NUCLEOLAR ORTHOPHOSPHATE IONS

Electron Microscope and Diffraction Studies

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ABSTRACT

Lead acetate (3–10%, pH between 4.3 and 7.0, alone or containing 2% glutaraldehyde), when used as fixative, has been demonstrated to produce an intracellular microcrystalline precipitate of lead orthophosphate, $Pb_5(PO_4)_3OH$ (lead hydroxyapatite). This confirms earlier work with the light microscope (6). In interphase cells the nucleoli are sharply delimited by the massive lead phosphate precipitate. Some diffuse precipitate is found in the nucleoplasm; it is always delimited by the nuclear membrane. Nucleolar localization of this orthophosphate pool is not a diffusion artifact; the pool is probably in a loosely bound state and is not retained by conventional fixatives. In maize root cells in advanced mitotic stages the lead phosphate crystals are seen distributed throughout the cytoplasm and also relatively concentrated on the late anaphase-early telophase chromosomes. This pool of inorganic phosphate anions may be involved in the mitotic cycle of chromatin condensation, and it may be partially responsible for the absence of mature ribosomes in the nucleolus through the chelation of divalent cations. It is evident that the siver-reducing component detected in the nucleoli of fixed cells (6) is a completely different substance.

INTRODUCTION

In earlier work at the light microscope level one of us (1–6) reported that when an aqueous solution of lead acetate is used as fixative a lead compound precipitates massively in the nucleoli of plant and animal cells. In further work, and on the basis of several lines of evidence, the author maintained the view that: (*a*) the lead deposited in the nucleolus is really a precipitate and not merely the result of an affinity of lead for the unfixed proteins or other macromolecular component; (*b*) the component precipitated by lead is a highly diffusible substance and could not be retained by conventional fixatives; (*c*) the lead precipitate is mainly, or exclusively, lead orthophosphate; and (*d*) the nucleolar location of precipitate is not a false location due to diffusion artifacts. It was concluded that a pool of soluble orthophosphate anions, probably in a relatively high concentration, is located in the nucleolus of plant and animal cells.

More recently, a similar procedure was carried out on cultures of HeLa cells by Studzinski and Love (7). However, those authors did not try to identify the exact nature of their staining procedure. Although they also found that the test must be made on unfixed cells, the question was raised whether the lead precipitate could be due to an easily denatured protein rather than to inorganic phosphate.

The problem was further complicated by the finding that the nucleoli of fixed cells possess a striking affinity for zinc (8, 9) and strongly reduce

silver salts (6, 10–15). However, the fact that lead "affinity" is found only in unfixed cells suggests that this affinity and the former ones are due to two completely different kinds of substances.

To resolve the problem, we investigated the lead acetate reaction at the electron microscope level. The evidence presented here conclusively demonstrates that lead acetate fixation of living cells produces a massive microcrystalline lead precipitate inside the nucleoli, which is exclusively lead orthophosphate, thus confirming earlier work with the light microscope. It is also obvious that the silver-reducing and the zinc-binding components detected in fixed cells are completely different entities.

MATERIALS AND METHODS

The investigations were carried out on vigorously growing maize and onion bulb root tips, as well as on rat liver. The roots which developed when the plant materials were put in contact with tap water at 22–25°C were allowed to grow until they reached 1 inch in length, and then segments about 4 mm from the tip were cut and immediately fixed. Rats were killed by decapitation and small slices of liver about 1 mm thick were immediately immersed in the fixatives.

Fixation Procedure

Neutral lead acetate $Pb(C_2H3O_2)_2 \cdot 3H_2O$ (Merck Chemical Div., Rahway, N. J., analytical reagent) and glutaraldehyde (50%; Fisher Scientific Company, Pittsburgh, Pa., biological grade) were employed. Deionized water, or water glass-distilled twice, was always freshly prepared before dissolving the lead salt. The effect of varying the composition of the fixing fluid was investigated with the following solutions, freshly prepared: (*a*) Lead acetate (pH 6.0–6.1): dissolved in water in concentrations from 4 to 10% (w/v). (*b*) Acetic acid-lead acetate (pH 4.2–4.3): a 10% solution of lead acetate containing 1–2% glacial acetic acid. (*c*) Acetic acid-lead acetate-glutaraldehyde: same as (*b*) but containing also 2% glutaraldehyde. (*d*) Picric acid-lead acetate (pH 5.4–5.5): prepared by mixing two volumes of 10% lead acetate and one volume of a half-saturated aqueous solution of picric acid. Picric acid reacts with lead acetate, and the fixative finally contains lead picrate (saturated), lead acetate, and acetic acid (1). (*e*) Lead acetate-glutaraldehyde-buffer (pH 7.0): a 3–4.5% solution of lead acetate in 0.2 M cacodylate-HCl buffer, pH 7.0, containing 2% glutaraldehyde. (*f*) Same as (*e*) but omitting the lead acetate. This is included as a control preparation and for comparison with (*e*).

The time of fixation was usually 3 hr at 4–6°C or at room temperature, but fixation can be left overnight in the refrigerator if desired. The tissues immersed in fixatives (*a*), (*b*) and (*d*), which do not contain aldehydes, are hardened by placing them in a 4% lead acetate solution in 2.5% glutaraldehyde for 3–12 hr at 4–6°C.

Washings and Embedding

After fixation, the tissues were immersed in an ice-cold solution of 4% lead acetate in 35% acetic acid for 1–3 hr (or overnight if desired) and then thoroughly washed for 30–60 min with several changes of ice-cold deionized water. This simple procedure has been shown to reduce considerably (or completely, in some cases) the nonspecific binding of lead to proteins and to dissolve any deposits of lead carbonate, which contaminate the preparations because of the air, although lead phosphate is completely unaffected (1, 4). The presence of lead acetate in the acetic acid decreases the solubility of lead phosphate as a result of the common-ion effect; therefore there is no risk at all that the lead precipitate will be washed out of the tissues, even by prolonged immersion in this acetic-lead solution. In a few cases a 2% acetic acid solution containing 4% lead acetate was used overnight with similar results. For comparison, this acetic acid step was entirely omitted in other cases, and the tissues were washed with water only; the results were essentially the same, but it was difficult to avoid entirely the deposition of lead carbonate.

After they were washed, the tissues were dehydrated with graded concentrations of cold ethanol, passed through cold propylene oxide, and embedded in Maraglas (Marblette Corp., Long Island City, N. Y.). It was found necessary to extend to 12–24 hr the time of immersion in the propylene oxide-Maraglas mixture and in Maraglas in order to assure a good penetration of the plastic into the plant material.

Electron Microscopy

The sections were cut with glass knives on a Porter-Blum microtome, mounted on Formvar-coated copper grids (100 mesh), and examined *unstained* with a Siemens Elmiskop I electron microscope. After this observation, the sections were stained with uranyl acetate, alone or followed by lead citrate (16), with little alteration of the lead reaction product itself. In some cases the sections were stained with a 1% solution of $KMnO_4$ (17).

Thick (1μ) sections were also cut, adhered to a microscopical slide, stained for 15 min with a freshly prepared 2% aqueous solution of potassium sulfide (Matheson, Coleman, and Bell, Cincinnati, Ohio), and washed with distilled water. In this manner any lead present in the tissue is converted into lead sulfide which is easily seen, under the light micro-

scope, as brown or yellow-brownish (depending on its relative concentration) sites. This step of conversion into lead sulfide was *never* used for electron microscope observations, since lead itself has enough electron opacity to be visualized directly and, furthermore, it interferes with the electron diffraction analysis. For this last reason, postfixation in osmium tetroxide was also omitted.

In order to visualize rapidly the extent of the reaction, the whole tissue was immersed (after washing) into potassium sulfide for 30–60 min, squashed, and observed in a drop of glycerine with the light microscope. If necessary, frozen sections were cut, immersed in lead acetate-acetic acid to remove lead carbonate, washed with distilled water, and stained with potassium sulfide.

Electron Diffraction

Selected-area electron diffraction was performed with a Siemens Elmiskop I electron microscope, by using a selecting-area aperture of 50 or 30 μ. The microscope was operated at 100 kv and the pole piece was used for a magnification of 80,000 diameters.

Pairs of micrographs of diffraction patterns from nucleolar areas with precipitate and from the lead phosphate precipitate obtained in the test tube, were taken at the same values of the lenses controls, one immediately after the other. The same procedure was used to obtain pairs of diffraction patterns from the nucleolar precipitate and "free" nucleoli from control roots. The interpretation of diffraction patterns was made according to the methods used by Wiesenberger (18) in his study of microchemical reactions. The use of selected-area diffraction is described by Hall (19).

Lead orthophosphate was obtained by adding a dilute solution of Na_2HPO_4 to a solution of 10% lead acetate in 40% acetic acid, centrifuging, and washing several times with 40% acetic acid and deionized water. A small amount of precipitate was put on a Formvar-coated grid with a small platinum loop.

X-Ray Analysis

X-ray powder patterns were kindly determined by Dr. Dora Bedlivy with Philips X-ray diffraction equipment (Philips Electronics and Pharmaceutical Industries Corp., N. Y.) in a Debye-Scherrer camera of 114.6 mm, using Cu- $K\alpha$ radiation with a nickel filter.

RESULTS

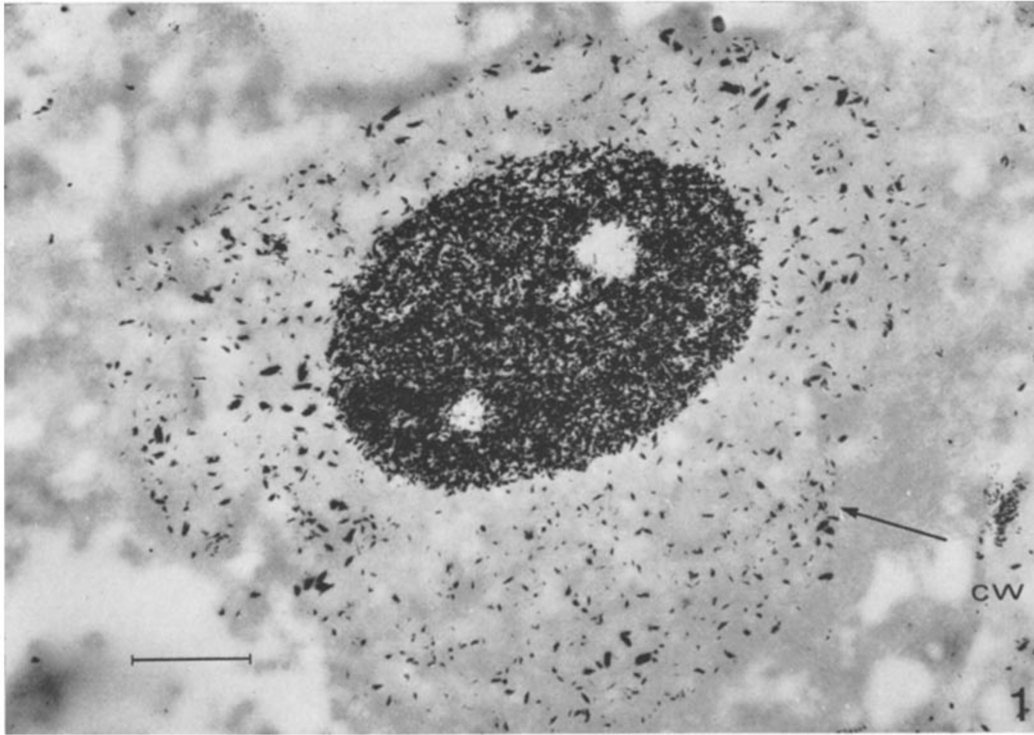
Unstained Sections

The most prominent feature to be observed in unstained sections of lead-fixed tissues is the presence of a microcrystalline precipitate, delimited by the nuclear membrane and massively deposited inside the nucleoli (Fig. 1). At higher magnifications the crystals are seen as small plates 900–1000 A in length and randomly distributed through the nucleolus (Fig. 2). Their appearance is similar to the lead orthophosphate precipitate obtained in the test tube (Fig. 4). A few crystals, in amounts that depend on the fixative used (see below), are scattered through the whole nucleus. The background of the sections is completely clear, indicating the effectiveness of the washing procedure. Some precipitate can be found also in the cellulose walls (Fig. 1). There is no precipitate at all in tissues fixed in glutaraldehyde alone and treated afterwards with lead acetate (compare Figs. 1 and 9 with Fig. 12), nor is there any enhancement of contrast of the tissue, after the latter treatment, that could be due to lead binding on the tissue proteins. As has been pointed out before (1, 4, 5), the substance precipitated by lead acetate could not be retained by any form of conventional fixation and must be a highly diffusible substance.

Identification of the Precipitate

ELECTRON DIFFRACTION: Fig. 3 compares side by side the diffraction pattern of an area of about 1 μ diameter of the precipitate found in the nucleolus and the diffraction pattern of the lead orthophosphate obtained in the test tube. The diffraction patterns are practically indistinguishable. Of course, the tissue sections give some additional diffraction halos which are mainly due to the plastic in which the tissues are embedded. These halos are made evident by focusing on the areas outside the nucleolus (or nucleus), and they are more diffuse than those due to lead phosphate. The same diffuse halos are obtained over the nucleoli of control sections, i.e. those without the lead precipitate (Fig. 5).

The pattern of the lead orthophosphate obtained in the test tube shows some diffraction spots in addition to the ring pattern; these spots are due to the presence of some larger crystals, since the precipitate obtained in the test tube is composed of microcrystals heterogeneous in size. Although the crystals obtained in the test tube are single crystals, the dense masses observed in the nucleoplasm in Fig. 2 are mainly crystalline aggregates. A point of interest is that diffraction spots have never been observed in patterns of the precipitate found inside the nucleolus, indicating



All the electron micrographs shown in these figures have been fixed in lead acetate-containing fixatives and embedded in Maraglas. Postosmication was always omitted.

FIGURE 1 Thin section of maize root tip fixed in lead acetate, pH 6.0. Electron-opaque, microcrystalline precipitates are present in the nucleus and also in the cellulose walls (*cw*). The crystals are massively deposited in the nucleolus; the nucleolus boundary is quite sharply delimited by the precipitates. Two nucleolar "vacuoles" are free of crystals. The rest of the precipitates in the nucleus are delimited by the nuclear membrane (arrow). Unstained. × 15,000. Mark: 1 μ.

the extremely small size and evenness of these crystals. In tissue sections the crystals are frequently seen disposed parallel to each other, about three to eight in number and with a spacing of about 100 A between them (Fig. 2). This is not seen in the lead phosphate obtained in vitro; at most, two or three plates are discernible with such a spacing. This difference could be due either to the effect of sectioning, which exposes the profile of the crystalline aggregates, or to the peculiarity of precipitation in the presence of the tissue macromolecules. Finally, the plant and animal nucleoli give the same diffraction pattern, which is *exclusively* that of lead orthophosphate.

X-RAY ANALYSIS: Fig. 6 compares side by side the X-ray diffraction pattern given by lead orthophosphate as obtained in the test tube and the X-ray diffraction pattern given by six onion roots fixed in acetic-lead acetate, washed in distilled water, and dehydrated in ethanol. Except for the weaker lines obtained with lead-fixed tissue, which are due to the extremely small size of the crystals and to the presence of organic material, the patterns show a good correspondence. This proves that lead orthophosphate is the *only* crystalline lead material found within the whole tissue. These data, when compared to those tabulated in the ASTM Index (20), indicate that the lead orthophosphate precipitate conforms to the lead hydroxyapatite, $Pb_5(PO_4)_3OH$. A total of 22 lines are exactly in the same position and give comparable intensities (Table I). Lead pyrophosphate and lead sulfate give completely different X-ray diffraction patterns (20). The possible presence of significant amounts of other lead precipitates besides lead orthophosphate can be discarded, as shown by paper chromatographic analysis of ice-cold trichloroacetic acid extracts of

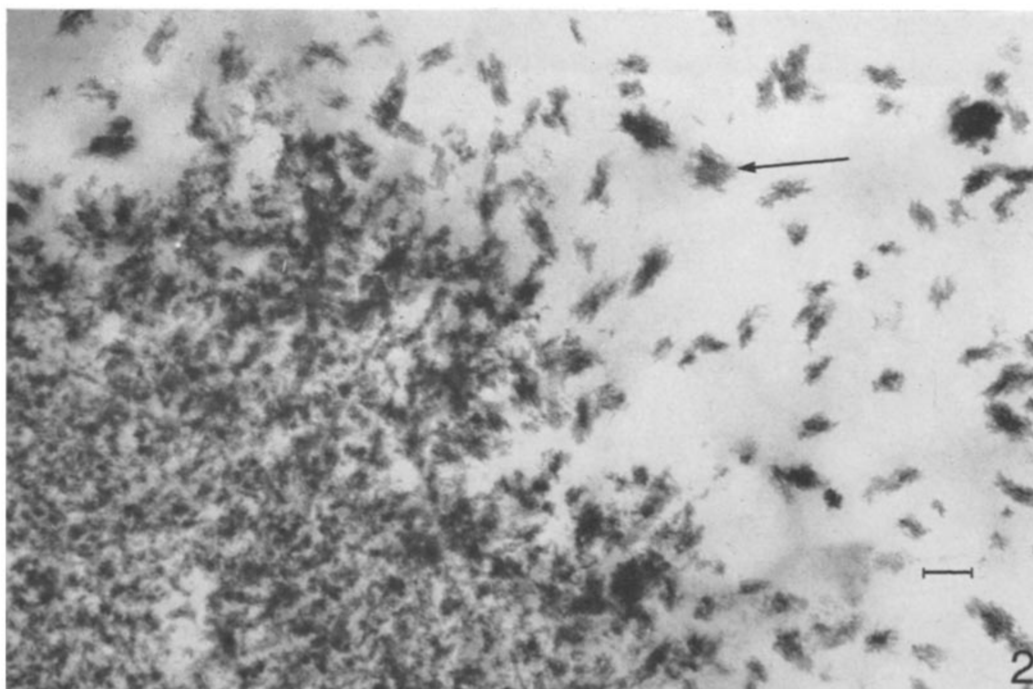


FIGURE 2 Same as Fig. 1 but fixed in lead acetate–glutaraldehyde, pH 7.0. Higher magnification. The part of the nucleolar area which can be seen at left of the micrograph has a greater concentration of the crystals, which are plates about 1000 A in length. The crystals are frequently disposed parallel to their lengths, about 3–8 in number and with a spacing of about 100 A between them (arrow). Unstained. × 60,000. Mark: 0.1 μ.

lead acetate–fixed root tips (before spotting, the acid is eliminated with ether in the presence of EDTA to prevent reprecipitation of lead salts) (4).

Preliminary observations were made with the electron microprobe (kindly performed by Dr. C. M. Libanati of the Commission of Atomic Energy) on sectioned maize root cells fixed with lead acetate-glutaraldehyde. Those observations, through an elementary scanning of the tissue area, showed a high concentration of phosphorus and lead in the nucleolar areas as compared with the controls fixed in glutaraldehyde alone.

Chemical Analysis

The question arises now whether the lead precipitate represents genuine cellular orthophosphate, or whether it is artifactually produced during the fixation procedure, i.e., either by decomposition of a labile organic phosphate compound, or by an enzymatic system acting on endogenous substrate (which, of course, must be an organic phosphate itself). Phosphate analysis on ice-cold extracts of onion and maize roots have shown that orthophosphate alone accounts for 60–65% of the total soluble phosphates (4). Similar high values for orthophosphate have been found also in barley and pea roots and seedlings (21). This indicates that inorganic phosphate is the only phosphate present in the roots in amounts appreciable enough to give a visible lead precipitate at the cellular level. Moreover, the lead acetate cytochemical reaction does take place at 0°C and at pHs between 4.2 (acetic acid added) and 7.4 (in cacodylate-HCl buffer). These results point to the conclusion that in the rootlets the lead precipitate represents genuine orthophosphate ions. Since a large number of plant and animal tissues respond similarly to the lead acetate test (1–4), the existence of a soluble pool of orthophosphate anions is very probably a universal property of all nucleoli.

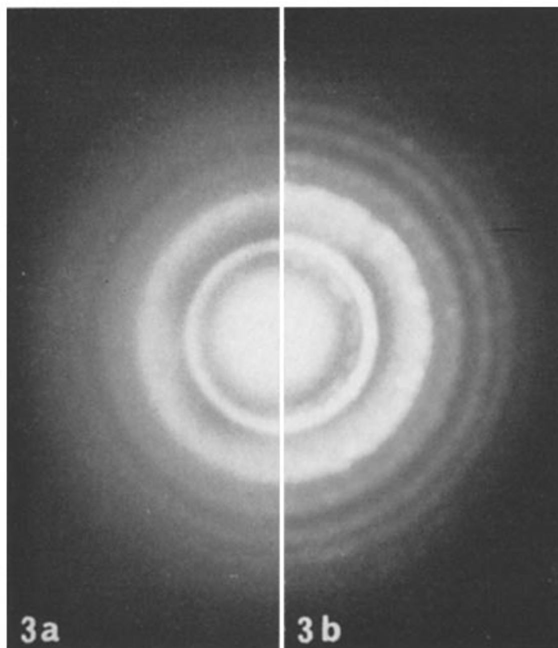


FIGURE 3 Side-by-side mounting of two electron diffraction patterns: (*a*) obtained from an area, about 1 μ diameter, over the precipitate found in the nucleolus of Fig. 1. (*b*) obtained from the crystals of lead orthophosphate prepared in vitro (Fig. 4). The patterns are identical. In (*b*) the [pattern] is somewhat stronger and also shows diffraction spots due to the presence of some larger crystals.

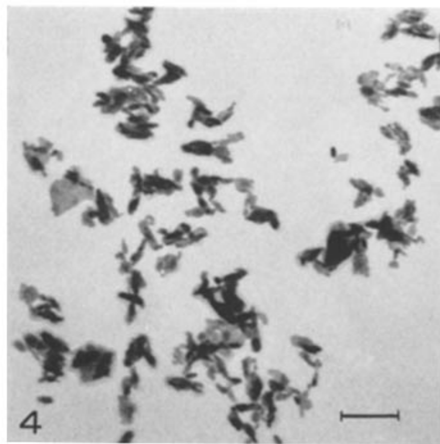


FIGURE 4 Electron micrograph of lead orthophosphate crystals obtained in the test tube and deposited on a Formvar-coated grid. × 72,000. Mark; 0.1 μ.

Influence of the Fixatives: Stained Sections

The localization of the precipitate is essentially similar with all the fixatives tested (*a* to *e*), but differs in some minor details. Diffusion artifacts inside the nucleus are larger with the acid fixatives (*b* to *d*) (compare Figs. 1 and 7 with Figs. 10 and 11). This is to be expected, since dilute acetic or picric acid is known to extract the soluble phosphates from the tissues (5). Probably part of this extranucleolar precipitate arises from the nucleolar phosphate, where the larger part of the precipitate is found. This is strongly suggested by comparison of the pictures given by the nucleolus itself after different fixatives. With the acid fixatives, the lead phosphate precipitate occupies only a portion of the nucleolar body, either as one large mass in the central part (Fig. 10) or as several small masses. Frequently a rim of the outer part of the nucleolus is negative (Fig. 10) or has a lower concentration of lead precipitate, suggesting that extraction occurs first at this outer region; this diffusing phosphate is then trapped elsewhere inside the nucleus. In many cases the picture directly suggests a diffusion from the nucleolus towards the outer parts of the nucleus, but no evidence ever was found to indicate diffusion in a reverse direction. When several small masses of precipitate are found, they give the appearance of "granules" inside the nucleolus, when stained with potassium sulfide and observed with the light microscope. Therefore, the granules observed by Studzinski and Love (7) in HeLa cells after fixation with lead at pH 5.5 are probably diffusion artifacts and cannot be considered an important difference between their and our

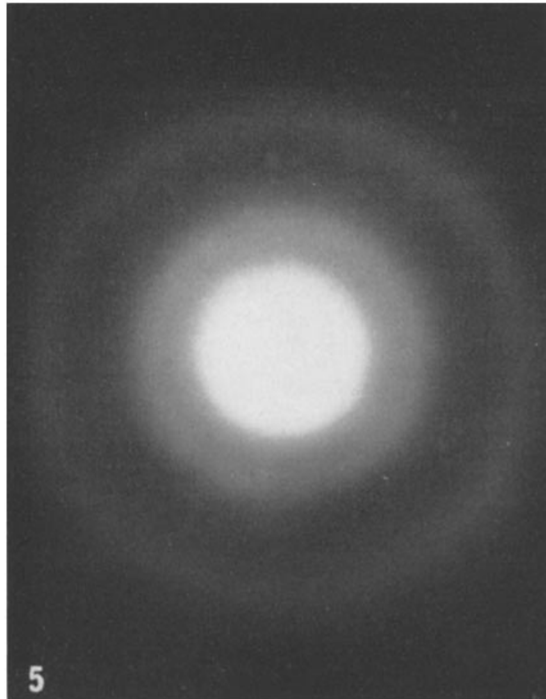


FIGURE 5 Electron diffraction pattern obtained from an area, about 1 μ in diameter, over a nucleolus free of lead precipitate. The diffraction halos are chiefly due to the embedding resin. Compare with Fig. 3.

procedures. Whether these zones of localized precipitate are more refractive to extraction and correspond to an ultrastructural component (fibrillar zones?) cannot be said with certainty. It is clear that near-neutral pH diffusion artifacts are diminished; in these cases, the only parts of the nucleolus that could be found free of precipitate are the vacuoles (Fig. 1), which are considered portions of the nucleoplasm intruding into the nucleolar body. The ultrastructure of the nucleolus remains intact with all the fixatives tested, as judged by the examination of stained sections from which the lead phosphate precipitate was eliminated by cold 5% trichloroacetic acid. The lead phosphate precipitate appears localized in both the granular and fibrillar parts of the nucleolus.

With the acid fixatives *b* and *d*, the chromatin is coarsely coagulated and some components of the nucleoplasm are probably extracted. In these cases the extranucleolar lead phosphate appears anchored largely on the chromatin masses (Figs. 10 and 11). However, at higher pH the chromatin is relatively free of precipitate (Fig. 7). In the rat liver the nucleolus-associated chromatin is also negative to the test, the precipitate being sharply restricted to the nucleoli (Fig. 8).

An interesting feature is the observation that with all the fixatives the lead phosphate crystals are always delimited by the nuclear membrane. This membrane appears double and continuous judged by examination of stained sections with uranyl acetate (Fig. 11) or $KMnO_4$. Even when the intranuclear diffusion is considerable, the lead precipitate is always found against the inner membrane; no precipitate is found between the two membranes, and rarely is precipitate found against the outer membrane (Fig. 11). It is interesting that with the more drastic fixative *d* the membranes of the endoplasmic reticulum as well as the ribosomes are relatively well preserved (Fig. 11). The mitochondria and plastids are damaged, but they are well preserved at neutral pH and with added glutaraldehyde (Fig. 9).

The Cell in Mitosis

When maize roots fixed in lead acetate-glutaraldehyde (pH 7.0) are stained with potassium sulfide and observed with the light microscope, the usual picture of unstained cytoplasm, black nucleoli, and light yellow-to-brownish nuclei is given by the cells in interphase, both in the meristem and in the vacuolized zones. This staining pattern corresponds, as shown by the electron

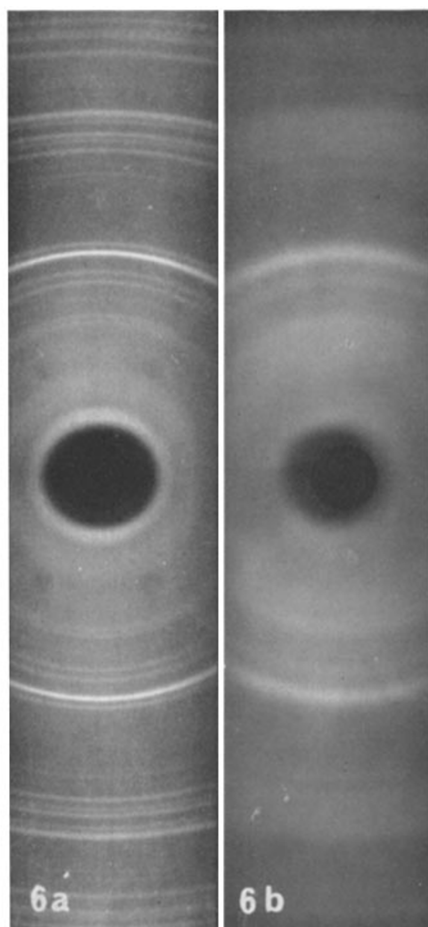


FIGURE 6 Comparison of X-ray diffraction patterns. (*a*) Obtained from the lead orthophosphate crystals of Fig. 4 (lead hydroxyapatite). (*b*) Obtained from six onion roots fixed in lead acetate.

microscope, to the distribution of the microcrystalline lead orthophosphate. In advanced mitotic stages the whole cell stains brown. This pattern is so clear-cut that the distribution of these mitotic cells can be visualized immediately at low magnifications. There is some darker stain on parts of the chromosomes, especially in late anaphase and telophase. The question arises whether this pattern also reflects the distribution of lead phosphate. That this is really so is shown by the electron micrograph in Fig. 13, which depicts a mitotic cell in metaphase or early anaphase; the whole cytoplasm is filled with a fine and homogeneous microcrystalline precipitate of lead orthophosphate, except for the mitochondria and starch granules, which are free of precipitate. There are some darker zones over parts of the chromosomes which correspond to a greater concentration of lead phosphate. This finding shows that lead acetate is able to precipitate the orthophosphate anions in the cytoplasm too, and strongly suggests a low concentration of phosphate ions in the cytoplasm of the interphase cells.

Fig. 15 shows a cell in early telophase stained with uranyl acetate and lead hydroxide. The

TABLE I

Comparison of the X-ray diffraction data of lead hydroxyapatite (a) (see ref. 20) and of the precipitate of lead orthophosphate (b)

hkl	(*a*)		(*b*)	
	I/IO	d	d	I/IO*
110	18	4,93		
200	26	4,28	4,26	m
111	40	4,11	4,07	m
002	10	3,71	3,67	vw
102	22	3,41	3,40	m
210	32	3,23	3,21	m
211	100	2,965	2,94	vs
300	38	2,852	2,83	s
202	<1	2,806		
220	<1	2,469		
310	<1	2,371		
302	3	2,261	2,26	vw
113	9	2,214	2,19	w
400	6	2,138	2,13	vw
222	18	2,056	2,04	m
312	13	2,000	1,988	w
213	18	1,956	1,947	m
321	16	1,898	1,883	w
303	10	1,868		
410	13	1,866	1,840	vs
004	20	1,856		
114	<1	1,737	1,74	vw
204	<1	1,704		
412	<1	1,670	1,68	vw
501	<1	1,666	1,65	vw
420	5	1,612	1,599	m
214	7	1,610		
304	6	1,555	1,545	m
502	7	1,553		
510	6	1,536	1,529	w
511	9	1,503	1,498	m

hkl: Miller indices; d: spacings in angstroms; I/IO: relative intensities of the powder lines.
* vs, very strong; s, strong; m, moderately strong; w, weak; vw, very weak.

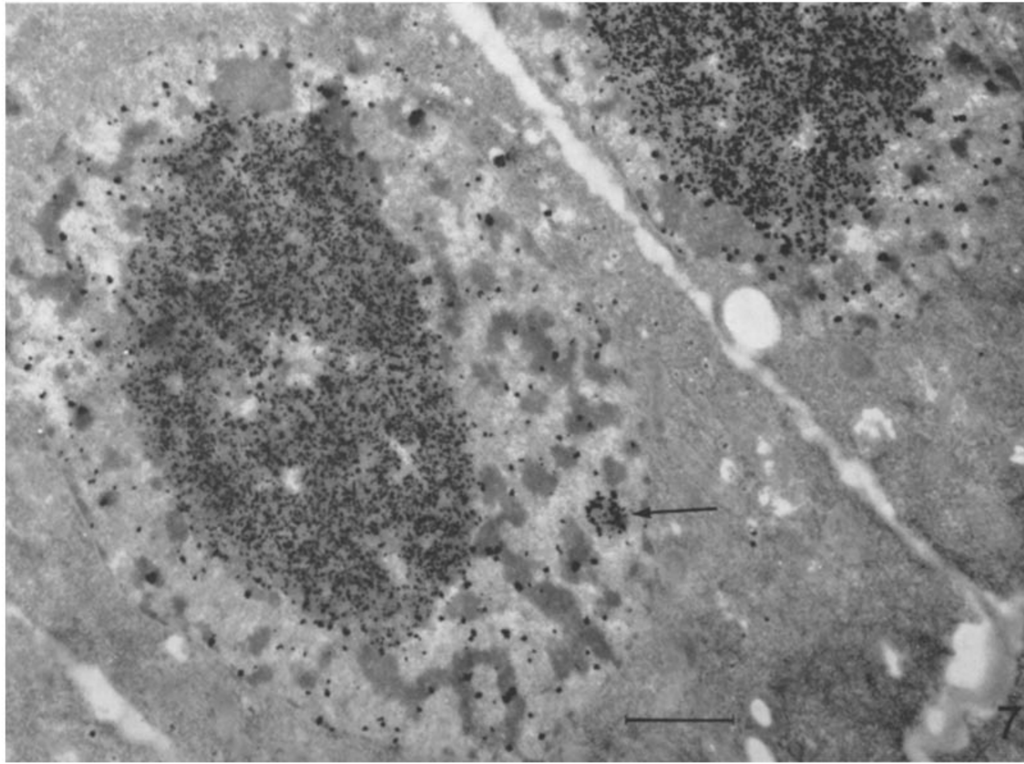


FIGURE 7 Thin section of maize root the fixed in lead acetate-glutaraldehyde, pH 7.0. The chromatin is relatively free of precipitate. The accumulation of crystals in a small spherical body, besides those of the large nucleolus, probably corresponds to a small nucleolus (arrow). Stained with uranyl acetate. × 14,000. Mark: 1 μ.

nuclear membrane is still incomplete. The ultrastructure of the developing nucleus exhibits two main zones: one fibrillar, which is the structure typical of chromatin, and the other consisting mainly of ribosome-like granules. The singular appearance of the chromosomes in the sections could be due to the fact that granular zones are mainly superficial; because of this, at those parts where the chromosomes are cut, the fibrillar structure is exposed. The most abundant lead phosphate precipitate, characteristic of the mature nucleoli, is found associated with the granular zones. This pattern is seen also on the (probably late) anaphase chromosomes in Fig. 14. In Fig. 15, a larger accumulation of both ribosome-like granules and lead precipitate can be seen in a spherical structure similar to that found in the interphase nucleoli, but smaller in size. The cytoplasm of these cells still shows some dispersed lead phosphate precipitate, which is characteristic of mitotic cells in advanced stages.

These findings deserve further study, but at present they give evidence—for the first time at the electron microscope level, we think—on the possible mode of formation of at least part of the nucleolus as described by several authors at the light microscope level (11, 12, 15; for further discussion refer to 22): a thin coating with some of the cytochemical characteristics of the mature interphase nucleolus appears in close contact with all the late anaphase, early telophase chromosomes, and abundant material in the form of small bodies subsequently accumulates within the early and middle telophase nuclei. At present there is no satisfactory evidence as to whether a fusion process occurs in these events or whether this nuclear material is a morphological forerunner of at least part of the nucleolar material (i.e. that part which

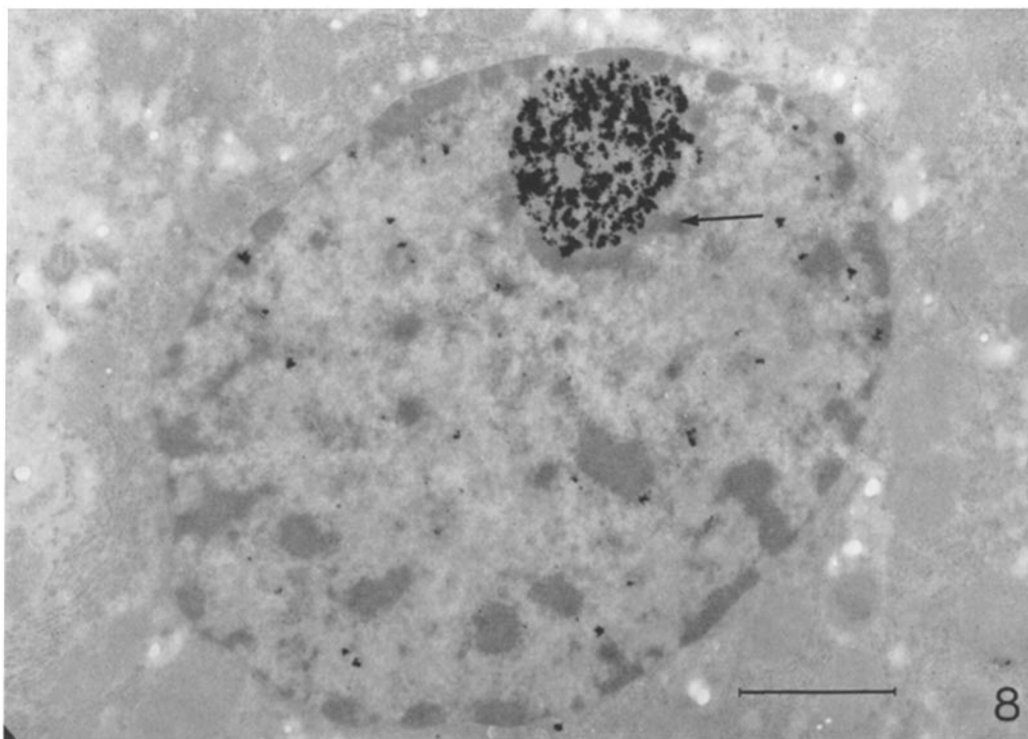


FIGURE 8 Electron micrograph of a nucleus of rat liver cell fixed as for Fig. 7. The nucleolus-associated chromatin (arrow) is also free of lead phosphate crystals, the precipitate being restricted to the nucleolar body. The crystals are grouped in small clumps when glutaraldehyde is added to lead acetate, in comparison to a more homogeneous distribution of crystals when lead acetate alone is used. Stained with uranyl acetate. × 20,000. Mark: 1 μ.

does not originate in the translation process of the organizer region).

Lead-Binding Sites in the Cytoplasm

This point must be briefly referred to here. In onion roots there are numerous cytoplasmic inclusions which bind lead and form a lead salt insoluble in lead acetate-acetic acid. A large part of these inclusions can be preserved by ethanol fixation and stained by the lead procedure at the light microscope level (1, 2). In the electron microscope and after fixation in lead acetate-glutaraldehyde (either at acid or at neutral pH), these inclusions appear as amorphous masses of different sizes in close contact with the membranes of the plant vacuoles and apparently hanging towards the interior of the vacuoles. This confirms earlier statements that they are probably inclusions connected with vacuoles (2). The substance responsible for this lead binding was not identified because it failed to give an electron diffraction image. These cytoplasmic inclusions are not present in maize root tips.

DISCUSSION

The localization of small, diffusible ions at the intracellular level, especially by means of precipitation with heavy metal salts, has not been generally judged as solid and well-based evidence. This is true on theoretical grounds and for simple models, e.g. in which the ions are embedded in gels of different consistencies; the addition of the precipitant leads to gross diffusion artifacts and to severe losses from the gel itself (see 23). On this basis alone, it should be impossible to produce a precipitate at the intracellular level, mainly because of differences in the velocities of diffusion. Even when a precipitate forms inside the cell, it is subject to the criticisms of false location. How-

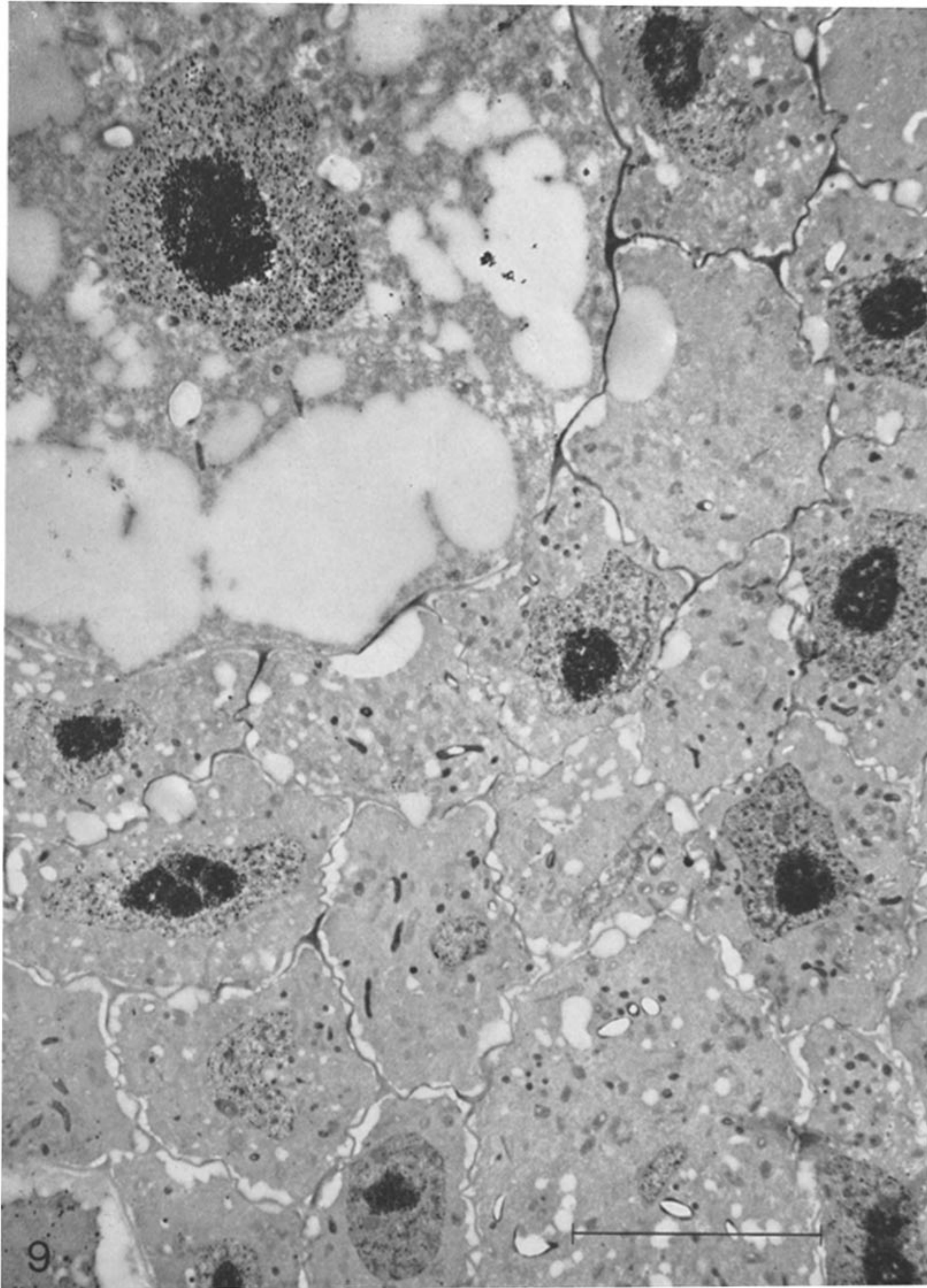


FIGURE 9 Survey electron micrograph of a thin section of maize root tip fixed as for Fig. 7, illustrating the generality of the reaction. The lead orthophosphate precipitate is deposited in the nucleoli and it is also delimited by the nuclear membrane. Mitochondria, plastids, and cell walls show contrast because of the staining of the section. Stained with uranyl acetate and lead citrate. × 3,600. Mark: 10 μ.

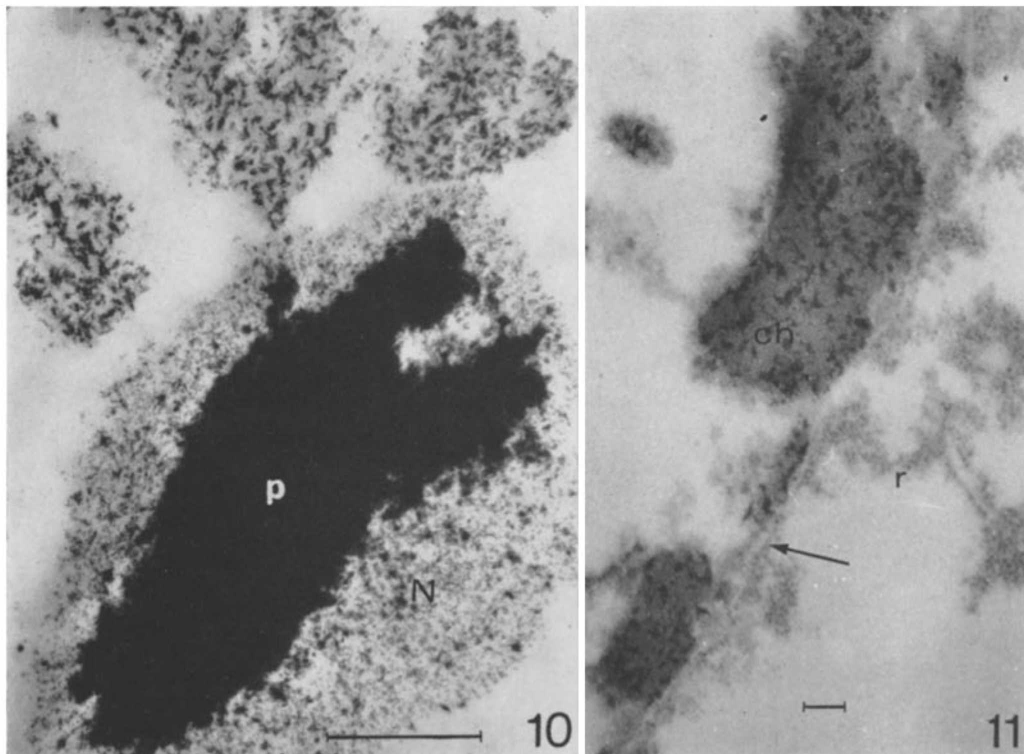


FIGURE 10 Electron micrograph of a nucleus of onion root tip fixed in picric acid-lead acetate, pH 5.4. The preservation is not good and diffusion artifacts are evident. The massive lead phosphate precipitate (*p*) occupies the central portion of the large nucleolus. Diffusion of phosphate ions occurred from the outer portion of the nucleolus (*N*), and precipitates appear over the coarsely coagulated chromatin (compare with Figs. 7 and 9). Unstained. × 20,000. Mark: 1μ.

FIGURE 11 Same as Fig. 10. The nuclear double membrane (arrow) is continuous and two layers can be discerned. The membranes of a fragment of endoplasmic reticulum and the ribosomes (*r*) are also relatively well preserved. Lead phosphate crystals are present over the chromatin masses (*ch*). Stained with uranyl acetate. × 48,000. Mark: 0.1 μ.

ever, we have conclusively demonstrated here that lead acetate fixation produces an intracellular precipitate of lead phosphate. This is a constantly reproducible result, and an explanation must be given for this phenomenon.

Evidence Against Diffusion Artifacts

The first point demonstrated here is that the precipitate is exclusively lead orthophosphate (lead hydroxyapatite) and that it is formed by precipitation of soluble phosphate anions. Since we are dealing with diffusible ions, the question might be raised whether the massive precipitate observed in the nucleoli truly represents a natural location of orthophosphate anions or whether it is the result of a diffusion artifact. If we consider the latter possibility, then a selective diffusion of phosphate ions from cytoplasm and nucleus *into* the nucleolus must occur during lead fixation.

Evidence has been presented at the light microscope level that when diffusion artifacts are induced—e.g. by a short prefixation with 2% acetic acid followed by fixation in lead acetate—the phosphate ions diffuse out of the nucleoli and are precipitated as scattered granules within the nucleus; at the same time, negative nucleoli or nucleoli partially filled with lead phosphate are produced (5). This showed that the phosphate ions which have diffused into the extranucleolar part of the nucleus do *not* move to the nucleolus

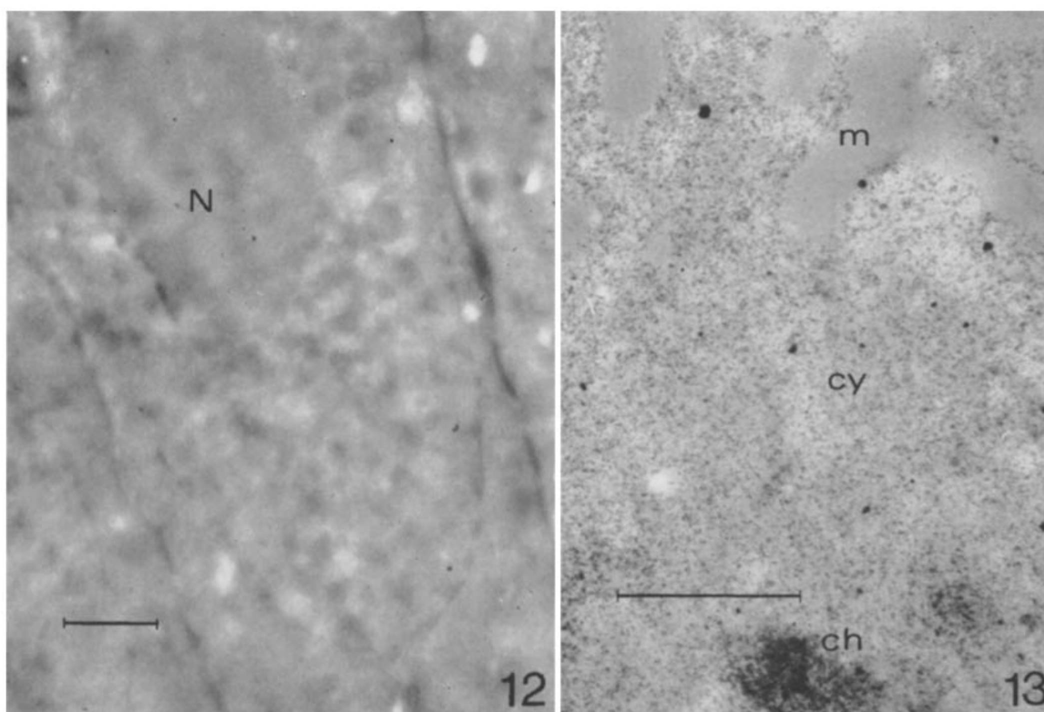


FIGURE 12 Electron micrograph of a control preparation of maize root tip fixed in glutaraldehyde *alone* (pH 7.0), immersed afterwards in lead acetate, and processed as usual. The electron-opaque deposit of lead phosphate is completely absent (compare with the lead-fixed sections). Lack of contrast indicates absence of lead-binding and the effectiveness of the washing procedure (*N*, nucleolus). Unstained. × 12,000. Mark: 1 μ.

FIGURE 13 Electron micrograph of a cell in mitosis fixed as for Fig. 7 (metaphase or early anaphase). The cytoplasm (*cy*) is filled with a fine crystalline lead phosphate precipitate, except inside the mitochondria (*m*) and plastids. (*ch*), chromosome. Compare with Figs. 7 and 9: in interphase and prophase cells the lead precipitate is restricted to the nucleus. Unstained. × 24,000. Mark: 1 μ.

when fixation is completed by immersion into lead acetate. This simple experiment makes highly improbable a diffusion of cellular orthophosphate into the nucleoli during lead fixation.

The present investigation at the electron microscope level strengthens the view that the direction of diffusion artifacts is from the nucleolus towards the cytoplasm, and not vice versa. The lead orthophosphate crystals are always sharply delimited by the inner membrane of the nuclear double membrane. This happens even with the more drastic acid fixatives, which show an increased deposition of lead phosphate crystals throughout the whole nucleus (Figs. 10 and 11). At the same time, the *outer* part of the nucleolus is free, or has a lower concentration, of crystals (Fig. 10). This observation strongly suggests that phosphate has diffused from the outer part of the nucleolus towards the nuclear membrane and has been subsequently trapped elsewhere in the nucleus (probably mechanical trapping of the crystals by the chromatin).

If the extranucleolar precipitate represents diffusing ions *in transit* towards the nucleolus, then one would expect to find the reverse picture, i.e. the crystals localized mainly at the outer part of the nucleolus but absent in its center. We conclude that the observed precipitate identifies a pool of soluble orthophosphate anions, probably in a relatively high concentration, located in the nucleolus.

The second point demonstrated is that all the precipitate is sharply delimited by the nuclear membrane (Figs. 1, 7, and 9), which apparently

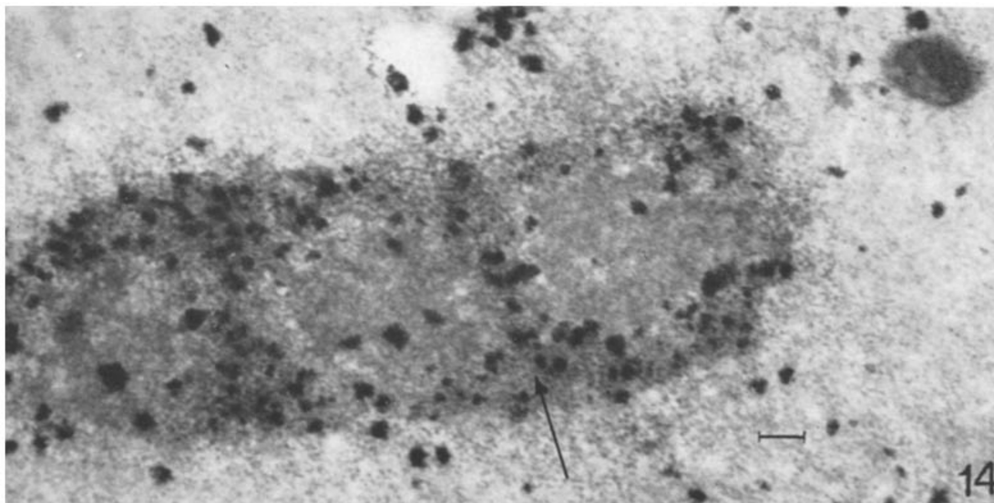


FIGURE 14 Electron micrograph of a segment of a late anaphase chromosome sectioned longitudinally. Maize root tip fixed as for Fig. 13. Two zones are evident: one fibrillar zone, typical of chromatin; and one zone rich in ribosome-like granules (arrow), where the lead phosphate crystals are concentrated. The singular appearance of the chromosome in the section could be due to the fact that the granular zone is mainly superficial. Stained with uranyl acetate and lead citrate. × 51,000. Mark: 0.1 μ.

behaves as a barrier against the rapid outflow of phosphate ions. No precipitate is usually found between the two membranes or against the outer membrane of the nuclear double membrane (Fig. 11). This fact strongly suggests that the nuclear membrane is partly responsible for the success of the cytochemical test. The velocity of movement through the membranes of the nuclear double membrane is shifted in favor of the lead ions during the fixation process. In this manner, and within certain limits, lead cations could reach the interior of the nucleus more quickly than the phosphate anions could escape from the nucleus. An intranuclear precipitate of lead phosphate is thus feasible. This answers the criticisms mentioned at the beginning of this section.

In the living cell the nuclear membrane may also play a role in the accumulation of phosphate anions from the cytoplasm. This seems more likely when one looks at mitotic cells in which the cytoplasm is filled with the lead phosphate precipitate (Figs. 13–15), whereas in the interphase cells this precipitate is restricted to the nucleus (Figs. 1, 7, and 9). This result suggests that the concentration of orthophosphate is low in the cytoplasm of interphase cells, though it is accumulated in the nuclei. In this connection, it is interesting that the nucleus has been shown to be intimately involved in the accumulation of iron by liver cells (24) and pea root cells (25). Of course, a negative result does not mean that orthophosphate is absent from the cytoplasm of interphase cells, since the degree of dispersion can affect its visualization very markedly. This means that "cytochemically detectable amounts" will be found only in those places where the concentration reaches a certain value, though this value might represent only a fraction of the total orthophosphate of the whole cell.

The third point demonstrated is the striking feature that the nucleoli are quite sharply delimited by the lead precipitate and show the highest concentration of orthophosphate ions in the cell. This requires an explanation since there is no membrane at the interphase with the nucleoplasm (and with the content of the nucleolar "vacuoles," Fig. 1). Accumulation of phosphate anions inside the nucleolus could be due to a Donnan type of equilibrium (which does not necessarily require the presence of a membrane), or to the existence of a "binder" in the nucleolus itself. This latter possibility could be considered either a binder for orthophosphate itself (e.g. the sulfate-binding protein, reference 26) or for a cation(s), which then binds the phosphate anions. In fact, it has been reported that the nucleolus has a striking

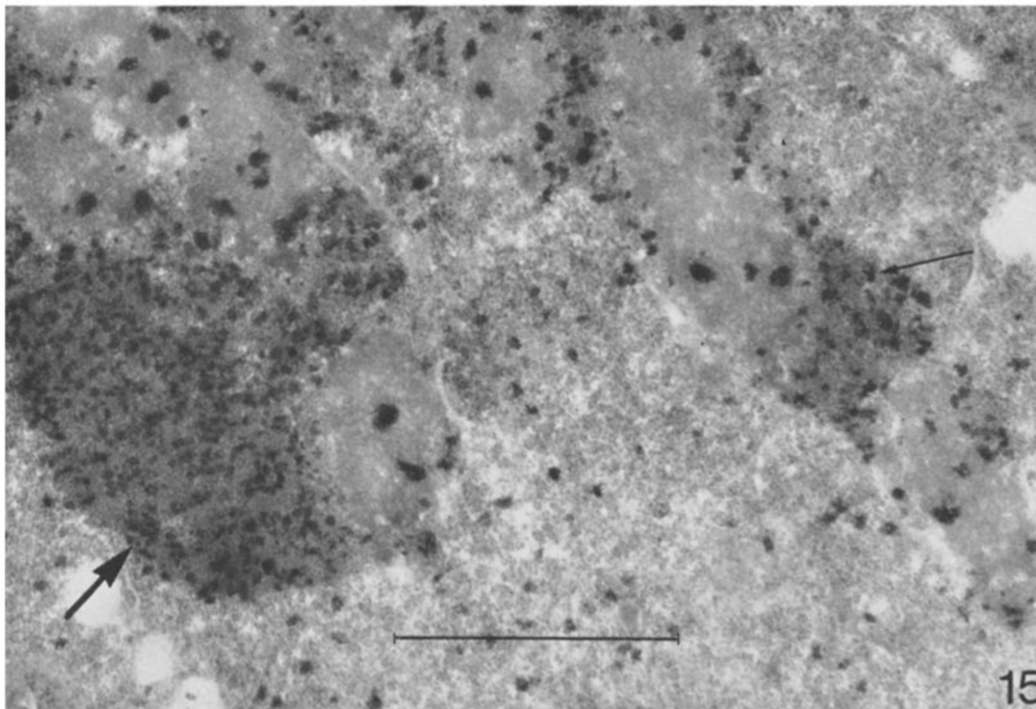


FIGURE 15 Electron micrograph of a portion of an early telophase nucleus. Maize root tip fixed as for Fig. 13. The nuclear membrane is still incomplete. The two zones of the chromosomes are clearly seen. The zone rich in ribosome-like granules (arrows) contains the greater concentration of lead phosphate crystals. These crystals are present, but more dispersed, within the cytoplasm. A larger accumulation of both ribosome-like granules and lead phosphate crystals, characteristic of the interphase nucleolus, can be seen in an oval structure (thicker arrow). Stained with uranyl acetate and lead citrate. × 37,500. Mark: 1 μ.

affinity for zinc (8, 9); other cations apparently have not been tried. If we assume that orthophosphate is held by a cation to some macromolecular component, it can be understood why lead ions behave successfully in the localization of phosphate ions: they replace the cation and at same time are a good precipitant of these anions. Furthermore, it would also explain why phosphate ions are found concentrated on zones of chromosomes at late anaphase-early telophase even when the nuclear membrane is absent or incomplete (Figs. 14 and 15).

Our work suggests that orthophosphate anions are in a bound state, which is apparently rather loose, since these anions are easily leached out of the cells and cannot be retained by any kind of conventional fixation, except lead acetate. Even in this last case, addition of the aqueous phase of organic solvents, which do not affect precipitation in the test tube, causes diffusion and a leakage of orthophosphate ions (5). The same result occurs when the osmolarity of the lead acetate solution is varied by adding sucrose or by increasing the lead concentration.

In testing the addition of other fixatives to the lead acetate, it must be kept in mind that we are dealing with water-soluble anions. Therefore, these anions are easily mobilized and extracted by aqueous fixatives, or even, in the aqueous alcohol, formed as the tissue is being dehydrated (1). As Studzinski and Love have mentioned (7), frozen sections cannot be used because damage to the cells allows the penetration of water, and the washing-out of phosphate ions occurs. This effect is followed by a great dilution of the concentration of these ions since the volume of the nucleolus is a small fraction of the total volume of the cell. In fact, the ion content may be so diluted that it

no longer is able to give a precipitate because it is now below the range of sensitivity of the reagent; negative results will be obtained. The addition of another substance or fixative to lead acetate will not affect the positivity of the reaction only when the lead ions precede the added compound, or when both reach the reactive sites simultaneously. In the last case, the particular fixative added will impose some new features of its own on the structures. Thus, addition of acetic acid causes the lead acetate to penetrate faster than it would alone (5), but some phosphate diffusion occurs. The addition of glutaraldehyde, which was successful in maize roots, produces a better general fixation image than lead alone. However, with a larger piece of tissue (e.g. the liver and spinal cord), the nuclei react more positively at greater depths with lead acetate alone than with added glutaraldehyde. This observation indicates that this aldehyde penetrates somewhat faster than lead ions.

Significance of Nucleolar Orthophosphate

The enzymology of the nucleus has been reviewed by Siebert and Humphrey (27). Definite proof has been given for the existence of glycolysis in nuclei. Orthophosphate could be required for this process as well as for the synthesis of nucleic acids. It is suggested here that the main function of the orthophosphate pool detected in the nuclei should be considered in relation to the particular site of the pool. This fact strongly points to a relation to some aspect of ribosome biogenesis, since this biogenesis is the main function associated with the nucleolus (see 28). An interesting correlation can be found in the virtual absence of completed ribosomes in the nucleoli—the granules are the larger subunit (29), or a precursor of it (30, 31)—and the dissociating effect of phosphate ions on ribosomes (32, 33). It is well established that the 70–80S ribosomes separated from different types of cells undergo dissociation under a certain balance of pH, ionic strength, and bivalent cation concentration. The phosphate ions, in concentrations from about 0.04 to 0.5 M, bind magnesium and cause dissociation of the ribosomes into their subunits (see 33). Therefore, the concentrated nucleolar pool of orthophosphate anions may be partially responsible for the virtual absence of (Mg-bound) ribosomes in the nucleolus through chelation of divalent cations. However, this reasoning does not explain the virtual absence of the smaller subunit in the nucleolus. The mechanism by which 18S RNA is very rapidly transferred from the nucleolus to the cytoplasm compared to the transfer of 28S RNA is unknown. In this connection, it is interesting to note that the dissociated ribosomes in high salt concentrations are unstable and that it is possible to reduce greatly the amount of the smaller subunit relative to the other subunit by such a process (34).

Whitfield and Perris (35) have shown that both inorganic phosphate and phosphoprotein cause reversible disaggregation of condensed chromatin structures. We have demonstrated the location of orthophosphate ions on the chromosomes at late anaphase-early telophase (Figs. 14 and 15). Therefore, this phosphate pool may also be involved in the mitotic cycle of chromatin condensation through the chelation of divalent cations.

Although it is logical to relate the location of the phosphate anions to the nucleolar function, it cannot be underemphasized that a considerable amount of calcium is present in many nuclei (36). It has been proved (37, 38) that divalent cations, specifically Ca^{++}, in millimolar concentrations produce a strong condensation of nucleohistone fibrils. It is interesting to note that, in the interphase nucleus, when the chromatin is largely noncondensed, the nucleolus is filled by the orthophosphate; but when mitotic chromosomes are fully condensed at metaphase, orthophosphate is only demonstrable in a diffuse state over the cytoplasm. Whether this fact is connected to a cycle in the release of Ca^{++} that could intervene in chromosomal condensation, may merit further study.

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Note Added in Proof:

In collaboration with Dr. C. M. Libanati (manuscript in preparation), the concentration of phosphorus in the lead acetate-glutaraldehyde-fixed maize root cells, relative to that of the tissue fixed

in glutaraldehyde alone, has been determined with the electron probe X-Ray microanalyzer. The large size of the nucleolus in these cells, relative to the nucleus and cytoplasm, permitted a good intranuclear resolution. The result indicated that the concentration of phosphorus over the nucleolus due to lead orthophosphate is about four to five times as high as the concentration of the organic phosphorus. Assuming that nearly all of the organic phosphorus is due to RNA and that this RNA represents 10% of the total dry matter of the nucleolus (which has a protein concentration of about 500 mg/cm^3), the concentration of inorganic orthophosphate in these nucleoli must be roughly 0.4 to 0.8 M. The concentration of inorganic orthophosphate in the total undiluted cell sap is about 10^{-2} M; this demonstrates that the nucleoli are centers of inorganic phosphate accumulation in the living cell. The high concentration of these anions (together with their cationic counterparts) points to their role in the solubilization of ribosomal proteins and some other types of protein located in the nucleoli which are known to be rather insoluble in dilute aqueous salt solutions but soluble in salt solutions of high ionic strength.

REFERENCES

1. Tandler, C. J. 1956. An acid-soluble component of the nucleolus; the cytochemical specificity of the lead acetate reaction. *J. Histochem. Cytochem.* **4**:331.
2. Tandler, C. J. 1957. A chemically specific technique for the intracellular localization of inorganic phosphate. *J. Histochem. Cytochem.* **5**:489.
3. Tandler, C. J. 1958. The localization of inorganic phosphate in the oocytes of *Bufo arenarum*. Heterogeneity of the nucleoli. *Exp. Cell Res.* **14**:408.
4. Tandler, C. J. 1960. The localization of intracellular orthophosphate. The role of the nucleoli. *Biochim. Biophys. Acta.* **44**:536.
5. Tandler, C. J. 1961. The detection of orthophosphate within the cell nucleus. *Exp. Cell Res.* **25**:246.
6. Tandler, C. J. 1966. Detection and origin of nucleolar components: a model for nucleolar RNA function. *Nat. Cancer Inst. Monogr.* **23**:181.
7. Studzinski, G. P., and R. Love. 1964. Nucleolar organelles shown by lead precipitation in unfixed cultured cells. *Stain Technol.* **39**:397.
8. Studzinski, G. P. 1965. Selective binding of zinc by basic proteins of the HeLa cell nucleolus. *J. Histochem. Cytochem.* **13**:365.
9. Studzinski, G. P., H. E. Reidbord, and R. Love. 1967. Enhancement of the affinity of nucleoli and chromosomes for zinc by treatment with chromic acid. *Stain Technol.* **42**:301.
10. Tandler, C. J. 1955. The reaction of nucleoli with ammoniacal silver nitrate in darkness; additional data. *J. Histochem. Cytochem.* **3**:196.
11. Tandler, C. J. 1959. The silver-reducing property of the nucleolus and the formation of prenucleolar material during mitosis. *Exp. Cell Res.* **17**:560.
12. Mazia, D. 1961. Mitosis and the physiology of cell division. *In* The Cell. J. Brachet and A. E. Mirsky, editors. Academic Press Inc., New York. **3**:77.
13. Das, N. K. 1962. Demonstration of a non-RNA nucleolar fraction by silver staining. *Exp. Cell Res.* **26**:428.
14. Das, N. K. 1962. Synthetic capacities of chromosome fragments correlated with their ability to maintain nucleolar material. *J. Cell Biol.* **15**:121.
15. Das, N. K., and M. Alfert. 1963. Silver staining of a nucleolar fraction, its origin and fate during the mitotic cycle. *Ann. Histochim.* **8**:109.
16. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
17. Lawn, A. M. 1960. The use of potassium permanganate as an electron-dense stain for sections of tissue embedded in epoxy resin. *J. Biophys. Biochem. Cytol.* **7**:197.
18. Wiesenberger, E. 1965. The use of the electron microscope in analytical chemistry. *Lab. Invest.* **14**:1026.
19. Hall, C. E. 1966. Introduction to electron microscopy. McGraw-Hill Book Company, New York. 200.
20. ASTM Index. 1963. Index (Inorganic) to the powder diffraction file. Published by The American Society for Testing and Materials. Philadelphia. Card No. 8–259.
21. Ts'O, P. O. P., and C. S. Sato. 1959. Synthesis of ribonucleic acid in plants. *Exp. Cell Res.* **17**:227.
22. Lafontaine, J. G. 1968. Structural components of the nucleus in mitotic plant cells. *In* The Nucleus. A. J. Dalton and F. Hagenau, editors. Academic Press Inc., New York. 151.
23. Lison, L. 1963. *In* Histochimie et Cytochimie Animales. Gauthier-Villars. Paris. 10.
24. Bass, R., and P. Saltman. 1959. The accumulation of iron by rat liver cell suspensions. *Exp. Cell Res.* **18**:560.
25. Possingham, J. V., and R. Brown. 1957. Intracellular incorporation of Iron-59 into the root cells of *Pisum*. *Nature.* **180**:653.

26. Pardee, A. B. 1966. Purification and properties of a sulfate binding protein from *Salmonella typhimurium*. *J. Biol. Chem.* **241**:5886.
27. Siebert, G., and G. B. Humphrey. 1965. Enzymology of the nucleus. *Advan. Enzymol.* **27**:239.
28. Birnstiel, M. 1967. The nucleolus in cell metabolism. *Ann. Rev. Plant Physiol.* **18**:25.
29. Narayan, S. K., W. J. Steele, and H. Busch. 1966. Evidence that the granular and fibrillar components of nucleoli contain 28 and 6S RNA, respectively. *Exp. Cell Res.* **43**:483.
30. Penman, S., I. Smith, and E. Holtzman. 1966. Ribosomal RNA synthesis and processing in a particulate site in the HeLa cell nucleus. *Science.* **154**:786.
31. Warner, J. R., and R. Soeiro. 1967. Nascent ribosomes from HeLa cells. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1984.
32. Chatterjee, S., and N. D. Gupta. 1963. Ultracentrifugal studies on the extracts from proliferating yeast cells. *Exp. Cell Res.* **30**:224.
33. Petermann, M. L. 1964. The physical and chemical properties of ribosomes. American Elsevier Publishing Co., Inc., New York. 148.
34. Ts'o, P. O. P., and J. Vinograd. 1961. Studies of ribosomes from reticulocytes. *Biochim. Biophys. Acta* **49**:113.
35. Whitfield, J. F., and A. D. Perris. 1968. Dissolution of the condensed chromatin structures of isolated thymocyte nuclei and the disruption of deoxyribonucleoprotein by inorganic phosphate and a phosphoprotein. *Exp. Cell Res.* **49**:359.
36. Steffensen, D. M. 1961. Chromosome structures with special reference to the role of metal ions. *Int. Rev. Cytol.* **12**:163.
37. Solari, A. J. 1965. The structure of the chromatin in sea urchin sperm. *Proc. Nat. Acad. Sci. U.S.A.* **53**:503.
38. Solari, A. J. 1968. The ultrastructure of chromatin fibers. I. The effect of spreading conditions. *Exp. Cell Res.* **53**:553.