BUFFERED OSMIUM TETROXIDE (OsO_4) FIXATION FOR CYTOLOGICAL AND FEULGEN MICROSPECTROPHOTOMETRIC STUDIES OF HUMAN RECTAL POLYPS*

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INTRODUCTION

The value of osmium tetroxide (OsO_4) as a superior fixative for the structure of cells and tissues has been recognized for some time (1). However, staining of OsO_4-fixed tissues with the usual histological stains such as hematoxylin and eosin (H and E) or with the Feulgen reaction has met with great difficulty in the past. Baker (2), who discusses OsO_4 fixation in great detail, states that there is no other fixative that leaves tissues in such an unstainable state. Furthermore, according to Baker, it is difficult or impossible to stain the chromatin a different color than the cytoplasm after OsO_4 fixation unless another fixative is added.

In the following report, evidence is presented that when human rectal mucosa and polyps are fixed with buffered OsO_4 according to the method of Palade (3),[1] staining of paraffin sections by basic and acidic dyes results in a distinct color difference between chromatin and cytoplasm. Furthermore, the Feulgen reaction also can be carried out successfully and such OsO_4-fixed Feulgen-stained sections are especially suitable for microspectrophotometric determinations of desoxyribose nucleic acid (DNA) in individual cells.

Material and Methods

Immediately after surgical removal, human rectal mucosa and rectal polyps were cut into pieces not thicker than ¼ mm., while immersed in buffered OsO_4. These minute tissue pieces were then fixed for 1 hour in fresh buffered OsO_4 after the method of Palade (3). At the same time, duplicate pieces were fixed for 1 to 2 hours in Lavdowsky's solution (10 cc. concentrated formalin, 50 cc. 95 per cent alcohol, 2 cc. glacial acetic acid, and 40 cc. distilled water). The

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OsO_4-fixed tissues were washed in several changes of the buffer, dehydrated in an ascending alcohol series, cleared in benzene, and embedded in paraffin. The Lavdowsky-fixed material was treated the same way with the exception that no buffer solutions were used. The total time from fixation until embedding did not require more than 6 hours. Sections were cut from the paraffin blocks at 1, 2, 4, and 6 μ using an ordinary rotary microtome. These sections were stained by a routine H and E method and by a standard Feulgen reaction (4). The estimation of the DNA content in individual cells by Feulgen microspectrophotometry was done as previously described (5).

RESULTS

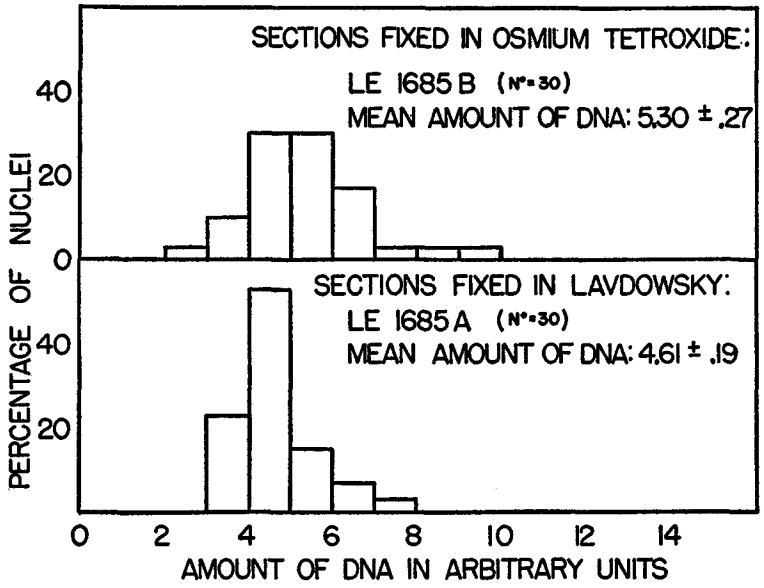
On microscopic examination, OsO_4-fixed rectal polyps and normal rectal mucosa exhibit superior preservation of all structures when compared with the same material fixed in Lavdowsky's solution. We had previously considered the latter as one of the best fixatives for rectal mucosa. Fig. 1 *a* shows a photomicrograph of glandular epithelial cells from a rectal polyp fixed in OsO_4 and stained by Feulgen–fast green. It is evident that OsO_4 fixation according to Palade's method prevents cell shrinkag to a marked degree. (Note that all the photomicrographs have been taken at the same magnification.) It can also be seen that the membranes of the nuclei and cells maintain a smooth outline, while in contrast, many nuclei after Lavdowsky fixation (Fig. 1 *b*) show a striking shrinkage of the cells and nuclei and a ragged appearance of their membranes.

The cytoplasmic inclusion bodies containing DNA which are depicted in Fig. 2 show much less shrinkage than similar bodies seen in a previous study (6) in which Carnoy's (3 parts absolute alcohol and 1 part glacial acetic acid), Lavdowsky's, and formalin fixatives were used. Attempts to photograph the differences in these bodies due to fixation, were unsuccessful because of the density and small size of the bodies. However, under the microscope, especially in thin sections (1 to 2 μ) and at high magnifications, the inclusion bodies after OsO_4 fixation show a definite structure while after other fixatives, they appear dense and homogeneous. Some indication of differences in density within inclusion bodies after OsO_4 may perhaps be noticed in the inclusion body designated by $\times$ in Fig. 2.

In addition to the excellent structural preservation of rectal mucosa and of rectal polyps after OsO_4, H and E–stained sections exhibit a sharp difference in color between the chromatin material in the nucleus and the cytoplasm. The chromatin stains dark blue while the cytoplasm stains distinctively red with the eosin.

If the Feulgen reaction is done on OsO_4-fixed sections, it is as positive and as specific for DNA as is the Feulgen reaction on Lavdowsky-, Carnoy-, or 10 per cent formalin–fixed sections. Control, OsO_4-fixed sections on which the Feulgen reaction is done without hydrolysis are completely Feulgen-negative. Consequently, estimation of DNA in individual cells by Feulgen microspectrophotometry can easily be done on such OsO_4-fixed material. DNA measure-

ments were carried out on 304 Feulgen-stained nuclei from cases of rectal polyps and normal rectal mucosa fixed in OsO_4, Lavdowsky's solution, Carnoy's solution, and 10 per cent formalin. No significant differences in the DNA content of individual nuclei were found in the tissues fixed in the various fluids. This was true for the rectal polyps as well as for the normal rectal mucosa. An example of these findings is given in Text-fig. 1. The DNA measurements presented in this histogram were done on the same rectal polyps photographed in Figs. 1 *a* and 1 *b*.



TEXT-FIG. 1. Amount of DNA (microspectrophotometry) in individual nuclei of glandular epithelium of human rectal polyp. N° = number of nuclei measured.

It can be seen that the amount of DNA in individual nuclei from the glandular epithelium of this rectal polyp fixed both in OsO_4 and in Lavdowsky's solution is essentially the same, although the mean DNA value of the OsO_4-fixed tissue is slightly higher than that of the Lavdowsky-fixed tissue.

DISCUSSION OF THE RESULTS

In view of the great difficulty which other workers have reported in staining tissues after OsO_4 fixation (1), our own observation warrants an explanation. Indeed the finding that paraffin sections of OsO_4-fixed human rectal polyps show such a distinctive stainability came as a complete surprise to us. It was an incidental observation made during the course of electron microscope studies of rectal polyp inclusion bodies (6). Previously, we also had not been able to stain OsO_4-fixed tissues differentially. However, such tissues were

usually fixed for 24 hours in unbuffered OsO_4. Perhaps, fixing minute pieces in buffered OsO_4 for 1 hour, only allows what Berg calls a primary fixation (7).

According to Berg, OsO_4 produces a primary and a secondary effect in tissues. The primary fixation involves the immediate combination of the whole molecule with the amino groups of the proteins and does not prevent staining. The secondary effect leads to an oxidation of the compound formed, after which the tissues are no longer stainable unless they are thoroughly washed.

The possibility of obtaining a specific Feulgen stain after the superior OsO_4 fixation of tissue structure, is of great advantage not only to the cytologist but also to the cytochemist who carries out microspectrophotometric analysis of DNA in individual nuclei. The measurement of nuclear diameters required by this technique is greatly facilitated by the good preservation of the nuclear sizes and of the nuclear membranes after OsO_4 fixation. After Lavdowsky fixation, some nuclei cannot be measured at all because of their extreme shrinkage and the distortion of their nuclear membranes (see Fig. 1 *b*). Another advantage for Feulgen microspectrophotometry is the homogeneous distribution of the absorbing material in polyps fixed in OsO_4. Perhaps this homogeneous distribution may be interpreted as a cause for the slightly higher mean DNA values which were occasionally found in OsO_4-fixed material (see Text-fig. 1). Since, on the other hand, the DNA content in OsO_4-fixed cells was sometimes identical with, or sometimes even slightly lower than that in Lavdowsky-fixed cells, the small DNA variability seems to be of random nature.

Since the question may arise whether the differential staining after Palade's OsO_4 fixation may possibly be limited to human tissues and in particular to rectal mucosa, we fixed and stained animal tissue such as mouse liver by the same methods. H and E staining of buffered OsO_4-fixed mouse liver shows excellent differential staining. The chromatin stains blue, the nucleoli blue or red, the cytoplasm red, and the erythrocytes orange. In mouse livers which show mitosis, the chromosomes are of deep blue color. The Feulgen reaction and Feulgen microspectrophotometry for DNA can also be carried out easily on such OsO_4-fixed livers. Analysis of the DNA content by Feulgen microspectrophotometry in buffered OsO_4-fixed polyploid livers gives 2 DNA, 4 DNA, and 8 DNA; that is, essentially the same results as obtained in Lavdowsky-, Carnoy-, or 10 per cent formalin–fixed livers (8).

It may be of interest to use the Palade buffered OsO_4 fixation technique for tissues other than the ones reported here.

SUMMARY

Evidence has been presented that when human rectal mucosa and polyps are fixed with buffered OsO_4 according to the method of Palade, staining of

paraffin sections by basic and acidic dyes results in a distinct color difference between chromatin and cytoplasm. Furthermore, the Feulgen reaction also can be carried out successfully and such OsO_4-fixed Feulgen-stained sections are especially suitable for microspectrophotometric determinations of DNA in individual nuclei.

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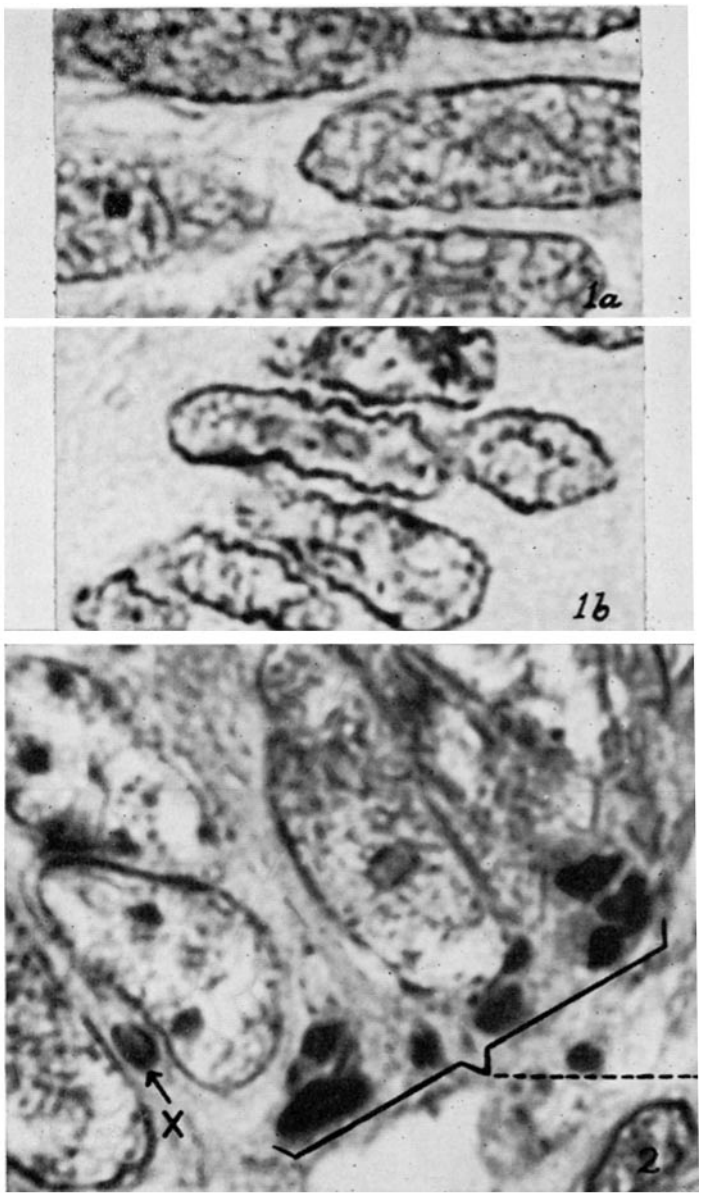
EXPLANATION OF PLATE 101

FIG. 1 *a*. Human rectal polyp (LE 1685 B), osmium tetroxide fixation (after Palade).

FIG. 1 *b*. Human rectal polyp (LE 1685 A), Lavdowsky fixation.

FIG. 2. Human rectal polyp (LE 1616 B), osmium tetroxide fixation (after Palade). Feulgen–fast green stain. Magnification 3500.



Cytoplasmic inclusion bodies containing DNA

(Leuchtenberger *et al.*: Microspectrophotometric studies of human rectal polyps)