

**The Use of OsO<sub>4</sub> as Fixative for Feulgen-Stained Preparations.** BY MARIA A. RUDZINSKA.\* (*From The Rockefeller Institute for Medical Research.*)†

In the course of an investigation of structural differences between young and old organisms in the protozoan *Tokophrya infusionum* (2-6), it became necessary to study the macronucleus by histochemical procedures (1). The macronucleus of *Tokophrya* is a conspicuous body (5 to 20  $\mu$  in diameter) which contains numerous refractile granules ( $\sim 0.5 \mu$  in diameter) clearly visible in the living organism (Fig. 3). Because of their staining properties these granules are called chromatin bodies. In electron micrographs of thinly

sectioned, OsO<sub>4</sub>-fixed specimens, the chromatin bodies appear as dense, oval profiles (Fig. 5) similar in shape and distribution to those seen in the living organism. It appears therefore that in this case, OsO<sub>4</sub> fixation insures the faithful preservation of a characteristic intranuclear structure that can be clearly observed *in vivo*. The finding deserves some attention in view of the opinion frequently expressed that OsO<sub>4</sub> is not a satisfactory fixative for nuclear structures in general.

Previous studies have shown that the macronucleus contains Feulgen-positive material (11), but the intranuclear location of the DNA responsible for this

\* Supported by a grant from the National Heart Institute, H-1350 (C2).

† Received for publication, July 7, 1955.

staining reaction could not be studied in detail, because the nuclear structures seen *in vivo* are usually altered greatly by Carnoy's fixative. The latter is, in recent years, one of the common fixatives for cytophotometric measurements of the Feulgen reaction (12). OsO<sub>4</sub>, the fixative of choice for electron microscopy, has apparently not yet been used in histochemistry for the Feulgen nuclear staining.

Histochemical information on the macronucleus is derived therefore from Carnoy-fixed material, whereas information on the fine structure of the organelle is obtained from OsO<sub>4</sub>-fixed specimens. The result is that structural details cannot be convincingly interpreted in histochemical terms. It is evident that a satisfactory correlation of morphological and histochemical observations requires the use of a common fixative for these two different technical approaches. For this reason it was decided to try the OsO<sub>4</sub> as a fixative for the Feulgen nuclear staining and to compare the results of this procedure with those obtained in the current histochemical technique that combines fixation in Carnoy's mixture with staining by Feulgen's reagent.

For this purpose cultures of *Tokophrya* were grown in hanging drops on coverslips. Since the adult *Tokophrya* is a sessile organism and attached firmly to the substrate (coverslip) the cultures may be easily fixed and stained on coverslips and thereafter examined in whole mount preparations. In the study herein reported, cultures prepared in hanging drops were divided into two groups; one was fixed with Carnoy's fluid (3 parts of absolute alcohol and 1 part of glacial acetic acid) for 10 minutes, the other with vapors of 2 per cent OsO<sub>4</sub> for 30 minutes. Both groups after the necessary washings were hydrolized together for 10

minutes and thereafter taken through the same staining and dehydration procedures.

The examination made after mounting disclosed that, although the intensity of the nuclear stain appeared to be the same in both groups<sup>1</sup> there were considerable and striking differences between the preparations fixed in OsO<sub>4</sub> and those fixed in Carnoy's fluid. The differences proved that OsO<sub>4</sub> fixation has many advantages over Carnoy's fixative for Feulgen's nuclear staining. First of all, it was found that after Carnoy's fixative, the Feulgen-stained macronucleus shows considerable shrinkage demonstrated by a large clear zone between it and the cytoplasm (Fig. 2). It contains a few irregular masses of deeply stained material. They may represent chromatin bodies clumped together by the shrinkage of the macronucleus (Fig. 2 A). In the preparations fixed with OsO<sub>4</sub> the macronucleus does not show shrinkage and retains its size and shape (Fig. 1). The chromatin bodies remain unchanged and can be clearly seen as separate granules (Figs. 1 A and 4). Their shape and distribution resembles those in the living organism (Fig. 3). OsO<sub>4</sub> fixation insures, in addition, a satisfactory preservation of the body shape, of the tentacles, and of the cytoplasmic organelles, whereas specimens fixed in Carnoy's mixture show body shrinkage, contraction or disappearance of the tentacles, and poor preservation of cytoplasmic detail.

This comparative study shows clearly that OsO<sub>4</sub> has definite advantages over the Carnoy's fluid as a preparatory fixative for the Feulgen reaction. It seems somewhat surprising and difficult to understand why OsO<sub>4</sub> known as a very

<sup>1</sup>No spectrophotometric measurements were taken.

good cytological fixative (7) has not been used so far in the Feulgen procedure. This might be explained by the complexity of the reaction and the precautions it requires. As is known, the Feulgen reaction uses a mild acid hydrolysis for splitting guanine and adenine from desoxyribonucleic acid, and liberating aldehyde functions which stain intensively reddish purple when treated with leuco-fuchsin. It is understood that in such a procedure every step is important. Feulgen himself was much concerned about the kind of fixative (8). He warned against the use of mixtures containing chromic acid because it might destroy the nuclear material. He was also against the use of formaldehyde because it might give a positive color reaction. He recommended sublimate-acetic fixative in spite of the knowledge that it produces swelling and vacuolization of the chromatin. Bauer (9) and Hillary (10), however, studied the influence of various fixatives on the Feulgen reaction and came to the conclusion that practically every fixative can be used if the tissues are thereafter properly washed. In this connection it is even more interesting to note that Bauer (9) was strongly against the use of Carnoy's fluid describing it as the least desirable fixative for the Feulgen reaction because it changes the structure of the chromatin. He recommended moreover Flemming's fluid without glacial acetic acid. One of the three components of the Flemming's fluid is  $\text{OsO}_4$  (1 per cent chromic acid, 30 parts; 2 per cent  $\text{OsO}_4$ , 8 parts; glacial acetic acid, 2 parts).

The finding that  $\text{OsO}_4$  is a good fixative for the Feulgen nuclear staining is of special importance in view of the fact that electron microscopy relies on this fixative. With a common fixative, it becomes possible to compare directly elec-

tron micrographs with photomicrographs of well preserved, Feulgen-stained material, and to interpret in histochemical terms structures seen in the electron microscope.

#### SUMMARY

$\text{OsO}_4$  has many advantages over Carnoy's fixative mixture for the Feulgen nuclear staining in the protozoan *Tokophrya infusionum*.

While Carnoy's fluid used prior to the Feulgen reaction produces shrinkage of the macronucleus and coarse clumping of its chromatin bodies,  $\text{OsO}_4$  preserves faithfully the size and shape of the macronucleus and its chromatin material.

This finding seems to be of special importance in view of the fact that electron microscopy relies on  $\text{OsO}_4$  fixation. The satisfactory preservation of structured detail in Feulgen-stained preparations is of importance for the correlation of histochemical and morphological information.

#### BIBLIOGRAPHY

1. Rudzinska, M. A., and Porter, K. R., Observations on the fine structure of the macronucleus of *Tokophrya infusionum*, *J. Biophysic. and Biochem.*, 1955, **1**, 421.
2. Rudzinska, M. A., and Porter, K. R., An electron microscope study of a protozoan, *Tokophrya infusionum*, *Anat. Record*, 1953, **115**, 363.
3. Rudzinska, M. A., and Porter, K. R., Submicroscopic morphology of structures involved in the feeding of *Tokophrya infusionum*, *Proc. Soc. Protozoologists*, 1953, **4**, 9.
4. Rudzinska, M. A., and Porter, K. R., The origin and fine structure of the brood pouch in *Tokophrya infusionum*, *J. Protozool.*, 1954, **1**, 7.
5. Rudzinska, M. A., and Porter, K. R., The fine structure of *Tokophrya infusionum* with emphasis on the feeding

- mechanism, *Tr. New York Acad. Sc.*, 1954, **16**, 408.
6. Rudzinska, M. A., and Porter, K. R., Electron microscope study of intact tentacles and disc in *Tokophrya infusionum*, *Experientia*, 1954, **10**, 460.
  7. Strangeways, T. S. P., and Canti, R. G., The living cell *in vitro* as shown by dark-ground illumination and changes induced in such cells by fixing reagents, *Quart. J. Micr. Sc.*, 1927, **71**, 1.
  8. Feulgen, R., und Rossenbeck, H., Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung vom Zellkernen in mikroskopischen Präparaten, *Z. physiol. Chem.*, 1924, **136**, 203.
  9. Bauer, H., Die Feulgenische Nuklealfärbung in ihrer Anwendung auf cytologische Untersuchungen. *Z. Zellforsch. u. mikr. Anat.*, 1932, **15**, 225.
  10. Hillary, B. B., Use of the Feulgen reaction in cytology. I. Effect of fixation on the reaction, *Bot. Gaz.*, 1938, **101**, 276.
  11. Jiroveč, O., Protozoenstudien. II, *Arch. Protistenk.*, 1927, **59**, 550.
  12. Moses, M. J., Quantitative optical techniques in the study of nuclear chemistry, *Exp. Cell Research*, 1952, **2**, 75.

## EXPLANATION OF PLATE 128

FIG. 1. Photomicrograph of young *Tokophrya infusionum* fixed with vapors of 2 per cent  $\text{OsO}_4$  and stained with Feulgen's reagent. The shape of the body is pyriform as in the living organism. The macronucleus (*m*) does not show shrinkage. The cytoplasm seems to be well preserved. The tentacles (*t*) retain their length.  $\times 2350$ .

FIG. 1 A. Underexposed photomicrograph from the same negative as in Fig. 1 to show the macronucleus only.  $\times 2350$ .

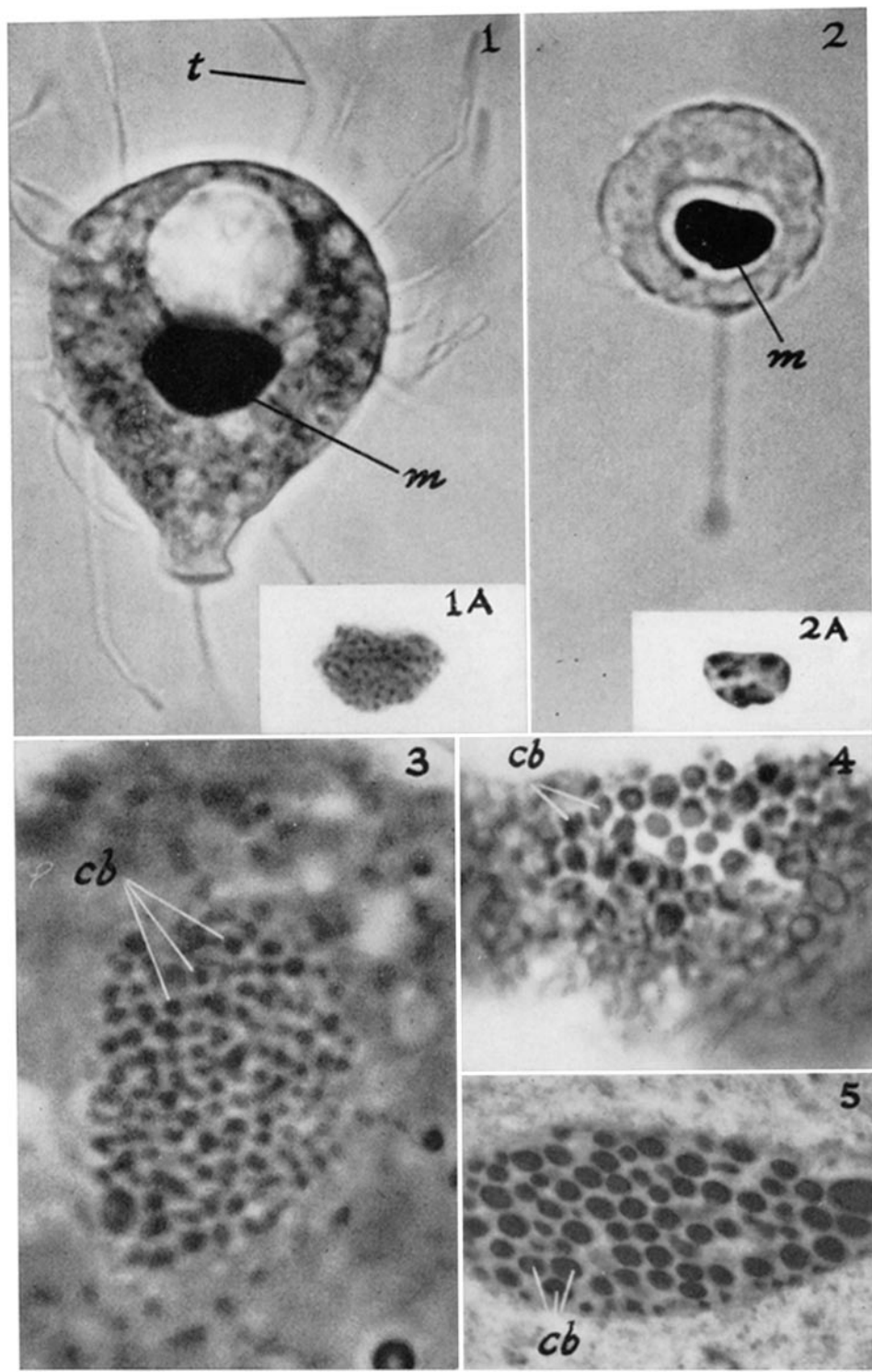
FIG. 2. Photomicrograph of young *Tokophrya* fixed in Carnoy's fluid and stained with Feulgen's reagent. The shape of the body is changed to one that is spherical. Between the macronucleus (*m*) and the cytoplasm a large clear area can be seen, indicating that the macronucleus underwent considerable shrinkage. Little detail is visible in the cytoplasm. The tentacles have contracted or disappeared.  $\times 2350$ .

FIG. 2 A. Photomicrograph showing the macronucleus of Fig. 2 only. The technique used was the same as in Fig. 1 A.  $\times 2350$ .

FIG. 3. Part of body of a living organism in phase contrast microscopy showing the macronucleus and its chromatin bodies (*cb*).  $\times 3420$ .

FIG. 4. Photomicrograph of macronucleus fixed with vapors of 2 per cent  $\text{OsO}_4$  and stained with Feulgen's reagent. The chromatin bodies (*cb*) are clearly visible and look like the ones seen in the living organism (Fig. 3).  $\times 3580$ .

FIG. 5. Electron micrograph of section through macronucleus; *cb*, chromatin bodies.  $\times 6000$ .



(Rudzinska: OsO<sub>4</sub> as fixative for Feulgen-stained preparations)