THE USE OF BISMUTH AS AN ELECTRON STAIN FOR NUCLEIC ACIDS

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ABSTRACT
Evidence is presented to show that bismuth combines in vitro with the phosphate of nucleic acids in a manner similar to its reaction with inorganic phosphate. When tested under similar conditions, protein exhibited no attraction for bismuth. The results of the in vitro experiments, which are of interest within themselves, may be indirectly applicable to in vivo staining. Dividing cells of onion root tips were fixed in OsO4, stained with bismuth, and examined in the electron microscope. The electron opacity of cell structures known to contain nucleic acids was enhanced by bismuth, while organelles known to lack appreciable quantities of DNA or RNA showed little, if any, change. Bismuth is particularly effective as a stain for the chromatin material during interphase and for the chromosomes during division.

INTRODUCTION
Heavy metals have, so far, proven to be indispensable for electron microscopy. Many are of value as general stains, and some have given evidence of significant specificity. We should like to introduce bismuth as a member of the latter group. Perhaps this metal has been passed over in previous studies because of the insolubility of commercially available salts (1). Bismuth, however, has several properties which convinced us that it might be valuable as an addition to the already imposing array of useful metals. First, it is one of the heaviest metals and thus adds near maximal density per attached atom. Secondly, and more important, bismuth forms highly insoluble complexes with phosphates (2).

Several successful attempts to stain nucleic acids have been published (1, 3–6). Despite the selective staining exhibited by these methods, all have been deficient in their capacity to show ultrastructure of chromatin in the intact tissue. This may be a result of the inherent complexity of chromatin and the inability of the electron microscope to resolve it, or it may be due to a limitation in our present methods of fixation and staining. It was this latter possibility that prompted the development of bismuth as a nucleic acid stain. Mitotic cells of the onion root tip were utilized throughout these studies, for it was deemed advantageous to compare our results with previously published pictures of similar tissue (7).

MATERIALS AND METHODS

Materials
Bismuth metal was purchased from Fisher Scientific Company, the highly polymerized salmon sperm DNA from the California Corporation for Biochemical Research, Inc., Los Angeles, and crystallized bovine plasma albumin from Armour Pharmaceutical Company, Kankakee, Illinois. The RNA was obtained from Schwarz Laboratories, Inc., Mount Vernon, New York, and purified further according to the procedure of Frisch-Niggemeyer and Reddi (10).

Analytical Procedures
Soluble bismuth was determined colorimetrically by the iodide method as described by Sandell (2). Phosphate was analyzed by the procedure of Allen (8). DNA and RNA were measured by their phos-
phorus content and by ultraviolet absorption at 260 and 280 m\(\mu\) based on the extinction coefficients of Warburg and Christian (9). The crystalline bovine plasma albumin was measured gravimetrically.

Precipitation of inorganic phosphate, DNA, RNA, and protein was carried out by adding a specified amount of these substances to a given quantity of bismuth dissolved in dilute \(\text{H}_2\text{SO}_4\). The solutions were adjusted so that the final volume was 2 ml (0.1 \(N\) with respect to \(\text{H}_2\text{SO}_4\)). The solutions were cooled in an ice bath for 15 minutes, centrifuged, and 1 ml of the supernatant analyzed for soluble bismuth, or, in the case of the inorganic phosphate, for both bismuth and phosphate. Four ml of acetone were added to the 2 ml bismuth-protein solution in an additional attempt to precipitate a bismuth-protein complex.

**Figure 1**
The binding of bismuth by inorganic phosphate to form a complex insoluble in dilute acid. Stoichiometric precipitation independent of the concentration of the constituents occurs when the phosphate concentrations are greater than \(5 \times 10^{-4} \text{M}\).

**Preparation and Staining of Tissue**
The micrographs contained within this report were obtained from cells within the first 2 mm of the growing root tips of *Allium cepa* (white onions). The 1 to 2 mm tips were cut at midday from roots 3 to 6 mm in length and placed directly in fixing solution. The root caps were then discarded and the tips split longitudinally. Fixation was carried out following the procedure of Millonig (11). The tips were placed in 2 per cent OsO4 in 0.1 \(m\) phosphate buffer (pH 7.3) and allowed to remain in the cold for 20 to 24 hours.

Bismuth stain was prepared by dissolving 20 mg of metallic bismuth in 0.2 ml of concentrated nitric acid (15.4 \(m\)). (Once the acid was added to the metal, care was taken to keep the bismuth covered with the acid in order to avoid oxidation of the metal.) This solution was then diluted to 80 ml. Four ml of 0.1 \(m\) citric acid was added and the solution adjusted to pH 7 with 1 \(N\) NaOH. (Slight cloudiness may occur about pH 2, but this should disappear at pH 4–5.) The volume was then increased to 100 ml and sucrose added so that its concentration was 0.2 \(m\). This solu-

**Figure 2**
Precipitation of bismuth by the phosphate of DNA and RNA. See text for explanation.

**Figure 3**
Two micrographs, each representing approximately one-half of a cell of the onion root tip during interphase. Photographic and printing conditions were as nearly identical as possible. Note the high density of the chromatin material in the upper bismuth stained micrograph. The electron opacity of the nucleolus and ribosomes of this cell has also been enhanced, but to a lesser degree. The cell in the lower micrograph and that in the upper micrograph have been treated in a similar manner except that the lower cell lacked bismuth in the staining solution. Upper micrograph \(\times 11,000\); lower micrograph \(\times 15,000\).
tion was then stored in the refrigerator and used as long as it remained clear. After fixation in OsO₄, the tissue was washed once with a control solution of 0.1 N HNO₃ buffered to pH 7 with sodium citrate (as above, but no bismuth), and then placed in the bismuth solution for 90 minutes at 0°C.

Dehydration was achieved by placing the tissue for 5 minutes in each of the following aqueous acetone solutions: 50, 75, 85, and 95 per cent. This was followed by six 5 minute washes in 100 per cent acetone and finally by a single 1 to 2 minute wash in 100 per cent propylene oxide. The specimens were then placed in a 1:1 propylene oxide–Epon mixture; the propylene oxide was permitted to evaporate overnight in the hood. The Epon mixture followed the recommendation of Luft (12), and specifically was the following: Epon Resin 812 (100 ml), methyl Nadic Anhydride (89 ml), and 2,4,6-tri(diethylaminomethyl)phenol (2 per cent). After the tissue had been allowed to remain at room temperature overnight, the specimens were removed, drained briefly on paper towels, and placed in fresh Epon in gelatin capsules. The capsules were then placed in a desiccator and evacuated with a water pump for 30 minutes. After removal of air bubbles, the tissue was moved to a desired location within the capsule and the capsules placed in a 60°C oven for 5 days.

Thin sections were obtained with the aid of a Porter-Blum ultramicrotome and an E. I. du Pont de Nemours and Company diamond knife. A RCA EMU-3D electron microscope was used throughout this study.

RESULTS AND DISCUSSION

In Vitro Experiments

In determining the effectiveness of histochemical stains, in vitro experiments with purified biochemicals are of limited value. This may be especially true in the experiments outlined below, for the in vitro measurements were facilitated by acidic solution, while the in vivo experiments, in order to better preserve the tissue, were routinely stained at neutrality. We should like to stress that, owing to this disparity in reaction conditions, these in vitro experiments should not directly be compared to in vivo staining. Nevertheless, we felt that prior to the staining of biological materials it would be worthwhile to see if bismuth would combine with organic phosphate in a manner similar to its complexing of inorganic phosphate.

The data of Fig. 1 demonstrate the stoichiometry for the precipitation of bismuth with inorganic phosphate. While bismuth was completely precipitated by excess phosphate, the reverse is not true. No precipitation was observed until the phosphate concentration approached 5 × 10⁻⁴ M. From the slope of the curve in Fig. 1 it is possible to determine that 1.07 atoms of bismuth were precipitated for each molecule of phosphate.

The experiments summarized by the curves of Fig. 2 were obtained by a procedure similar to that used to obtain the data of Fig. 1. However, the curves of Fig. 2 present the results of the precipitation of bismuth by DNA and RNA. The precipitation of bismuth by added DNA was more or less stoichiometric until the concentration of bismuth was reduced to less than 4 × 10⁻⁵ M, at which point the addition of increasing amounts of DNA had little further effect. However, by extrapolating to complete precipitation of bismuth, one could calculate that, for the initial linear portion of the curve, about 1.08 bismuth atoms were precipitated for each phosphate added as DNA. This figure compared favorably with that obtained for inorganic phosphate and encouraged us to hope that bismuth would be bound by cellular DNA.

The results obtained from the in vitro precipitation of bismuth with RNA phosphate were not so clear. RNA phosphate did not precipitate stoichiometrically. Increasing amounts of RNA precipitated less and less bismuth per added phosphate, although eventually the bismuth was completely removed from solution. By extrapolating (broken line) through the first two points of the RNA curve, one can determine that, at even the lower RNA concentrations, little more than 1 bismuth atom was bound for every 2 RNA phosphate groups added. Although the possibility is not unlikely, it is hard to say from this data whether

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Figure 4

Portions of two cells of the onion root tip. A part of an interphase nucleus (n) may be seen at the lower left and the majority of a prophase nucleus dominates the upper portion of this micrograph. A nucleolus (n) is evident in each nucleus, as is the more densely stained chromatin (c) material. The heavily stained objects adjacent to the nucleus of the upper cell are lipid (l) bodies. X 8,500.
one would expect less staining of RNA than of DNA in histological preparations.

Bovine plasma albumin was utilized to determine the ability of a phosphate-free protein to precipitate bismuth. Even an 0.5 per cent solution of this protein failed to precipitate any bismuth. Adjustment of this solution so that it became 67 per cent with respect to acetone still yielded no loss of bismuth from the solution. It is possible that the bismuth combined with the protein without precipitation. Nevertheless, this was a particularly encouraging result for, although bismuth could not determine decisively between DNA and RNA, it had distinct possibilities of yielding contrasting densities in the electron microscope between nucleic acids and proteins, and perhaps between nucleic acids and phospholipids, for phospholipids contain far less phosphate in proportion to their total weight than do the nucleic acids.

In Vivo Staining

Efforts were made to stain the tissue with bismuth both prior to and simultaneously with osmium tetroxide fixation. It was found, however, that best tissue preservation was achieved by applying the bismuth subsequent to fixation. Since our in vitro experiments were carried out under acidic conditions, staining of the tissue was attempted at hydrogen ion concentrations ranging from pH 2-9. Not only was the tissue better preserved in neutral solutions, but the deposition of bismuth seemed to be more nearly maximal. Staining of thin sections was not successful, presumably because of failure to penetrate the plastic. It was difficult to prevent precipitation of the bismuth at the high pH levels found to be advantageous for the staining of sections with lead (13).

The increased contrast due to bismuth, as seen in Figs. 4 to 7, made necessary only visual inspection of a section in the electron microscope in order to determine whether the tissue had in fact been treated with bismuth or whether it was an osmium-fixed control section. Control sections and stained tissue were prepared in an identical manner except that in the case of control sections bismuth was omitted from the staining solution. Fig. 3 contains two micrographs, each containing approximately one-half of a cell (and nucleus) in interphase. The photographic and printing conditions were as nearly identical as possible.

In Figs. 4 to 7 we have presented a series of electron micrographs depicting onion root tip cells in various stages of mitosis. All the plates were obtained from tissue previously stained with bismuth. Fig. 4 contains the image of a portion of a cell in interphase (lower left) and the majority of an adjoining cell in early prophase. The nuclear membrane and nucleolus of this cell are still clearly discernible. The densely stained structures in the cytoplasm located near the edge of the nucleus are presumably lipid bodies. Such bodies are found to be heavily stained even in osmium-fixed controls.

The cell shown in Fig. 5 has been sectioned transversely through the metaphase spindle at the level of the chromosomes. The chromosomes at this stage of division stain particularly well with bismuth. The cell pictured in Fig. 6 has reached anaphase. The poles of the spindle (not seen by this method) are below center to the left of this micrograph and to the right center, but presumably not in the plane of this section. Finally, in Fig. 7 a cell is depicted which is in telophase. Considerable quantities of lipids at either end of the newly forming cell plate are clearly seen. There are also several bodies along the cell plate which resemble the phragmosomes previously described by Porter et al. (7, 14). During telophase the chromatin appears to be more highly compacted than at any other time. The nucleolar material once again becomes easily distinguished during this terminal stage of division. Portions of this material seem to be derived from the chromatin of each dispersing chromosome. This nucleolar material will soon coalesce into one or sometimes two nucleoli.

It is noteworthy that, as a result of the bismuth treatment, the chromatin was densely stained in all stages of mitosis. Throughout division, even at high magnifications (high resolution studies will be presented in a later report), the chromatin

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**Figure 5**

Micrograph of transverse section through metaphase spindle at level of chromosomes (ch). The chromosomes at this stage of division stain particularly well with bismuth. × 10,500.
maintains a characteristic appearance. The nucleolar material, which is also stained by bismuth, is discernible whenever it is present in somewhat condensed form; and it may be distinguished from the chromatin by its slightly lower density. The ribosomes also seemed to have achieved some increase in density due to bismuth, but perhaps not so much as one would expect on the basis of their high RNA content. It is possible that the RNA in the ribosomes is bound in such a manner that its phosphate is not available to the bismuth (see discussion of in vitro experiments). It is known that the RNA of intact ribosomes is resistant to RNase. The cell wall remained unstained by bismuth and thus, on a comparative basis, must contain little phosphate. That bismuth leaves considerable material in the wall was easily demonstrable by counter staining a section with lead hydroxide. The lead rendered the cell walls of such bismuth-stained sections extremely dense. The cell pictured in Fig. 8 was obtained from a section of a bismuth-treated tissue. The section had, however, subsequently been stained with lead by the method of Millonig (13). The contrast in the mitochondrion, ribosomes, and various membranes has been greatly enhanced so that they are now the equivalent of the chromatin and nucleolus. The prophase chromatin and the nucleolus are considerably more granular in texture than are bismuth-stained sections untreated with lead. The granularity of the nucleolus is reminiscent of the granularity of the ground cytoplasm owing to the presence of ribosomes. Evident, too, are interchromatin strands, which are considerably more difficult to distinguish in the absence of lead staining. This may be an indication that these strands are not composed of nucleic acids but of some substance more densely stained by lead than by bismuth.

The results of these experiments have indicated to us that bismuth is indeed another of the growing list of heavy metals with considerable selectivity in its affinity for binding to the chemical constituents of cells. Of these, the nucleic acids, particularly DNA, appear to have the greatest ability to bind bismuth. Perhaps the most important property of this metal that would enable it to be of value as an electron-opaque stain is its relative lack of affinity for proteins. This characteristic should aid in the elaboration of chromatin ultrastructure by allowing one to observe DNA through a veil of closely associated histones.

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FIGURE 6
Oblique section through a cell in anaphase. The spindle poles appear to be to the lower-left of the micrograph and the right center. The chromosomes (ch) remain clearly discernible. Vacuoles (v), dictyosomes (d), mitochondria (m), and proplastids (p) are visible as a result of OsO4 fixation, but have received little or no enhancement from the bismuth treatment. X 8,500.
FIGURE 8
Shows a portion of a cell in prophase. The tissue was fixed with OsO₄, stained with bismuth, and the section subsequently counterstained with lead. Note the granular appearance of the nucleolus (ncl) and chromatin (chr). Also shown are the nuclear envelope (ne), a mitochondrion (m), and endoplasmic reticulum (er) with associated ribosomes. Note, too, the now evident interchromatin strands (ics). X 33,500.

FIGURE 7
Division at the time of cell plate (cp) formation. Portions of two telophase nuclei (nse) are shown. The chromatin (chr) material is more densely compacted, and thus stained, than at any other stage of division. The nucleolar (ncl) material of each nucleus is also evident. Shown, too, are several phragmosomes (ph), frequent dictyosomes (d), and considerable lipid (l) material. X 10,500.