METHODS FOR THE USE OF INDIUM AS AN ELECTRON STAIN FOR NUCLEIC ACIDS

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ABSTRACT

Methods are presented for the staining of blocks of tissue with trivalent indium so that good contrast and good specificity for nucleic acids is achieved for the electron microscope. The tissue is fixed in organic fixative, dehydrated, subjected to reduction by lithium borohydride, acetylated by acetic anhydride, stained with trivalent indium dissolved in organic solvent, and embedded. The embedding material may be either Vestopal or butyl methacrylate especially handled to eliminate the "explosion" phenomenon. Numerous new problems encountered are discussed and a brief description of the findings is included.

INTRODUCTION

To our knowledge, no electron stain for intact tissue has been described as specific for nucleic acids for which the claim is supported on chemical grounds. The present work describes such a technique. Huxley and Zubay (1) indicate that aqueous uranyl complexes specifically with ribonucleic acid (RNA) in a purified cell fraction of ribonucleoprotein (RNP) particles. A short note on staining virus nucleic acid with ferric ion has been published by Bernstein (2) with some chemical information on complexing with nucleic acid, but no data on other cell components. There are indications that staining with aqueous uranyl (3, 4) or lead hydroxide (unpublished data) of sections of tissue fixed with organic fixative may yield fair specificity for nucleic acids. We know of no published information, however, concerning the physical and chemical mechanisms involved in the staining of sections for the electron microscope.

Some features of the aqueous chemistry of trivalent indium with particular reference to indium–nucleic acid complexes and the use of indium in the isolation of nucleic acids have been discussed by one of us (5). As an outgrowth of these studies we recently made a preliminary report on methods for the use of indium as an electron stain with specificity for nucleic acids (6). Further investigation has forced many modifications of these methods before indium could be considered a reliable stain for electron microscopy. Described here are procedures for the staining of nucleic acids with indium which yield good contrast and good specificity. The general chemical reasoning behind these procedures is given together with examples of the results obtained.

DESCRIPTION OF METHODS

In order to achieve the most intense staining together with the highest degree of specificity for nucleic acids, a number of factors must be considered. Since some of them are novel to electron microscopy as well as essential to the procedure, it is desirable to discuss certain of the steps in detail before presenting the actual protocol of tissue preparation. The procedure may be summarized as follows: (*a*) fixation in organic fixative; (*b*) dehydration; (*c*) reduction

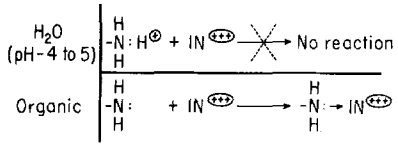
by $LiBH_4$ in pyridine; (*d*) acetylation by acetic anhydride in pyridine; (*e*) infiltration and staining by indium trichloride in acetone; (*f*) embedding and sectioning; (*g*) microscopy.

Fixation

In general, every effort is made to reduce to as low a contrast as possible those tissue components which are not stained by indium. For this reason, it is necessary to eliminate from the procedure all traces of heavy metal other than indium. Thus metal-containing fixatives cannot be used, and only organic fixatives are permitted. Of the various known organic fixatives, we have had experience with formalin, acrolein (7), 70 per cent ethanol, and mixtures of methanol and acetic acid. The best of these for electron microscopy appear to be acrolein and methanol containing 40 per cent acetic acid. For general purposes acrolein will probably be preferred. Acetic-methanol is not suitable for many tissues because it introduces considerable shrinkage of various components. Occasionally, however, it is useful for more distinct discrimination between certain nucleic acid–containing components in the nucleus (examples of this will be presented here) and because it can be expected to retain even small polynucleotides which might be lost in other fixatives.

Blocking Reactions

In our preliminary work (6), staining with indium was carried out in aqueous medium. It has since been found desirable to perform the staining in organic solvent (see below under Staining). In organic solvent, the relatively high specificity of trivalent indium for nucleic acid phosphate, which could be predicted with some confidence in aqueous medium, is markedly reduced. Ionization of many groups in aqueous medium either prevents the approach of, hence reaction with, In^{3+} or otherwise alters the group so that it is not reactive with indium. It may be noted that a condition for specificity was that the reaction was carried out at pH 4 to 5. As a simple example, the situation is illustrated in the case of an amino group:



In organic solvent, as indicated in the example given, such ionization is almost completely suppressed so that $—NH_2$, —OH, —COOH, —CO—, —CHO—, and $—SO_4$ as well as $—PO_4$ will complex with indium. Sulfhydryl groups are not considered significant because it has been reported by van Duijn (8) that they are blocked by treatment with acrolein, while preliminary results suggest that $—SO_4$ may not be a problem (9). The only compounds containing the listed groups which will be of importance in reducing specificity for nucleic acid phosphate will be those which are not washed out of the tissue during fixation and dehydration before exposure to indium. Thus, we are not concerned with inorganic ions including phosphate or with many small organic phosphates because these will be soluble in the fixative and lost from the tissue. Some of these phosphates and others, even if present and incompletely esterified, may not react because the stereochemistry of the molecule is involved and may be unfavorable for chelation. Most "salts" of organic molecules involving indium are chelates (10, Vol. I, pp. 458–489). Glucose-6-phosphate and sodium hexametaphosphate are examples. Although on the basis of conductimetric data a water-soluble indium complex forms with glucose-6-phosphate (9), it is not precipitated. Sodium hexametaphosphate forms a water-insoluble complex with indium (9). However, from organic solvent (acetone) no indium is bound by either compound (9).

Trivalent indium, in contrast to uranyl (11), is only slightly soluble in esters and does not form addition compounds with esters (10, Vol. I, pp. 458–489). Since indium will react only with phosphate which is not completely esterified, we might be concerned with phospholipids whose phosphate is in this condition. Membranes are well known to contain phospholipid; however, it appears that both acrolein followed by dehydration and acetic-methanol remove almost all such phospholipid. As will be seen, after the blocking reactions to be described, membranes do not stain. A considerable amount of phospholipid is not extracted even by treatment with chloroform-methanol. This phospholipid must be firmly bound to structural proteins, probably through a phosphodiester linkage with hydroxylated amino acids or associated polymeric carbohydrates (12, pp. 297–299; 13, 14).

There remain numerous compounds, notably protein and carbohydrate, which contain the reactive groups listed above and which we do not desire to stain. It is therefore necessary to use reactions designed to block these groups from complexing with indium. Remembering that indium does not react with esters, we find that a number of possible blocking reactions exist, but of these the most promising appears to be acetylation or methylation. In our hands, methylation has so far not proved effective. Acetylation results in the formation of an amide linkage with $—NH_2$ (15) and an ester linkage with —OH, including phenolic —OH but not tertiary

—OH (16, p. 189). It is important to note, because of their presence in nuclear histone, that basic amines also are blocked by acetylation (17). The procedure used here is that most fully investigated by Lillie (17) and involves incubation of tissue in anhydrous pyridine containing 40 per cent acetic anhydride with a trace of anhydrous sodium or potassium acetate to catalyze the reaction. Such treatment is expected not to affect appreciably carboxyl, carbonyl, or aldehyde groups. Carbonyl and aldehyde groups, however, are readily reduced to the alcohols by exposure to a saturated solution of lithium borohydride in cold, anhydrous pyridine (16, p. 116). Of other hydrides which might be used, lithium aluminum hydride forms aluminum hydroxide (16, p. 116), which is insoluble in pyridine and might be confused with indium in the electron microscope; sodium and lithium hydrides are too reactive for safety, and the remainder either contribute density problems or are insufficiently reactive. After reduction and acetylation, we are left with carboxyl groups which are mainly unblocked and with possible, although probably very small, amounts of mixed higher anhydrides formed by exchange reactions between tissue components and acetic anhydride.

Staining

As noted above, staining with indium as originally reported by us was carried out for reasons of specificity in aqueous medium. It has since appeared, however, that the amount of indium incorporated under these conditions was insufficient to provide generally useful contrast. It was found that contrast obtained when the staining was performed in alcohol was greatly increased over aqueous staining and that a correspondingly greater increase resulted when the solvent was acetone. This increase in contrast proved of such value that staining in acetone was adopted despite the added requirement of blocking reactions described above. The reason for the added contrast appears to lie in the tendency for indium to form polymers in organic solvent (10, Vol. I, pp. 458–489). It has been suggested, consistently with our observations, that indium forms a dimer in alcohol (18) and a tetramer in acetone (10, Vol. I, pp. 458–489), whereas in water only the monomeric form is present as long as the pH is not raised above 4 or 5 (19).

Embedding

Only three embedding materials appear to be in general use by electron microscopists: polybutyl methacrylate, Vestopal, which has been described as a polyester (20), and various Epoxy resins. Of these, we can recommend only the first two, butyl methacrylate, in our experience, being preferred if certain precautions are taken. Epoxy resins as they are currently used all contain an anhydride which effectively removes indium from the tissue. Much of the indium is removed after polymerization, so that although a freshly polymerized block may show adequate contrast, after a week or two it has become useless.

Tissue may be embedded in Vestopal by standard methods such as those suggested by Ryter and Kellenberger (20); however, special precautions must be taken in the case of methacrylate. Tissues fixed in organic fixative are notoriously difficult to embed in methacrylate because of the well known "explosion" phenomenon, in which a part or all of the tissue greatly increases in volume during the course of polymerization. This apparently is due to accelerated polymerization within the tissue. Polymer inside forms a gel at an early stage which swells greatly as monomer from the relatively unpolymerized plastic outside enters and dissolves in it. Two methods are suggested which will prevent this. One method is to add an agent, divinyl benzene in our case, which cross-links the polymer as it forms and renders it insoluble in monomer. The second method is to use as initiator α,α'-azodi-iso-butyronitrile rather than the usual peroxide initiator. Why this prevents "explosions" is not clear, but it may have to do with a higher rate of polymerization which successfully competes with possible accelerating groups within the tissue. By both methods, polymerization is carried out at room temperature under ultraviolet light. In the latter case it is complete in 2 or 3 hours. The α,α'-azodi-iso-butyronitrile has proved to be extremely allergenic to one of us and is not used in this laboratory.

Both these methods for embedding in methacrylate present certain mechanical difficulties which we cannot at present eliminate. Blocks of cross-linked methacrylate tend to split vertically during the course of polymerization. This split never passes through the tissue and does not interfere with sectioning provided both parts are held in proper register when the block is placed in the chuck of the microtome. When the nitrile initiator is used, the rate of polymerization is so rapid that numerous bubbles form although not inside the tissue. Bubbles which form directly beneath the tissue and which might render it unstable during sectioning can be filled with paraffin wax. Bubble formation can be much reduced by carrying out polymerization at $-20°C$ or lower.

Tissues which have undergone the acetylation procedure are greatly toughened so that regardless of the embedding material used they are relatively difficult to section. Experience with glass knives indicates that they are rapidly dulled, but that a new edge will cut satisfactory sections. We do not have experience with diamond knives.

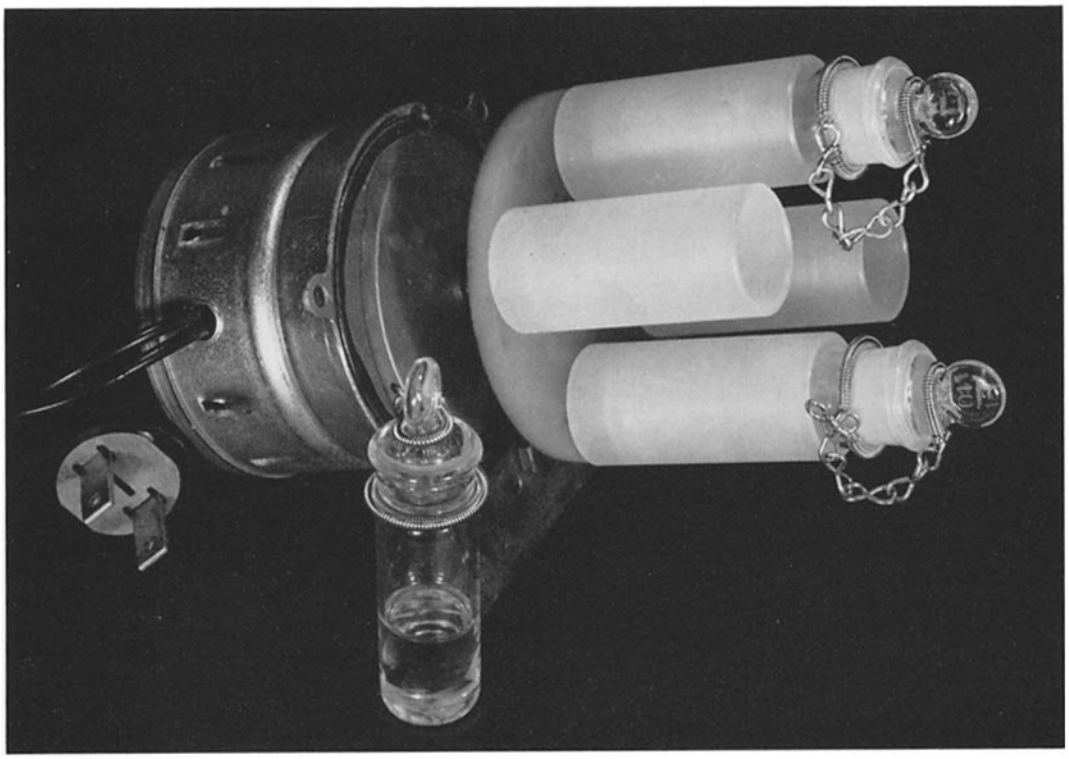


FIGURE 1

Stirring device for agitating specimens during preparation. A synchronous clock motor rotating at 10 to 30 RPM is supported so that the rotor is inclined at about 30 degrees from the horizontal. On the rotor is mounted a turret of polyethylene designed to hold four specimen bottles parallel to the axis of the rotor. The power requirements of the motor are sufficiently low so that it may be operated in a small refrigerator.

Microscopy

Contrast between different areas in an electron micrograph depends (other things being equal) roughly on the differences in physical density between corresponding areas in the specimen. For our present purposes, an ideal embedding material would have the same physical density as the various components of unstained tissue. Such components would then be invisible (for practical purposes) in the electron microscope. Addition of heavy metal to the tissue would result in an increase in density of those tissue components which complexed with the metal atoms. Such components, and only those, would become visible in the microscope. If unstained tissue were also visible, the method would lack specificity because we would be uncertain as to which elements were stained and which were not. In this regard, Vestopal is almost ideal. Its density approximates that of tissue, while exposure to the electron beam has little effect on it. In the case of methacrylate, however, matters are different. Although the density of polymethacrylate is also about the same as that of dry tissue, but slightly lower than that of Vestopal, roughly 50 per cent of it is lost from a section on exposure to ordinary beam intensities (21). This means that the effective density of the embedding material is only about half that of the tissue. In our hands, this difficulty is almost completely eliminated by sandwiching the section between the carbon substrate with which the grid is covered and a film of Formvar (22). Just why this is so effective in reducing the undesirable effects of sublimation is not known, but probably it involves increased retention of embedding material. Methacrylate is preferred in the present work because the contrast obtained from a sandwiched section of methacrylate is higher than that from an unsandwiched Vestopal-

embedded section. This may be due mainly to the higher density of Vestopal. In addition, methacrylate is considerably easier and more rapid to use.

Materials

Sources for the various materials necessary are as follows: Acrolein, pyridine, acetic anhydride, α-terpineol (note: not terpinol), styrene, α,α'-azodi-iso-butyronitrile, and benzoyl peroxide may be found in the list of Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York. Where more than one grade is listed, the purest is preferred. Lithium borohydride, analytical grade, is supplied by Metal Hydrides, Inc., Beverly, Massachusetts. Indium trichloride, anhydrous, analytical grade, is supplied by the Indium Corporation of America, Utica, New York. A mixture containing about 50 per cent divinyl benzene can be obtained from Monomer-Polymer Laboratories, The Borden Chemical Company, Philadelphia, Pennsylvania. For ultraviolet polymerization the source used is a Blak-Ray model XX-15 long wave lamp manufactured by Ultra-Violet Products, Inc., San Gabriel, California, and obtainable from laboratory supply houses. Vestopal W is supplied by Martin Jaeger, Chemin de Mancy, Vésenaz/Geneva, Switzerland.

Procedures

Throughout the preparation procedures it has been found essential to agitate the tissue blocks to ensure thorough exposure to the various solutions. This is also of value during infiltration with embedding materials. Two devices as shown in Fig. 1 were constructed using electric clock motors rotating at 10 and 30 revolutions per minute, respectively. The slower speed is useful for viscous embedding materials such as Vestopal. The motors are Hurst synchronous motors, type SM-10 and SM-30, respectively, and may be obtained from Allied Radio Corporation, 100 North Western Avenue, Chicago 80, Illinois. They are fitted with polyethylene rotors on which are mounted four polyethylene containers to hold the specimen bottles. Polyethylene is used partly for thermal insulation and partly for its resistance to the reagents used. One of these devices is kept in the refrigerator for those steps which must be carried out in the cold, and the other is kept at room temperature.

The procedures now recommended for staining of tissues with indium are presented below. Percentages given are based on volume measurements or, in the case of solids or liquids, on weight of solid to volume of liquid assuming a density of unity. Tissue blocks should be less than 1 millimeter on edge to ensure good embedding.

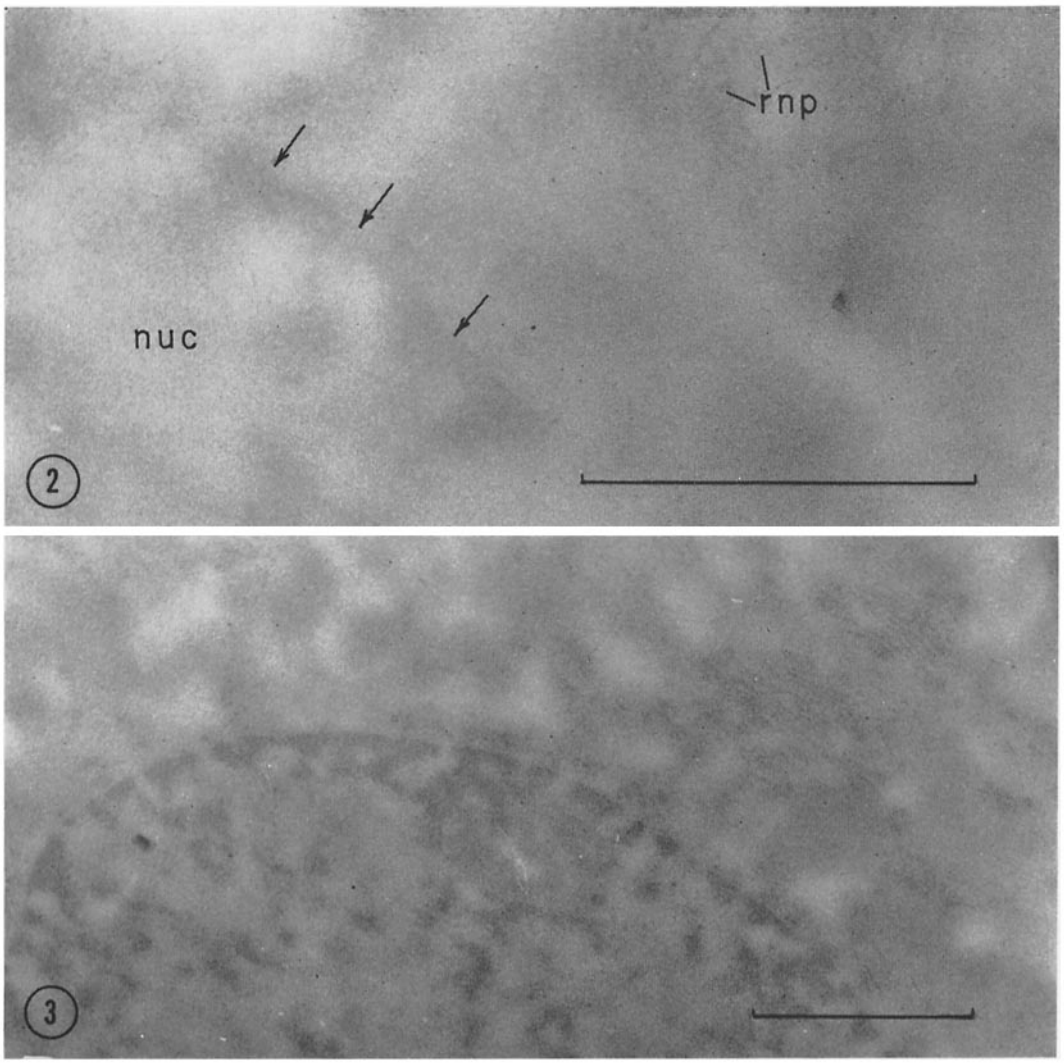
1. Fix at 0–5°C either in methanol containing 40 per cent glacial acetic acid for 15 to 30 minutes or in 10 per cent acrolein in water for 30 minutes.
2. Transfer acetic-alchol–fixed tissue to 2 washes in absolute methanol of 10 minutes each at 0–5°C.

Dehydrate acrolein-fixed tissue for 5 minutes each in 25, 50, 75, and 90 per cent acetone in water, and absolute acetone at 0–5°C.

3. Add pyridine in small amounts to the tissue in acetone or methanol so that it is brought to pyridine in 3 steps of 5 minutes each at 0–5°C.
4. Wash 3 times in pyridine for 10 minutes each at 0–5°C.
5. Incubate in pyridine saturated with $LiBH_4$ for 2 hours at 0–5°C. The saturated solution should be made up some hours before use and kept at 0–5°C.
6. Wash 3 times in pyridine for 10 minutes each at room temperature.
7. Incubate in pyridine containing 40 per cent acetic anhydride saturated with a trace of anhydrous sodium or potassium acetate overnight at room temperature. The acetylating mixture must be made up not more than 1 hour before introducing the tissue.
8. Wash 3 times in pyridine for 10 minutes each at room temperature.
9. Wash once in 50:50 pyridine-acetone for 5 minutes at room temperature.
10. Wash 3 times in acetone for 10 minutes each at 0–5°C.
11. Incubate in acetone containing 25 mg/ml anhydrous $InCl_3$ for 2 hours at 0–5°C. This solution is stable at room temperature.
12. Wash twice in acetone for 15 minutes each at 0–5°C.
13. Wash in 50:50 acetone–butyl methacrylate mixture for 5 minutes at room temperature. For cross-linking, use distilled *n*-butyl methacrylate containing 3 per cent divinyl benzene solution (see list of sources), 3 per cent α-terpineol, and 1 per cent benzoyl peroxide. Or use 1 per cent α,α'-azodi-iso-butyronitrile alone if cross-linking is not desired. It is important that dibutyl phthalate, widely used as a plasticizer in methacrylate embedding, not be used with indium. It strongly complexes with indium and will remove it from the tissue.
14. Wash 3 times in methacrylate solution (above) for 60 minutes each at room temperature.
15. Polymerize in gelatine capsules under ultraviolet at room temperature.

Specimen capsules are mounted about ½ inch below the ultraviolet lamp, and beneath them is placed aluminum foil to reflect radiation back into the capsules. Polymerization is complete in 12 hours for cross-linked methacrylate or in 2 to 3 hours when the nitrile is used.

Indium stained tissue embedded in methacrylate



The bar in each micrograph indicates 1 micron.

FIGURES 2 AND 3

Appearance of acrolein-fixed rat liver embedded in cross-linked methacrylate and sandwiched to minimize loss of embedding material, but not stained with indium. These micrographs illustrate the extremely low contrast afforded by unstained tissue and serve to emphasize the importance of sandwiching to maintain this low contrast when the embedding material is methacrylate.

FIGURE 2

This section was mounted on a carbon film and sandwiched with Formvar exhibiting silver to gold interference color on water. The surface of the nucleus (*nuc*) is indicated by arrows, and a number of RNP particles (*rnp*) are just discernible in the upper right corner of the micrograph. × 65,000.

FIGURE 3

This section was sandwiched with a layer of Formvar thinner than that in the previous micrograph. The thinner sandwich and the lower magnification make tissue details more easily seen. Nevertheless, contrast is still much lower than is required for practical microscopy. × 23,000.

as described may be sectioned onto water containing about 15 per cent acetone and spread with xylene vapor. However, to avoid possible extraction of indium the sections should not be allowed to remain wet any longer than necessary.

If it is desired to embed in Vestopal instead of methacrylate, the procedure which follows has proved satisfactory. Vestopal appears to contain styrene mixed with another component. Since the polymerization of styrene is strongly inhibited by the presence of dissolved oxygen, the monomer should be kept in sealed containers. Convenient dispensers are 50 ml hypodermic syringes provided with caps. Best results have been obtained when the tissue is passed through a rinse of styrene monomer between the last acetone and the Vestopal. The Vestopal readily polymerizes under ultraviolet light at room temperature without the addition of activator or initiator supplied by the manufacturer. Since these substances may complex with indium and affect the intensity of staining and since they contribute nothing to the polymerization if ultraviolet is used, we prefer to omit them. The procedure for Vestopal embedding after step 12 above is as follows:

13. Wash in 50:50 acetone-styrene (monomer without removal of inhibitor) for 5 minutes at room temperature.

14. Wash twice in styrene for 15 minutes each at room temperature.

15. Wash in Vestopal containing about 30 per cent styrene for 30 minutes at room temperature.

16. Transfer tissue to Vestopal without added styrene, bringing over as little styrene as possible, and leave overnight at room temperature with agitation.

17. Blot tissue blocks on a paper towel to remove excess Vestopal, place in the bottom of gelatine capsules, and fill the capsules with fresh Vestopal. Polymerize with ultraviolet light as described above.

The tissues used to obtain the accompanying micrographs were removed from laboratory stock rats anesthetized with ether. They were sliced into blocks less than 1 millimeter on edge under cold fixative on a polyethylene surface using a razor blade. Micrographs were made with the Siemens Elmiskop I electron microscope operating at 80 kv and provided with a 50 micron objective aperture. Eastman Kodak Contrast lantern slides were used and developed 3 minutes in Eastman D-19 developer. Prints were made by conventional procedures.

RESULTS

Contrast Considerations

Figs. 5 through 15 show the typical appearance of sections of tissue stained in the block with indium. We can state with some assurance that

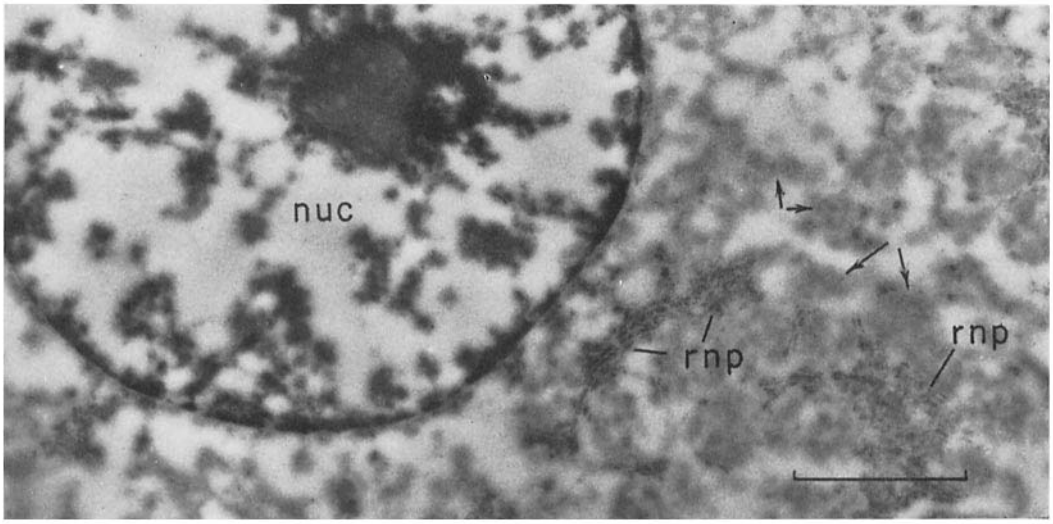
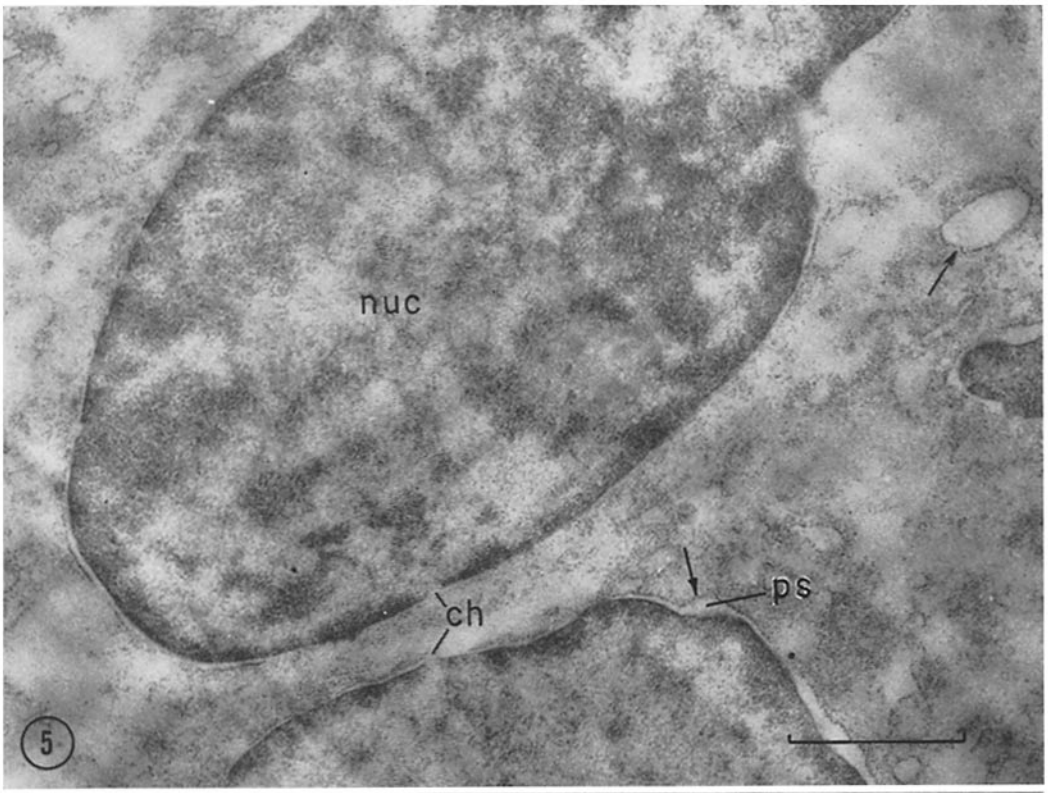


FIGURE 4

Section of rat liver fixed with acetic alcohol, stained with indium, and embedded in cross-linked methacrylate. This section was unsandwiched, so that sublimation of embedding material has revealed many tissue elements (arrows) which were unstained and would otherwise be nearly invisible as in Fig. 3. In addition to unstained tissue, nucleic acid in the nucleus (*nuc*) stands out sharply, and masses of RNP particles (*rnp*) are seen in the cytoplasm. × 23,000.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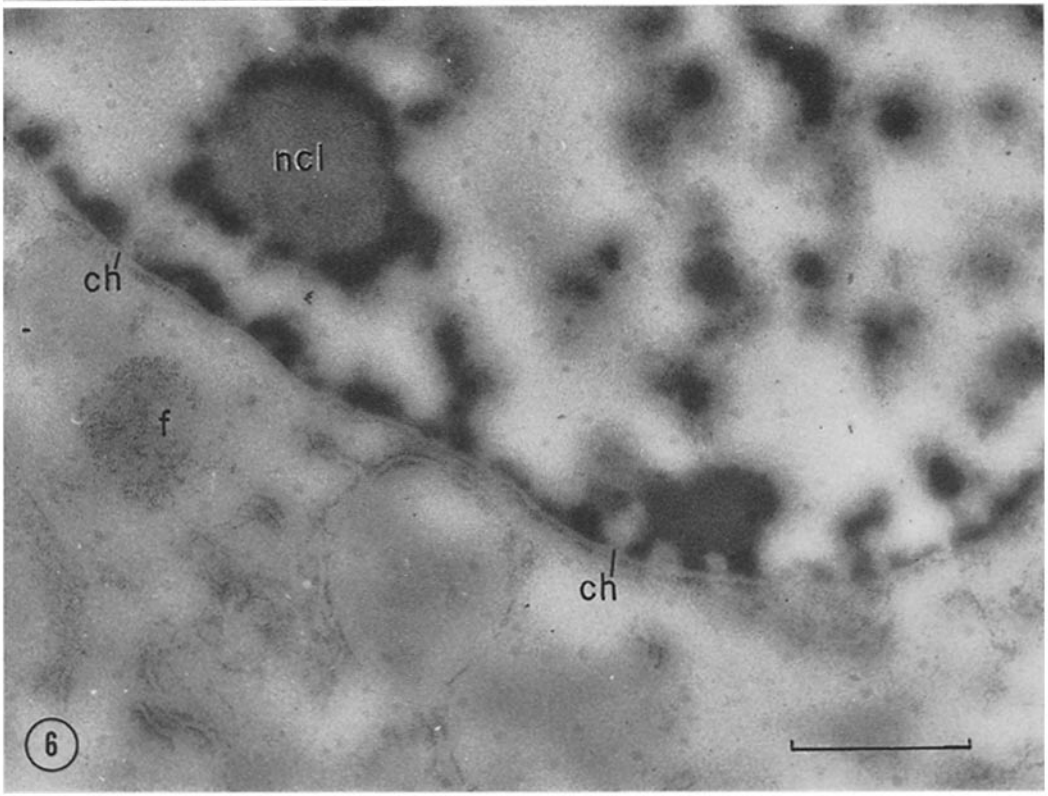
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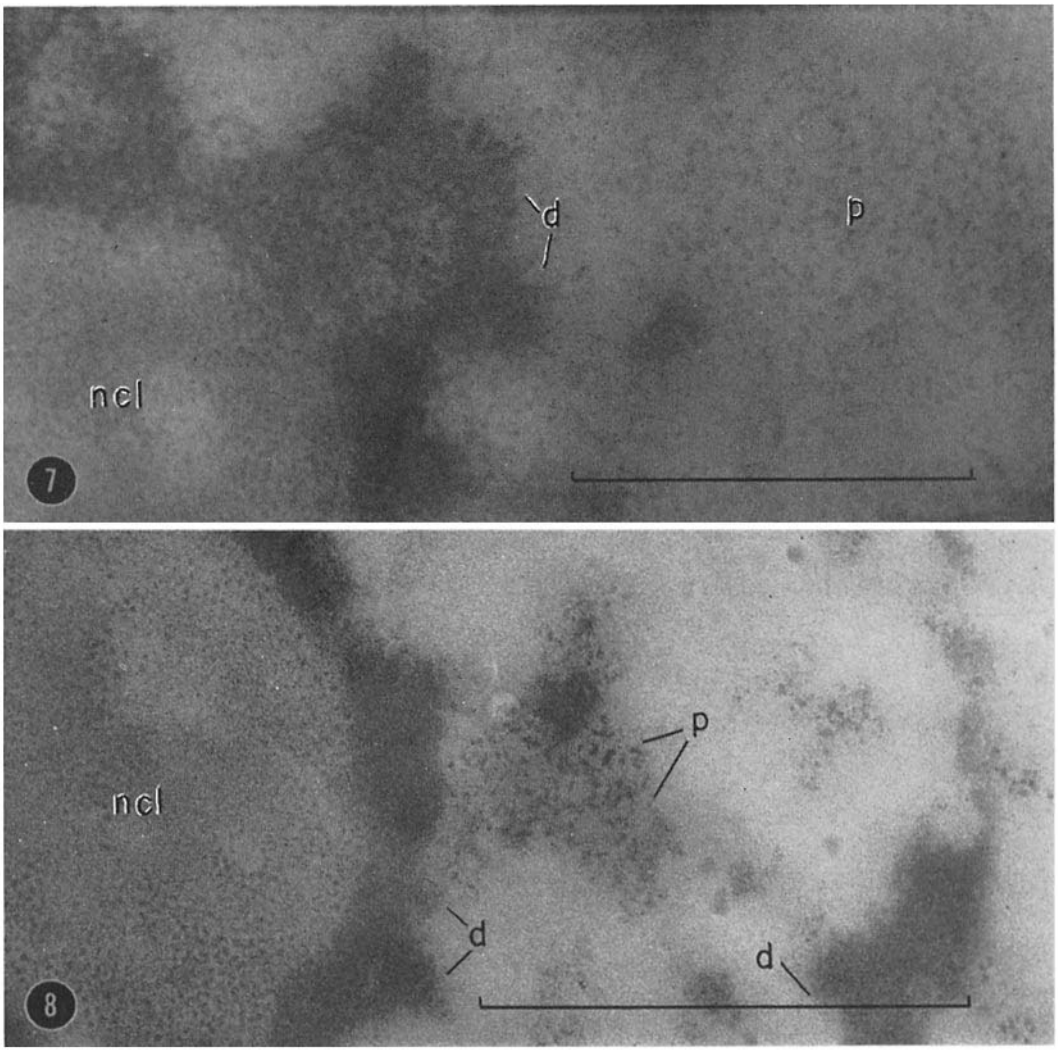

FIGURES 5 AND 6

Comparison at low magnification of tissues fixed with acrolein and with acetic alcohol and then stained with indium.

FIGURE 5

Rat mammary tissue fixed with acrolein, stained with indium, and embedded in Vestopal. The relatively diffuse appearance of staining material in the nucleus (*nuc*) is typical of acrolein fixation and somewhat resembles osmium fixation in this respect. In the cytoplasm, numerous RNP particles are present which in some areas outline "vesicular" regions so densely as to give the appearance of membranes at this low magnification (arrows). The perinuclear space (*ps*) is limited on one side by RNP particles and on the other by chromatin. Channels in the nuclear chromatin which mark the sites of pores appear at *ch*. × 23,000

FIGURE 6

Rat liver fixed with acetic-alcohol, stained with indium, embedded in cross-linked butyl methacrylate, and sandwiched. Details in the cytoplasm are much the same as in the previous micrograph; however, it is evident

the only tissue elements readily visible in these micrographs are those which have been stained. As evidence for this, we note the extremely low contrast of unstained tissue in a properly sandwiched section (Fig. 2). This micrograph is at relatively high magnification, where phase contrast effects can be expected to provide an important part of image contrast. At lower magnification, where loss of electrons by scattering out of the objective aperture is important, some contrast of large, unstained tissue components will exist despite sandwiching. In addition, if the sandwiching layer of Formvar is thin as in Fig. 3, we can expect to see small tissue components as well. Nucleic acids are particularly evident in this micrograph because, owing to shrinkage during dehydration and the presence of phosphate, they have rather higher density than proteins. It is desirable, therefore, that the Formvar film used for sandwiching methacrylate sections be thick enough, and we recommend a film which exhibits silver to silver-gold interference when viewed on water.

One can imagine situations where it would be important to see not only the indium-stained tissue elements, but unstained tissue as well. For example, one may wish to examine mitochondria for the possible presence of small amounts of nucleic acid. It is likely that mitochondria are present in some areas of Figs. 5, 6, and 10, but one cannot precisely define their limits. If the sandwich is deliberately omitted (Fig. 4), then a sort of "counterstaining" results whereby unstained elements are sufficiently visible so that their limits can be defined. Thus, examination of unsandwiched sections may sometimes be valuable. "Counterstaining" of this sort is not possible with Vestopal, which is resistant to sublimation.

Fixation

Whether to use acrolein or acetic-alcohol as fixative depends on the type of information desired. Acrolein (Figs. 5, 7, and 9 to 15) provides the best general morphology, while acetic-alcohol (Figs. 6 and 8) provides greater distinctness of nucleic acid as well as some assurance that everything larger than small polynucleotides is retained in the tissue. With both these fixatives the general disposition of nucleic acids is similar. For example, as described earlier (6), there are in the nucleus: (*a*) the nucleolus, which contains dense

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FIGURE 6 *continued*

that strong clumping of nuclear material has occurred. Despite this, channels (*ch*) which are part of the pore complex can be seen as in the previous micrograph, and the nucleolus (*ncl*) is essentially unchanged (*cf.* Figs. 8 and 9). What is probably a ferritin-containing body appears in the cytoplasm at *f*. × 23,000.

FIGURES 7 AND 8

Comparison of acrolein with acetic-alcohol fixation at moderate magnification. Rat liver stained with indium, embedded in cross-linked methacrylate, and sandwiched. In both cases, only the interior of the nucleus is shown. The nucleolus appears at the left (*ncl*) and is seen to contain dense particles about the same size as RNP particles (*ca.* 150 A) embedded in a rather homogeneous matrix which also appears to stain with indium. Characteristically, in normal rat liver the nucleolus is nearly surrounded by a mantle of densely staining material (*d*) which also tends to concentrate at the surface of the nucleus (see, for example, Fig. 6). Scattered about outside the nucleolus, but within the nucleus, are loose clumps of rather irregularly shaped particles (*p*) which also stain with indium. These particles can be seen with both fixatives, but are more distinct and easily identified after acetic-alcohol fixation.

FIGURE 7

Acrolein fixation. × 65,000.

FIGURE 8

Acetic-alcohol fixation. × 65,000.

particles about 100 A in diameter embedded in a more or less homogeneous but staining matrix; (*b*) clumps of dense and possibly stranded material which lie on the surface of the nucleolus and also congregate at the surface of the nucleus, in general following the pattern of Feulgen-positive material; and (*c*) loose clusters of granules or short rodlets scattered about outside the nucleolus. These appearances can be found both in acrolein (Fig. 7) and in acetic-alcohol (Fig. 8) fixed tissues. Ribonucleoprotein (RNP) particles follow the same general patterns in the cytoplasm with both fixatives.

Specificity

An important part of this investigation has been the reduction of non-specific staining by acetylation. The expectation, discussed in the Introduction, of such interference when staining is carried out in organic solvent has been amply justified. Figs. 9 and 12 show the appearance of unacetylated tongue muscle and testis, respectively. Among the structures which stain non-specifically are, in Fig. 9, collagen, muscle filaments and Z band, mitochondria, and cell membranes, and, in Fig. 12, the chromatoid body, mitochondria, and cell membranes. Acetylation does not completely eliminate staining of these structures, but reduces it to practical limits. Thus, collagen and muscle filaments are barely visible (Figs. 10 and 11), while mitochondria and cell membranes (Fig. 10) cannot be seen at all. In the testis, the chromatoid body and mitochondria are much reduced in intensity (Fig. 13), and the cell membrane is almost invisible.

Even after acetylation, certain tissue components presumed not to contain nucleic acid still stain strongly with indium. Among these are the keratohyalin granules of keratinous tissue (Fig. 14), mast cell granules (Fig. 15), and the nine "satel lites" which surround the ciliary filaments of mammalian sperm tails. This is in agreement with the finding of Lillie (17) that the staining of keratohyalin and mast cell granules by basic dyes is not eliminated by acetylation. This may be due to the presence of unesterified phosphate, carboxyl, or a reactive sulfate, or to some special configuration of other groups so that they are refractory to the acetylation procedure.

DISCUSSION

The density contributed by indium staining is adequate for moderately high magnifications, *i.e.*, 100,000 times. It does not appear to be sufficient, however, to detect readily detail below 20 or 30 A. Much of the contrast obtained arises in part from the shrinkage of nucleic acid during dehydration, which results in high concentration of nucleic acid in small regions of the section Since the stained structures, in general, are small, best contrast at the higher magnifications results when the sections are as thin as possible. Thin sections can be obtained with the sharpest knives, but must be collected rapidly to minimize possible extraction of indium in the collection trough, which would be emphasized in thinner sections.

Experience with the contrast afforded by indium has forced us to observe carefully the visibility of unstained tissue components in relation to stained ones. Although many unstained structures are faintly seen at low magnification in sandwiched, methacrylate-embedded tissues, the contrast is low enough so that phase contrast effects so useful at high magnifications contribute little to their visibility. For this reason, unstained tissue, visible at low magnification, cannot be discerned at high magnification, whereas phase contrast permits stained components to be visible distinctly at high magnification. Thus, we can test for the presence of stain by examining the structure in question at a magnification of 20,000 times on the viewing screen, for example with a 10 × magnifier. If it is stained, it will show sharp detail.

At present there is no way to make quantitative estimates of the amount of nucleic acid present in sections of tissue stained with indium. Such estimates will depend on the increase in density of stained over unstained tissue elements. The sublimation of embedding material in the electron beam produces a formidable obstacle to making such estimates from sections, but this constitutes only part of the problem. Even with purified nucleic acids the amount of indium complexed from organic solvent is probably not uniform. Nucleic acids after dehydration are greatly condensed, so that steric hindrance can be expected to make some otherwise acceptable reaction sites unavailable. In intact tissues, before or after fixation, nucleic acids are complexed in various degrees with protein. Such complexes can also be

expected to make some of the nucleic acid unavailable for reaction with indium. Thus, in sections of indium-stained tissue, we probably do not see all the nucleic acid present. Whether such effects simply reduce the general intensity of staining or blank out resolvable masses of nucleic acid entirely is not known. Finally, it should be remembered that we are depending for much of our contrast on the hypothesized formation of indium polymers in organic solvent. These polymers may be of somewhat variable composition and, in any case, may be partially removed during processing after incorporation with the nucleic acid. This is not intended to imply that the results using the suggested methods are highly variable; they are not.

It seems likely that other heavy metals forming insoluble phosphates might be used in place of indium for staining nucleic acids. Much of the contrast problem would be eliminated if elements of higher atomic weight or polymers could be used. The problem here is that general non-specificity tends to increase with atomic weight. Uranyl, which has been proposed by others as a nucleic acid stain, was tried in the present investigation as a stain in alcohol. In this case embedding was carried out in Vestopal because methacrylate readily removes uranyl from the tissue. It behaved to some extent like indium with respect to acetylation, but proved so ready to complex that acetylation did not reduce non-specific staining to acceptable limits. This appears to be traceable to the ease with which ring compounds with the uranyl ion can be formed (10, Vol. II, pp. 1075–1079). It seems likely that the specificity of uranyl in water solutions would be much higher than in organic solvent because of ionization. The use of aqueous uranyl has been reported by Huxley and Zubay (1) in the staining of RNA in whole mounts of RNP particles. In acrolein-fixed tissue, however, we have found that aqueous uranyl at pH 4.0 stains muscle and collagen and is therefore under these conditions non-specific for nucleic acids. Such non-specificity might be reduced by blocking reactions except that the use of blocking reactions in aqueous medium may introduce serious distortion of structure.

The application of enzymes represents a power

FIGURES 9 TO 11

Micrographs showing the effect of tissue reduction and acetylation on the distribution and intensity of indium staining. In all cases, the tissue was fixed in acrolein, embedded in cross-linked methacrylate, and sandwiched.

FIGURE 9

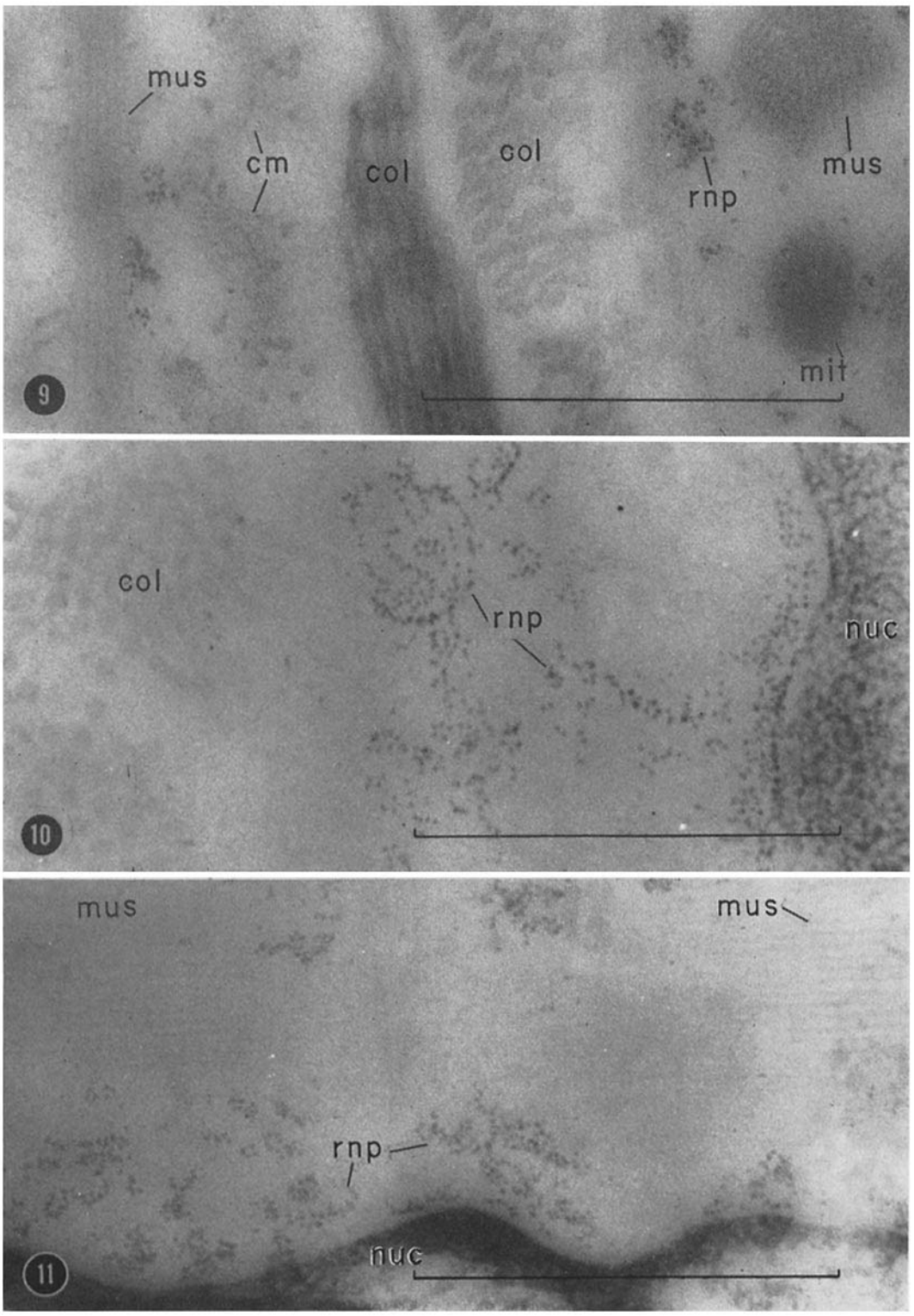
Region of rat tongue bordering on two muscle cells in the left and right thirds of the micrograph. In the central region is collagen (*col*). This tissue was not reduced or acetylated. Bundles of muscle filaments (*mus*) lie parallel to the section on the left and perpendicular to it on the right. A mitochondrion (*mit*), cell membranes (*cm*), and RNP particles (*rnp*) are present. All these structures stain obviously with indium, but only the RNP particles are presumed to contain amounts of nucleic acid readily detectable by indium staining. × 65,000.

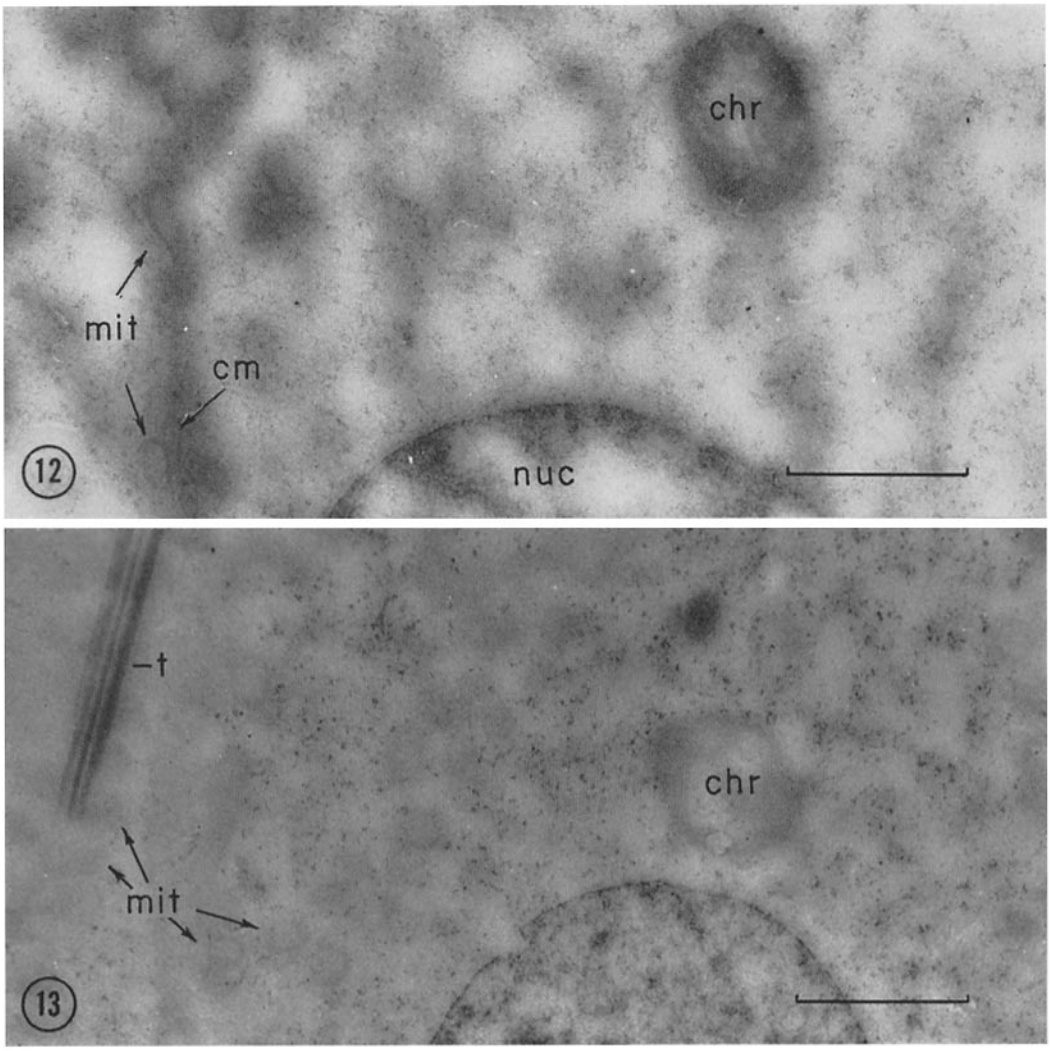
FIGURE 10

Rat tongue after reduction and acetylation. Part of a fibroblast appears at the right, and collagen (*col*) running perpendicular to the plane of section is just discernible on the left. The reduction in the intensity of collagen staining is evident. *rnp*, RNP particles. × 65,000.

FIGURE 11

Rat tongue muscle after reduction and acetylation. Muscle filaments (*mus*) running across the top half of the micrograph can be detected only on close inspection. *nuc*, nucleus; *rnp*, RNP particles. × 65,000.


mus
cm
col
col
rnp
mus
mit
9
col
rnp
nuc
10
mus
mus
rnp
nuc
11



FIGURES 12 AND 13

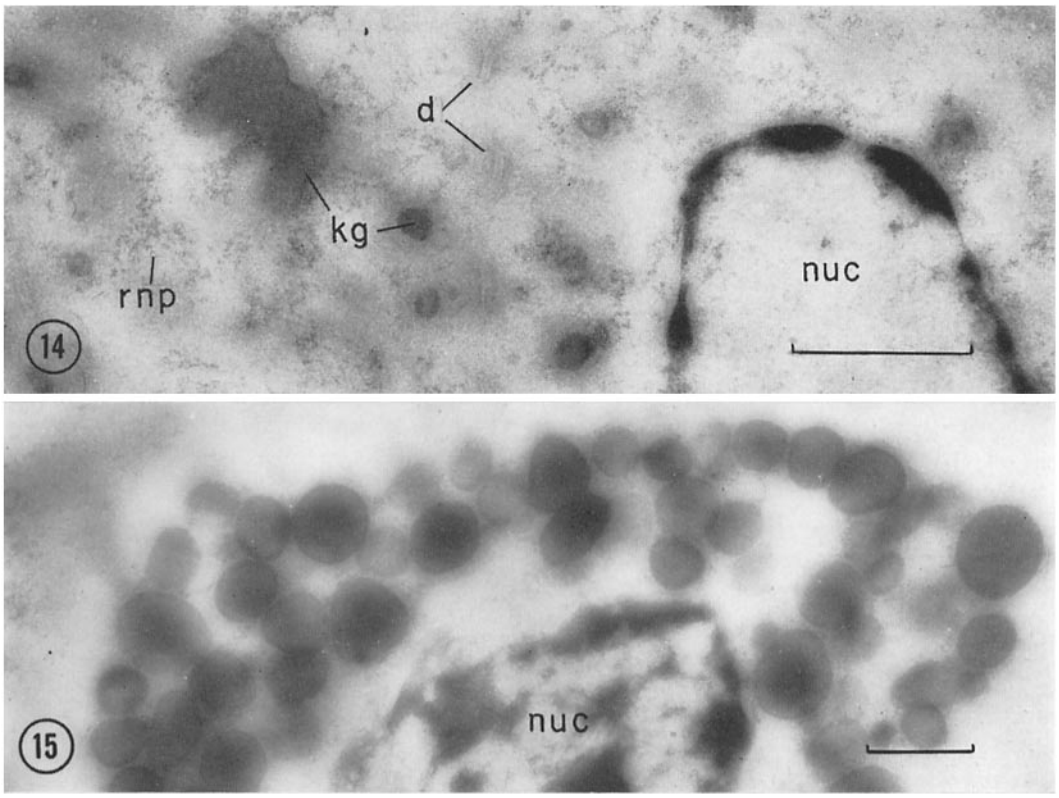
Comparison of structures in spermatids of rat testis before and after reduction and acetylation. Tissue was fixed in acrolein, embedded in cross-linked methacrylate, and sandwiched.

FIGURE 12

Before reduction and acetylation. Mitochondria (*mit*), cell membrane (*cm*), and the chromatoid body (*chr*) are well stained with indium. It is useful here and in the following micrograph to compare the staining of such elements with that of the nucleus (*nuc*). × 23,000.

FIGURE 13

After reduction and acetylation. Mitochondria (*mit*) and probably cell membrane are almost invisible. Staining of the chromatoid body (*chr*) is much reduced. Elements within the sperm tail (*t*) stain strongly even after acetylation, although the mitochondrial sheath does not. × 23,000.



FIGURES 14 AND 15

Micrographs showing two elements which stain strongly with indium even after reduction and acetylation. Tissue was fixed in acrolein, reduced and acetylated, stained with indium, embedded in cross-linked methacrylate, and sandwiched.

FIGURE 14

Keratin-producing cell near the surface of rat tongue. Staining are the nucleus (*nuc*), RNP particles (*rnp*), keratohyalin granules (*kg*), and, faintly, desmosomes (*d*). × 23,000.

FIGURE 15

Mast cell in rat tongue. The granules stain strongly in comparison with the nucleus (*nuc*). × 15,000.

ful aid for the evaluation of specificity of histochemical procedures for light microscopy. Such application is usually made on sections where the geometry is highly favorable for enzymatic attack. In the present case, since staining is carried out before sectioning, enzymes must also be applied to the tissue block. It remains to be seen whether effective enzymatic action is possible under these conditions. In addition to nucleases, perchloric acid and a number of other reagents have frequently been used to extract both nucleic acids or, under less drastic conditions, RNA from sections (see ref. 12 for a brief discussion of these methods). It seems likely that such methods can be usefully coupled with the indium technique.

In evaluating these procedures it is important to remember that indium at its best is specific not primarily for nucleic acid, but for unesterified phosphate. Most of such phosphate in cells is associated with nucleic acid, phospholipid, small organic and inorganic phosphates, polyphosphate and phosphorylated polysaccharides, and proteins

such as casein. Of these, small organic and inorganic phosphates appear to be lost during fixation and dehydration, as also does phospholipid containing unesterified phosphate. Polyphosphates do not appear to stain with indium under present conditions. Phosphorylated polysaccharides and proteins where present will stain if the phosphate is not fully esterified. However, they are uncommon. Thus we see that by far the major phosphate in cells which will stain with indium is nucleic acid. It is on this basis, neglecting interfering groups, that we claim specificity for indium as a nucleic acid stain. Despite the blocking reactions presented here, other interfering groups, which in the main are probably not phosphate, do remain in large amounts in a few structures (keratohyalin granules, mast cell granules, and sperm tails) and in satisfactorily low amounts in other structures (muscle filaments and collagen). It is hoped that other blocking procedures now under investigation may eliminate these. Thus, indium staining at the present stage of development represents a procedure of fair specificity for nucleic acids which, if used circumspectly, should prove of value in biological research.

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