Brief Notes

The Use of Mercuric Bromphenol Blue as a Stain for Electron Microscopy.

BY PATRICIA HARRIS AND DANIEL MAZIA.* (*From the Department of Zoology, University of California, Berkeley.*)‡

The search for suitable staining methods for use in electron microscopy has led to many and diverse attempts to increase the opacity to electrons of different structures in tissue sections. The most obvious approach has been the attempt to bind heavy metals to specific sites, either to increase the general contrast or to identify the chemical nature of the structures so stained. Other than the use of osmium tetroxide as a lipide stain, methods for binding heavy metals have included treatment with phosphotungstic acid or the incorporation of lanthanum nitrate into the fixing medium, and more recently Watson (1958 *a*, *b*) has reported the use of salts of several other heavy metals. While such treatment has shown indication of specificity, for the most part the effect has been to increase the general contrast. More specific cytochemical procedures have been described by Lamb *et al.* (1953), and recently there has been a reevaluation of organic dyes as possible stains for electron microscopy. Isenberg (1956, 1957) reported the use of a number of histochemical stains on frozen-dried materials following digestion with ribonuclease, deoxyribonuclease, or amylase, and has calculated the theoretical density characteristics of several organic dyes. It has been pointed out, however, that the contrast observed may not necessarily be due to the stain, but rather to the inherent density of the substance taking the stain. Favard and Carasso (1957) criticized the tendency to consider all electron dense structures in osmium fixed material as being "osmiophilic," when, in fact, many of the structures show the same density characteristics when fixed by other means. Thus, the protein granular structure which they describe in the oocyte of *Planorbis corneus* appears to be independent of the presence of a heavy metal in the fixing medium, since osmium-fixed material showed little difference in contrast from that fixed in 10 per cent formol. It was also pointed out that melanin, which contains a phenolic structure, has similar density characteristics.

In the present study of the slime mold, *Physarum polycephalum*, osmium-fixed material was used. The stain, mercuric bromphenol blue, was originally devised by Durrum (1950) and has been used for the identification of protein spots in chromatographic and electrophoretic studies. The reactions with proteins and amino acids have been described by Kunkel and Tiselius (1951) and Geschwind and Li (1952), and the application of mercuric bromphenol blue to cytochemical staining and measurement of protein was reported by Mazia, Brewer, and Alfert (1953). Such a stain seemed particularly suitable for electron microscopy since it binds at several different sites by at least two different mechanisms, and also for the obvious reason that it incorporates a heavy metal.

While such common cytological fixatives as Carnoy's, formalin, Schaudinn's and Bouin's solutions did not affect the staining characteristics of the material for light microscopy, Mazia, Brewer, and Alfert found there was interference when osmium-containing fixatives were employed. Presumably this interference was due to the masking of the stain by the increased opacity to light of the osmium-fixed material. However, the reaction of osmium tetroxide with proteins is little known, reviewed briefly by Baker (1950) and discussed in a more detailed study by Porter and Kallman (1953), but it appears to involve linkages at sites of double bonds. It is possible that the specificity of the mercuric bromphenol blue staining reaction may be altered somewhat by osmium fixation. Whether this is the case, is not known.

These studies, done several years ago, suffer from the disadvantages of older sectioning methods, but are presented at this time as a contribution to the current developments in electron staining methods.

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Methods

The slime mold was encouraged to crawl across the clean surface of a Petri dish, and when sufficiently spread out, was flooded with a solution of 2 per cent osmium tetroxide in H_2O. The tissue was fixed for 1 hour, followed by washing for 1 hour in several changes of distilled water. At this point it was cut up into smaller fragments and divided into two batches, the first being carried directly through graded alcohols for dehydration and embedded in the usual manner in *n*-butyl methacrylate. The second batch was immersed in an aqueous solution of mercuric bromphenol blue (10 gm. of HgCl and 100 milligrams of bromphenol blue per 100 ml.) for 1 hour, washed in distilled water, dehydrated in graded alcohols, and embedded.

The sections were cut with a modified Spencer microtome, and by virtue of this fact were relatively thick—approximately 0.1 microns. They were picked up off the dioxane-water surface on a glass microscope slide, the embedding material was removed with amyl acetate, and the slide was then immersed in a dilute solution of collodion in amyl acetate. The dried film was then stripped off onto a water surface and mounted on an electron microscope specimen screen.

OBSERVATIONS AND DISCUSSION

An examination of the cytoplasm of the unstained material reveals numerous dense spherical particles which presumably are lipide or fat droplets. On further examination, however, one can also find many barely discernible ovoid bodies considerably smaller than the nuclei and containing some dense particles that look very much like crystals. They occur in fairly great number and seem to be distributed quite evenly throughout the cytoplasm.

The stained preparation, on the other hand, shows a striking contrast. While the fat droplets are still present and identifiable, the crystal-containing bodies have been replaced by dark staining ovoid structures of about the same order of magnitude and with the same distribution throughout the cytoplasm as those in the unstained material. It is very tempting to equate the two, but regardless of whether or not they are identical, the fact remains that something in the cytoplasm is being visualized by a staining method that is in common use for the identification of proteins. The objection that dense structures may owe their contrast to the inherent density of the material rather than that of the stain is obviated by the use of a control section that is already osmium fixed. Thus any density differences between the two sections can be attributed to the staining procedure. The following question, however, remains to be explored: How much of the increased opacity is due to the mercuric ions and how much to the organic dye that is coupled to proteins by these ions? It is conceivable that even if the observed density is attributable to the mercuric ions alone, more of these are combined when they serve as ionic bridges between charged groups on the protein and anionic groups on the dye.

The granular material in the cytoplasm of the stained tissue may be residual stain which was incompletely washed out. On the other hand, since it occurs only in the cytoplasm and not in the nuclei, it may represent stain bound to normally soluble proteins which are lost during the usual preparation procedures.

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EXPLANATION OF PLATE 146

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Explanation of Plate 146

Fig. 1. Section of osmium-fixed, unstained *Physarum*, showing two of the ovoid bodies (arrows) distributed throughout the cytoplasm. × 2400.

Fig. 2. Same as Fig. 1. × 10,000.

Fig. 3. Osmium-fixed material treated with mercuric bromphenol blue, showing densely staining structures (arrow) in the cytoplasm. × 2400.

Fig. 4. Same as Fig. 3. The apparent difference in nuclear size between Figs. 2 and 4 is a result of the different levels at which the nuclei were sectioned. × 10,000.

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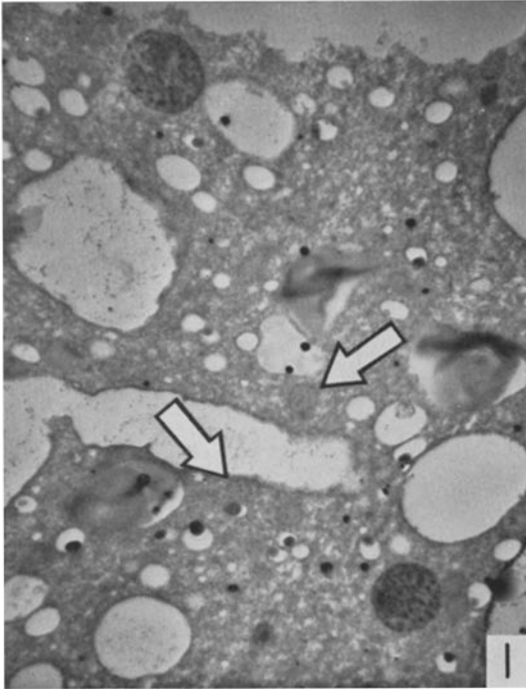

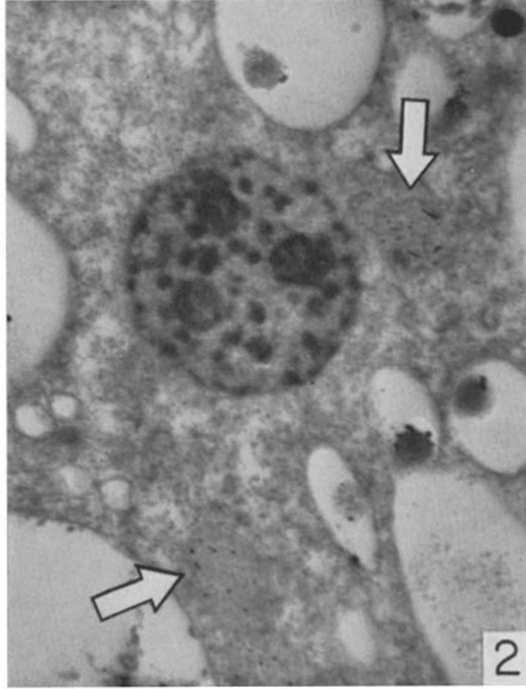

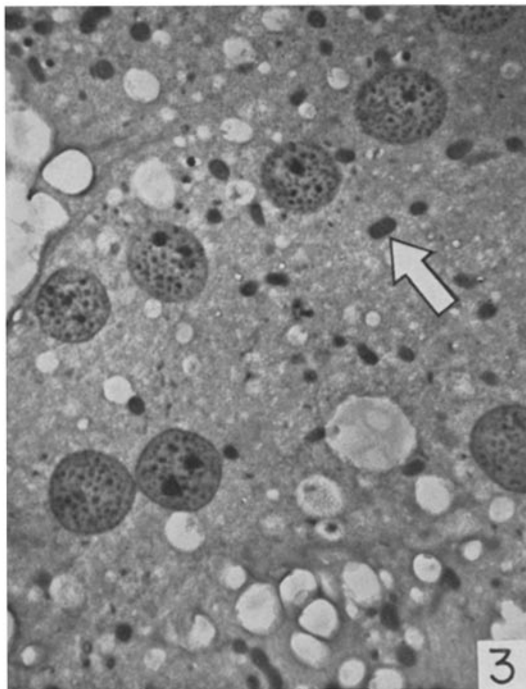

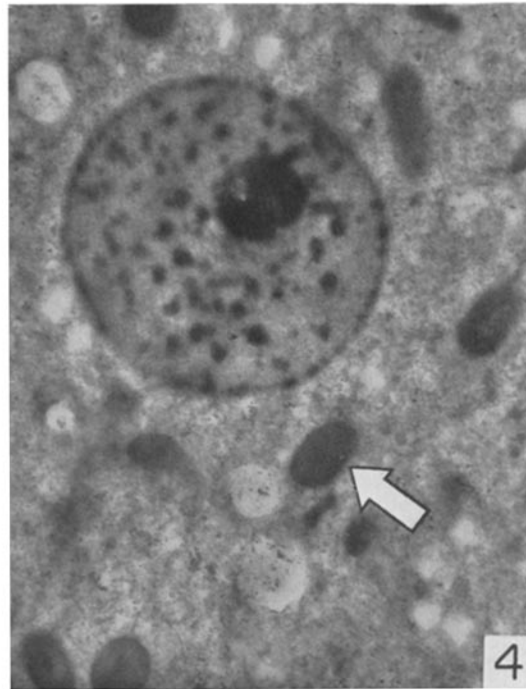

(Harris and Mazia: Electron microscope stain)

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