 5% might not be used as a constituent of a fixative mixture intended for use in studies of mitochondria, provided that steps were taken to render the mitochondria insoluble in fluids that would have to be used subsequently.

Osmium tetroxide is a reliable fixative for mitochondria, if allowed to act for sufficient time. It was therefore decided to try various fixative mixtures containing acetic acid at 5%, and subsequently to treat the tissues with osmium tetroxide solution before dehydrating and embedding them.

Material and Methods

Most of the work has been done with the epididymis, pancreas, and anterior part of the intestine of the mouse. A wide range of tissues from mammals, urodeles, and various invertebrate groups has also been studied.

The following fixatives were used: Zenker's fluid (1894); Flemming's strong fluid (1884) with full acetic; Hermann's fluid (1889, the fluid designed for mammalian tissues); acetic-osmium (Hermann's fluid without chloroplatinic acid); and Mann's mercuric-osmium (1894), with the addition of acetic acid at 5%. All these fixatives contain acetic acid at exactly or almost exactly 5% v/v. The last three are conveniently made up in accordance with the table on p. 427.

Pieces of tissue about 2 mm or less in thickness were used. The strand connecting the caput with the cauda of the epididymis was used, because it is so conveniently narrow. It was found best to leave a short piece of the intestine in the fixative for 5 min before opening it longitudinally. If this organ be opened before it is put in the fixative, it tends to turn inside out and the villi are then likely to be damaged during dehydration or embedding.

The pieces were fixed for 24 h. They were then washed in water and trans-

ferred to 2% osmium tetroxide solution. The period of washing, the period in the osmium tetroxide solution, and the temperature of osmication were varied. After osmication the pieces were washed for a few hours in changes of distilled water and then passed either through graded ethanols and toluene into paraffin or else through cellosolve into estax (Chesterman and Leach, 1956).

	Hermann's fluid (mammalian formula)	*Acetic-osmium*	*Mann's fluid plus acetic acid*
	(ml)	(ml)	(ml)
Distilled water . .	0·8	1·1	..
Chloroplatinic acid, 5% aq.	0·3	..	..
Osmium tetroxide, 2% aq.	0·4	0·4	0·5
Acetic acid, 20% aq. .	0·5	0·5	0·5
Mercuric chloride, sat. sol. in 0·75% NaCl . .	..	..	1·0
	2·0	2·0	2·0

Sections were generally cut at 5 μ and mounted in Canada balsam or DPX. Dyeing was unnecessary, since the cytoplasmic inclusions, if present, could be seen as grey or black objects.

Results

Mitochondria were usually visible, at any rate in some part of the piece of tissue, and they were often blackened if the period and temperature of osmication had been right for this purpose. They were often changed from filaments or rods into spheres, however, and in some cases the ground cytoplasm was darkened, so that they did not show very clearly. Satisfactory results were not obtained when Zenker's or Flemming's fluid was used as fixative.

Remarkably clear preparations of mitochondria were obtained when Hermann's fluid was used as fixative. It was found best to wash the pieces of tissue thoroughly after fixation. Short or careless washing resulted in poor osmication. Running water should be used, but since various ions interfere with the reduction of osmium tetroxide, it is a good plan to give a final wash in distilled water, though this is not necessary if the tap-water is reasonably pure. The osmium tetroxide must be warm if the mitochondria are to be blackened. At room temperature even 36 days' osmication does not blacken them, though some of them become pale grey. Less good results are obtained at 37° C than at 34°. At the higher temperature the cytoplasm usually becomes grey, so that the mitochondria do not stand out nearly so clearly as when the slightly cooler fluid is used. It is curious that such a small difference in temperature should have such a marked effect on the result. Six days' osmication suffices to give black mitochondria; this period may sometimes be halved. If pieces of tissue of the proper size be used, one may osmicate with only 0·5 ml of solution. If the fluid becomes grey or yellowish during osmication, it must be replaced.

The procedure may be summarized thus:

(1) Fix for 24 h in Hermann's fluid (with full acetic).
(2) Wash thoroughly in running water for 24 h. Give a final wash in distilled water.
(3) Leave in 2% osmium tetroxide at 34° C (in a small, tightly closed specimen-tube in the dark) for 3 to 6 days. (It is best to try 6 days first.)
(4) Wash for several hours in changes of distilled water.
(5) Embed in paraffin or estax.
(6) Cut sections at 5 μ (or less).
(7) Mount in Canada balsam or DPX.

Since the essential parts of this method are fixation in Hermann's fluid and postosmication, it may be called the Hermann PO or HPO technique. It gives very clear preparations of mitochondria, which appear in black against a nearly white background; chromatin is just touched with yellow from the chloroplatinic acid. Mitochondria are not shortened or broken up into rows of globules by this method, but they are sometimes considerably thickened. This is the easiest and most certain of all ways of making permanent preparations of mitochondria. The pancreas (fig. 1, A) and intestine of the mouse are among the easiest tissues from which to make striking preparations. The method usually succeeds with cells in which the mitochondria are rather difficult to show by the usual methods (the nerve-cells of mammals, for instance). It fails, however, with the nerve-cells of the cricket (*Acheta domesticus*), which are badly fixed, strangely enough, by Hermann's fluid. The large mitochondria of the first convoluted tubules of the mammalian kidney are not very well shown.

If the acetic acid is reduced to 2·5% or omitted, the result is not nearly so good. The acetic acid opposes the tendency of the chloroplatinic acid to shrink the cells.

If the chloroplatinic acid is omitted from Hermann's solution (or, to put it in another way, if acetic-osmium is substituted for Hermann), the result is entirely different, though the whole of the rest of the technique remains unaltered. The mitochondria are seen only vaguely or not at all, and the cytoplasm is grey. It is to be presumed that chloroplatinic acid acts upon lipoprotein complexes in such a way as to separate or 'unmask' the lipid constituents, and that these are fixed and blackened by osmium tetroxide.

Some of the various objects commonly called 'Golgi apparatus' are also blackened by the HPO technique. This applies to the material surrounding the developing secretion-globules in the epithelial cells of the epididymis and in the Paneth cells of the crypts of Lieberkühn of mammals. The so-called Golgi apparatus of the intestinal epithelium of mammals is evidently of a different nature, for it remains invisible by this technique. It may be recollected that Kolatchev (1916), in his studies of the 'Golgi apparatus' in the

FIG. 1 (plate). A, pancreas of mouse. Hermann's fluid; postosmicated for 6 days at 34° C. B, epididymis of mouse. Mann's fluid with the addition of 5% of acetic acid; postosmicated for 3 days at 34° C.

neurones of gastropods, sometimes fixed in a modified Hermann's fluid and postosmicated. In his fixative, however, the concentration of acetic acid was greatly reduced.

When chloroplatinic acid is omitted from Hermann's solution, the 'Golgi apparatus' is still blackened in the epididymis. It is evident that no unmasking process is necessary in this case. Indeed, the material blackened is probably not lipid (see Christie, 1955). Such preparations are poor, however, because the cytoplasm is grey.

If Mann's mercuric-osmium, with the addition of 5% of acetic acid, be substituted for Hermann's, it is best to reduce the period of osmication at 34° C to 3 days, in order to avoid darkening of the ground cytoplasm. The change of fixative profoundly modifies the picture. Mitochondria are now poorly shown in most tissues, but some of the various objects to which Golgi's name is usually attached are shown with astonishing clarity. The epithelial cells of the epididymis of the mouse provide a good example (fig. 1, B). The intestinal epithelium, on the contrary, reacts in rather a patchy way. A fairly characteristic picture of the Golgi apparatus is seen in some of the cells of the anterior mesenteric ganglion of the rabbit, but it appears to be made up mostly of separate threads. It is possible that these are in reality thickened mitochondria. It may be recalled that several authors, especially Monti (1915), have claimed that the Golgi apparatus of the neurone of vertebrates actually represents the mitochondria.

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