# THE DISTRIBUTION OF THE WATER-SOLUBLE INORGANIC ORTHOPHOSPHATE IONS WITHIN THE CELL: ACCUMULATION IN THE NUCLEUS

## Electron Probe Microanalysis

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ABSTRACT

Lead acetate treatment of unfixed cells immobilizes the intracellular water-soluble, inorganic orthophosphate ions as microcrystalline lead hydroxyapatite precipitates (see reference 1). These precipitates have been analyzed with the electron microprobe. A much higher concentration of phosphorus has been found in the nucleoli of maize root tip cells fixed in lead acetate–glutaraldehyde (organic phosphorus plus inorganic orthophosphate), as compared to the nucleoli of roots fixed in glutaraldehyde alone (organic phosphorus). The concentration of the inorganic orthophosphate pool in these nucleoli is three to five times as high as the concentration of the macromolecular organic phosphate. Since nearly all of the latter is in RNA, the concentration of inorganic phosphate in the nucleolus is calculated to be roughly 0.5–0.8 M. About 30%—and up to 50%—of the total cellular inorganic phosphate is accumulated in the nucleolus since the mean concentration per cell is about $10^{-2}$ M. In the extranucleolar part of the nucleus the mean concentration was estimated by densitometry to be roughly six times less than in the nucleolus ($\leq 0.1$ M), and appears more concentrated in the nucleoplasm than in the condensed chromatin. While there is no direct evidence for the concentration in the cytoplasm, it certainly must be much lower than the mean cellular level (i.e., $< 10^{-2}$ M) since the nucleus is about 10% of the total cell volume. The implications of this compartmentation in the intact cell are discussed in connection with (*A*) the availability of orthophosphate ions for the cytoplasm in those processes in which these ions affect the rate of enzymatic reactions, and (*B*) protein–nucleic acid interactions within the nucleus and nucleolus.

## INTRODUCTION

Inorganic orthophosphate, as a water-soluble anion, is well known to be an important constituent of all living plant and animal cells. Previous work in our laboratory (1) has lent support to the concept that the distribution of this ion within the cell is not homogeneous. The nucleus and especially the nucleolus have been demonstrated to possess a relatively high concentration of water-soluble inorganic phosphate ions (1).

An exact interpretation of this work, however, was hampered by lack of quantitative data. The following questions remained to be answered: What is the concentration of inorganic phosphate in the nucleolus and nucleus? How much of the

total inorganic phosphate of the whole cell is present in the nucleolus and nucleus?

To resolve the problem, we investigated the quantitative distribution of phosphorus with the electron probe X-ray microanalyzer. The evidence presented here conclusively demonstrates that the concentration of inorganic phosphate in the nucleolus is, indeed, very high and that the amount present in the whole nucleus probably accounts for a large (more than 50%) proportion of the total inorganic phosphate of the whole cell.

## MATERIAL AND METHODS

### *Electron Microscope*

Maize root tip cells were selected for this work because of the large size of the nucleolus. The fixatives used were: (*A*) a 4.5% solution of lead acetate, $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ (Merck Chemical Division, Rahway, N.J., analytical reagent) in 0.2 M cacodylate-HCl buffer (pH 7.0) containing 2% glutaraldehyde (50%; Fisher Scientific Company, Pittsburgh, Pa., biological grade), or (*B*) a 7.5% solution of lead acetate in glass-distilled water (pH 6.0). The roots were fixed by immersion, washed, and embedded in Maraglas as described before (1). Control sections were obtained from roots fixed in glutaraldehyde alone (in 0.2 M cacodylate-HCl buffer, pH 7.0), immersed afterwards into 4.5% lead acetate, washed, and embedded as before. Thin and thick (1-$\mu$) sections were cut with glass knives on a Porter-Blum microtome, mounted on formvar-coated copper grids, and examined *unstained* with a Siemens Elmiskop I electron microscope.

### *Electron Microprobe*

The X-ray microanalyzer used in this work is a sensitive instrument, although its resolution is limited to about 1 $\mu$ diameter. The atomic elements can be determined qualitatively and, in our case, also quantitatively if identical preparation procedures are followed. The instrument has been described in the literature (2, See also reference 3), and its application to biological samples is illustrated in more than 80 papers published since 1959 (4).

The procedures used in this work were as follows: the Maraglas-embedded 1-$\mu$ sections of maize root tips mounted on copper grids as described above were covered, in vacuum, on both sides with an aluminum film about 100 A thick. The high thermal and electrical conductivity of this simple arrangement made possible the scanning for long periods during measurement without burning of the specimen. The distribution of the elements in the sections was studied with a CAMECA model MS 46 microprobe with an accelerating voltage of 10 kv (for phosphorus and sulfur) and 30 kv (for lead) and a probe current of 70–100 nA. The $K\alpha_1$-radiation was used for studying the distribution of phosphorus and sulfur while lead was scanned with the L$\alpha$-radiation. As the electron beam sweeps across the tissue section the emission of characteristic X-rays by the element present in the section is picked up by a crystal spectrometer equipped with a suitable counting system, and the output is displayed on an oscilloscope; in this manner, the relative distribution of phosphorus, lead, and sulfur in a given area is recorded in micrographs (e.g., Figs. 8–10). It is also possible to display the electrons transmitted by the section on an oscilloscope and to photograph the image obtained (e.g., Fig. 7). Thus, by comparison, the topography can be correlated with the presence of the particular elements. Scans are obtained by moving the specimen slowly along a line; the amplitude of the trace is proportional to the concentration of the element present at different points in the traverse (Figs. 11 and 12). The emission from a given element is independent of the crystal structure of the compound containing it (5). Thus, the phosphorus in the crystalline lead hydroxyapatite precipitate can be measured accurately in our sections, as well as the phosphorus in the organic material. The degree of penetration of the beam varies with the density of the specimen; however, the thickness of the section is certainly less than the depth of penetration.

## RESULTS

### *Electron Microscope*

The experimental basis of these studies lies in the finding that lead acetate fixation effectively immobilizes the intracellular inorganic orthophosphate anions as microcrystalline lead precipitates, and that these precipitates accurately reflect the localization which exists in the living cell (1). Electron and X-ray diffraction data have demonstrated the chemical uniformity of the precipitates, which is due exclusively to lead hydroxyapatite $Pb_5(PO_4)_3$ OH (1). Fig. 1 illustrates the striking specificity of localization in an unstained section of maize root tip cells fixed in lead acetate-glutaraldehyde. The lead phosphate crystals are massively deposited inside the nucleoli, and the nucleolus boundary is quite sharply delimited by them. The rest of the precipitate is found within the nucleus and is delimited by the nuclear membrane. These precipitates were completely absent in roots fixed in glutaraldehyde first and treated afterwards with lead acetate, indicating the washing out of the water-soluble

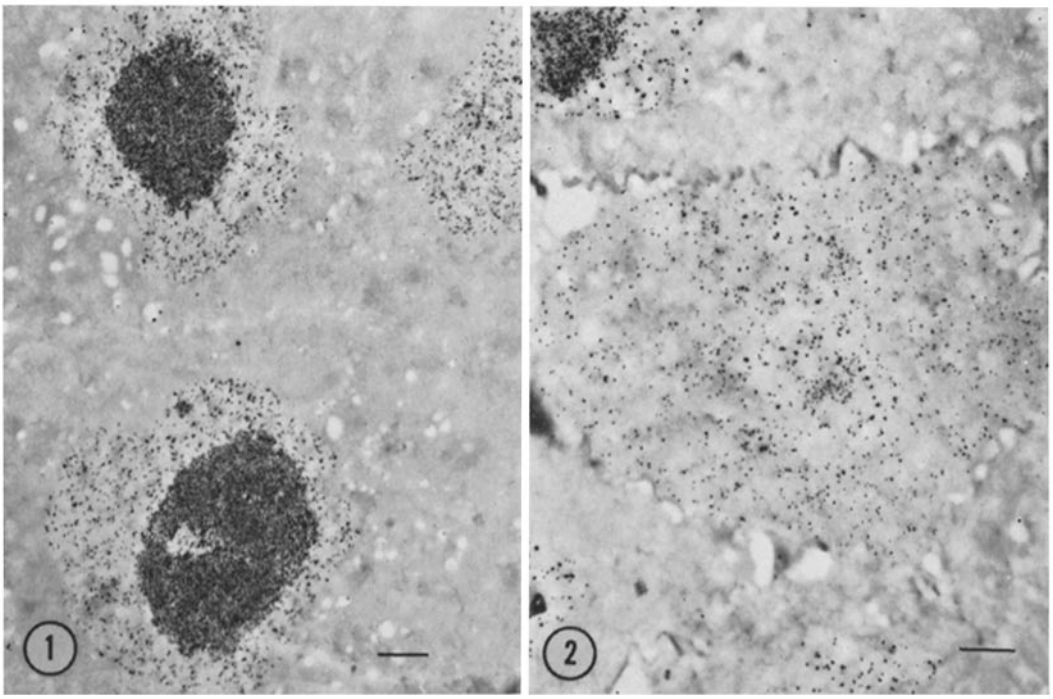


FIGURE 1 Electron micrograph of a thin section of maize root tip fixed in lead acetate–glutaraldehyde, pH 7.0. The electron-opaque microcrystalline precipitate of lead orthophosphate is massively deposited in the nucleoli. The rest of the precipitate is in the nuclei and is delimited by the nuclear membrane. Unstained. Scale mark, 1 $\mu$. × 6,000.

FIGURE 2 Electron micrograph of a thin section of maize root tip fixed as for Fig. 1. The cell in the center of the micrograph is in mitosis (metaphase or early anaphase); the cytoplasm is filled with the lead phosphate crystals. A part of two interphase nuclei can be seen at left of the micrograph. Unstained. Scale mark, 1 $\mu$. × 6,900.

orthophosphate pool by the aqueous glutaraldehyde fixative. Fig. 2 shows an unstained thin section through several cells, one of which is in mitosis (metaphase or early anaphase, as shown by uranyl-acetate staining of this same section). In this case—and contrary to what is found in interphase cells—the cytoplasm is filled with lead phosphate crystals. This evidence, that the water-soluble inorganic phosphate ions are detectable in the cytoplasm only when the nuclear membrane is absent (or incomplete), strongly suggests that the concentration of these ions in the cytoplasm of the interphase cell is quite low, and probably below the sensitivity range of the reagent. An independent confirmation, however, is needed to prove this point conclusively, and the quantitative analysis with the electron microprobe was, therefore, made with these same sections.

### *Electron Microprobe*

QUALITATIVE DETERMINATIONS: Fig. 3 shows the absorbed electron image from a lead acetate-glutaraldehyde–fixed section. The outlines of three nucleoli with their nuclei are evident in Fig. 3. This image also shows a nucleus without a nucleolus, owing to the plane of sectioning, and two "light" cellulose walls crossing at about the middle of the micrograph. This topography can be visualized in more detail in Fig. 1, which shows an image through the electron microscope.

Fig. 4 shows the lead L$\alpha$-emission image of the same specimen. The white specks are clearly distributed at the site of the nucleoli. The background specks over the cytoplasmic areas are similar in density to those found over an area free of tissue and are attributable to "noise" in the

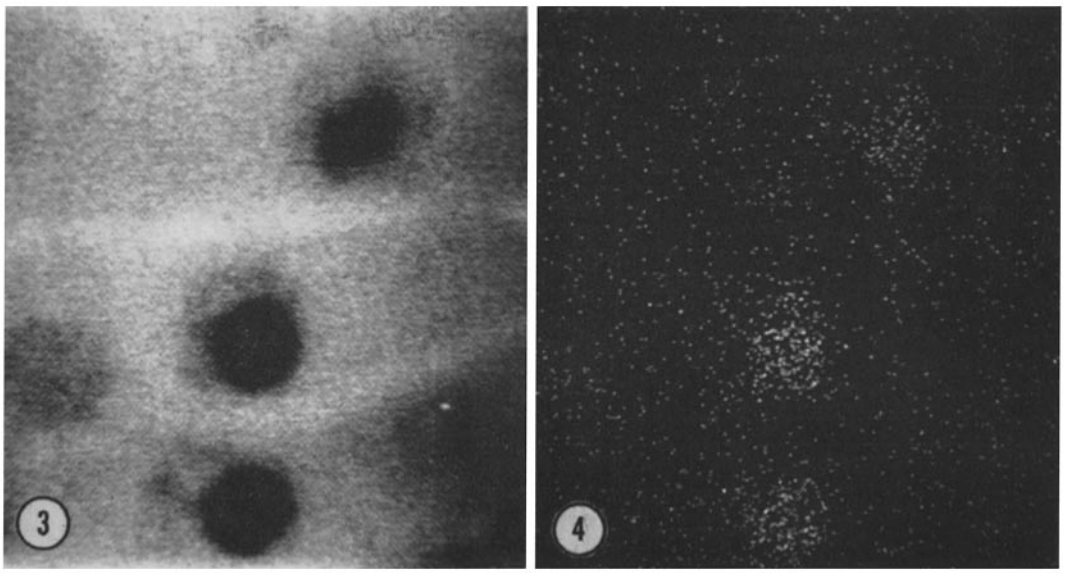


FIGURE 3 Absorbed electron image taken with the microprobe from a thick 1-$\mu$ section of maize root tip fixed in lead acetate–glutaraldehyde, pH 7.0. The outlines of three nucleoli with their nuclei are evident. The image also shows a nucleus without a nucleolus, and two "light" cellulose walls crossing at about the middle of the micrograph. × 3,800.

FIGURE 4 The image for the L$\alpha$-emission wavelength of lead on the same area of the section shown in Fig. 3. × 3,800.

instrument; they do not indicate presence of any element. Evidently the lead distribution closely corresponds to the electron-opaque microcrystalline precipitate found in the nucleoli of Fig. 1, as observed with the electron microscope.

Fig. 5 shows the phosphorus K$\alpha_1$-emission image of the same specimen. In this case, the white specks are more concentrated at the sites of the nucleoli than they are over the cytoplasm. The outlines of the cellulose walls can be discerned in this micrograph, more or less delimited by the white specks. It is evident that this emission image corresponds to the phosphorus distribution of both the lead phosphate and the organic phosphate (nucleic acids, etc.).

Fig. 6 shows the phosphorus K$\alpha_1$-emission image of a "control" section obtained from roots fixed in glutaraldehyde *alone*. This section represents the phosphorus distribution due to the organic material only: the white specks are more or less evenly distributed and there is no difference in phosphorus concentration over nucleoli and cytoplasm. It is evident, therefore, that the great concentration of phosphorus at the nucleoli in Fig. 5 is due mainly to the lead microcrystalline precipitate. This is the direct proof for the presence of phosphorus in the nucleolar lead precipitate and constitutes a further confirmation of the electron and X-ray diffraction evidence which showed that this crystalline precipitate is due exclusively to lead hydroxyapatite (1). Moreover, the sulfur K$\alpha_1$-emission images of the same section as Fig. 3 failed to show any difference in concentration at the nucleoli, indicating the absence of this element in the lead precipitate.

Fig. 10 shows the sulfur K$\alpha_1$–emission image of the same "control" section as Fig. 6 and shows that, as is the case with phosphorus, sulfur is more or less evenly distributed over nucleolus and cytoplasm.

Fig. 7 shows the absorbed electron image of a section from roots fixed in lead acetate alone (omitting the glutaraldehyde in the fixative). One large nucleolus is outlined lying inside the elongated nucleus. Figs. 8 and 9 show, respectively, the lead and phosphorus distribution in this same specimen. There is no doubt that with this fixative, too, the phosphorus is heavily concentrated

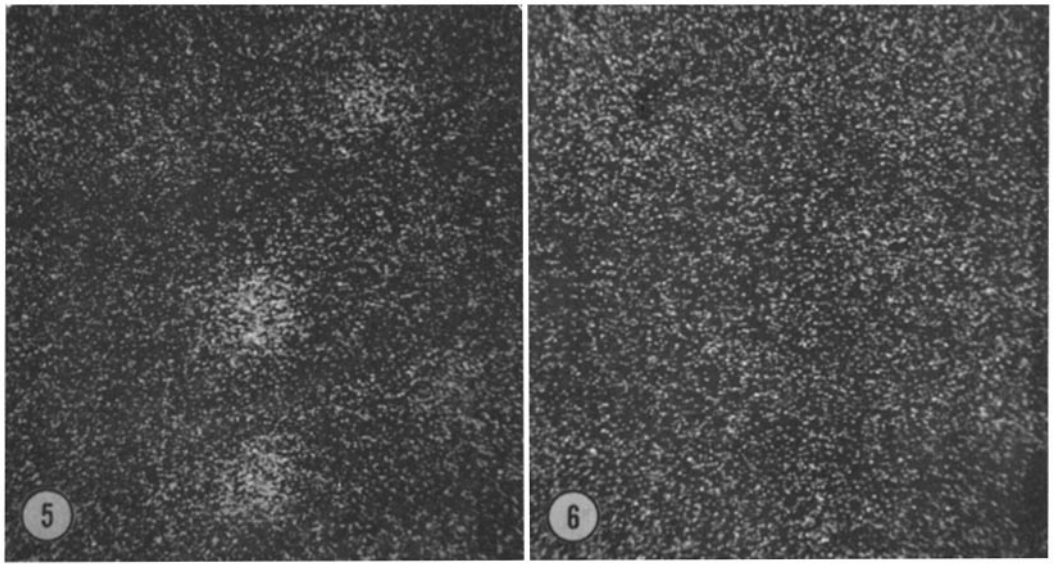

FIGURE 5 The image for the $K\alpha_1$-emission wave length of phosphorus on the same area of the section shown in Figs. 3 and 4. It represents the phosphorus distribution due to both the organic material plus the lead orthophosphate precipitate; the highest concentration is in the nucleoli (compare with Fig. 6). The outline of the cellulose walls can be seen delimited by the white specks. × 3,800.

FIGURE 6 The image for the $K\alpha_1$-emission wave length of phosphorus of a "control" section fixed in glutaraldehyde alone. It represents the phosphorus distribution due to the organic material only, and there is no difference in concentration over nucleoli and cytoplasm. × 3,800.

over the nucleolus and that the larger part of it corresponds to the lead orthophosphate precipitate. There is no doubt that the good intracellular localization obtained with the probe in Figs. 5 and 9 can be attributed both to the relatively large size of the nucleoli (2–5 $\mu$ in diameter) and to the high concentration and specificity of localization of the lead phosphate precipitate.

QUANTITATIVE DETERMINATIONS: Fig. 11 shows the phosphorus concentration along a line traversing several nucleoli. This is the same specimen as Fig. 3 and corresponds to roots fixed in lead acetate–glutaraldehyde. It is interesting that the intensity in the peaks is approximately equal, indicating that about the same amount of lead phosphate was precipitated per unit volume in all these nucleoli. The average ratio counts per second (cps) of nucleolus to counts per second of cytoplasm was 5.2, with values ranging between 4.0 and 6.3 (corrected for background of 2.8 cps over the nucleolus and 1.0 cps over the cytoplasm).

Fig. 12 shows the phosphorus concentration along a similar traverse line of the same "control" section as Fig. 6 and corresponds to roots fixed in glutaraldehyde alone. It is evident that the intensity due to organic phosphorus does not differ greatly along the trace. This indicates that the phosphorus concentration due to the organic material (per unit area) at the nucleolus and at the cytoplasm is nearly the same. Of course, this result applies to cells of the meristematic zone: in these cells—and contrary to those located in the vacuolized zone—the cytoplasm shows a strong basophilia due to RNA and is very rich in ribonucleoprotein granules, as observed with the electron microscope (1).

By comparing Figs. 11 and 12, the relative concentration of phosphorus as lead phosphate precipitate can be determined. In both cases, the concentration of organic phosphorus must be the same and the lead phosphate crystals are absent from the cytoplasm of the interphase cells (Fig. 1). Therefore, the relative concentration of phosphorus as lead phosphate precipitate can be expressed as the intensity ratio between nucleolus and cytoplasm of Fig. 11 and subtracting from it the intensity ratio for organic phosphorus (equal to *1*, Fig. 12). This gives an average ratio of about 4.2

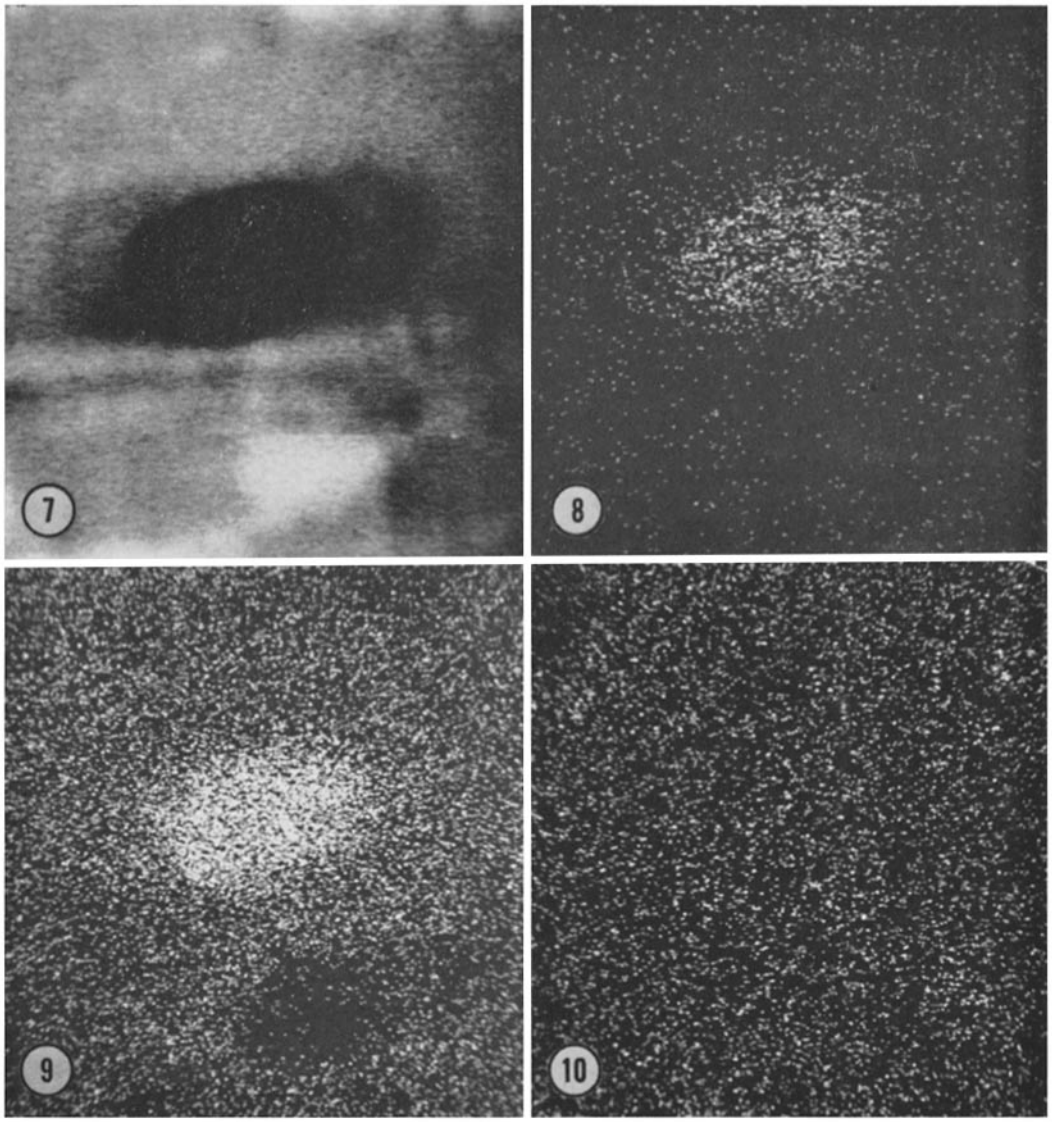


FIGURE 7 Absorbed electron image taken with the microprobe from a thick 1-$\mu$ section of maize root tip fixed in lead acetate, pH 6.0. The outline of one large nucleolus lying inside the elongated nucleus is evident. × 3,800.

FIGURE 8 The image for the L$\alpha$-emission wavelength of lead on the same area of the section shown in Fig. 7. × 3,800.

FIGURE 9 The image for the K$\alpha_1$-emission wavelength of phosphorus on the same area of the section shown in Figs. 7 and 8. × 3,800.

FIGURE 10 The image for the K$\alpha_1$-emission wavelength of sulfur of the same area of the "control" section shown in Fig. 6. × 3,800.

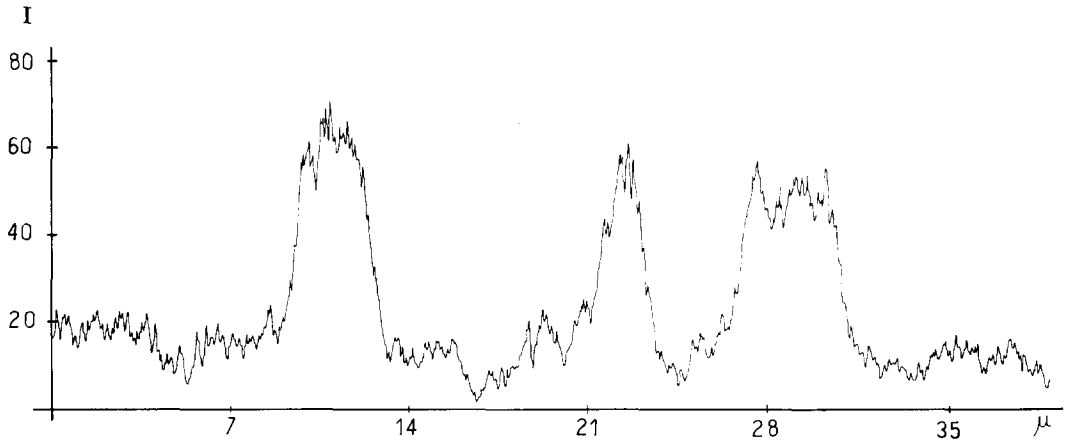


FIGURE 11 Phosphorus concentration along a 39-μ traverse line on the same area as Figs. 3 and 5. The peaks correspond to a much higher concentration in the nucleoli which is due mainly to lead orthophosphate (compare with Fig. 12). The height of the trace corresponds to the relative concentration of element present. *I*: X-ray intensity (counts per second).

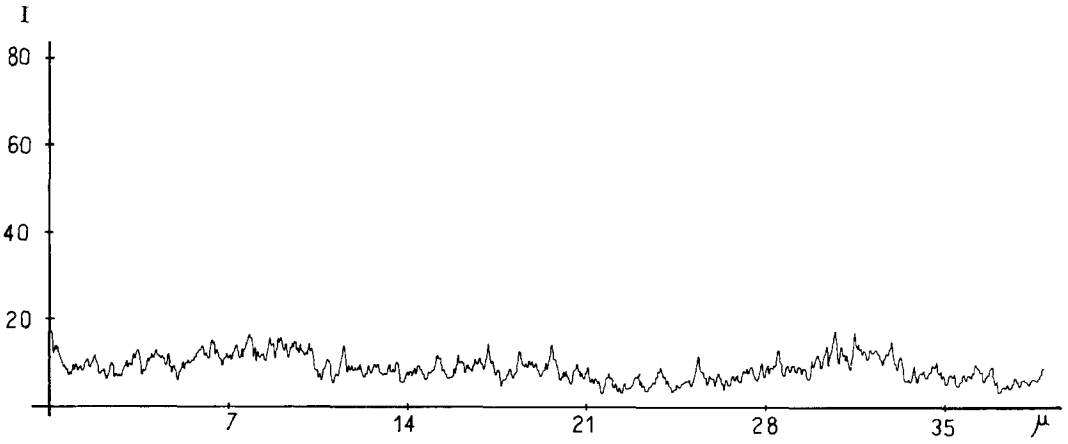


FIGURE 12 Phosphorus concentration along a 39-μ traverse line on the same "control" area as Fig. 6, and corresponds to the organic phosphorus only. *I*: X-ray intensity (counts per second).

(with values ranging from 3.0–5.3) for inorganic phosphorus relative to the organic phosphorus.

CALCULATIONS: There is no doubt, therefore, that lead acetate fixation preserves a large amount of phosphate, which is not retained by the conventional fixatives, e.g., glutaraldehyde alone versus glutaraldehyde plus lead acetate (1). In the nucleolus, the phosphate due to a water-soluble inorganic orthophosphate pool is three to five times greater than the phosphate due to the macromolecular organic phosphate. Some calculations can be made, assuming that practically all the organic phosphate in the nucleolus is due to RNA (6) and taking published values from the literature. The concentration of protein in the nucleolus is about 500 mg/cm³ (7) (which corresponds roughly to a dry matter of 40%). The RNA concentration is about 10% of the total dry weight, that is, about 50 mg/cm³, or 5 mg/cm³ as RNA phosphorus (taking 10% as the average phosphorus content of RNA; 8). Therefore, the concentration of the nucleolar inorganic phosphate pool, which is three to five times higher, gives a value of 15–25 mg/cm³ (as phosphorus) or about 50–80 mg/cm³ (as phosphate), which corresponds roughly to a molarity of 0.5–0.8 M. Although the exact value is not known, owing to the uncertainties in the value taken as representative for organic phosphate (or

for RNA, too), there is no doubt that the concentration is indeed very high.

The concentration of inorganic phosphate in the rest of the nucleus is somewhat difficult of estimation with certainty. The lead phosphate crystals, although delimited by the nuclear membrane (Fig. 1), are unevenly precipitated within the nucleus (1). An approximate value was calculated, from the micrograph of Fig. 1, by integration of the height of the densitometric tracings per unit length over nucleolus and nucleus (and over the nucleus without a nucleolus, Fig. 1), using a Chromoscan MK II Densitometer (Joyce, Loebl & Co., Ltd., England) (magnification of the micrograph, × 10,000; slit, 0.5 mm in width). The uneven dispersion of the phosphate crystals affected the values obtained. The average concentration ratio of nucleus to nucleolus found by this procedure was about 1/6, ranging from 1/4 to 1/9 at different sites within the nucleus. Therefore, the average inorganic phosphate concentration in the nucleus would correspond roughly to $\leq 0.1$ M.

The water-soluble inorganic orthophosphate concentration of the tissue, estimated by direct chemical analysis of the undiluted sap, is about $10^{-2}$ M (9). Taking this value as representative for the mean concentration of the whole cell, one can calculate approximately how much inorganic phosphate is present in the nucleolus and nucleus. These structures constitute, respectively, about 1 and 10% of the total cell volume, and their inorganic phosphate concentration is roughly 0.5–0.8 M and $\leq 0.1$ M, respectively. Therefore, about 30%—and up to 50%—of the inorganic phosphate of the whole cell is accumulated in the nucleolus. This means that more than 50% of the inorganic orthophosphate ions of the cell are accumulated—and unevenly distributed—in the nucleus. Of course, this is a very rough estimate, but there is no doubt that the concentration in the cytoplasm is quite low compared to that in the nucleus. There is no direct evidence for its exact value, but it is presumably much lower ($< 10^{-2}$ M) than the mean concentration, since this phosphate concentration should have been detected. What can be said with certainty is that the cytochemical test truly represents the sites in which important amounts of inorganic phosphate are *concentrated* in the living cell.

## DISCUSSION

### *Significance of Inorganic Phosphate Compartmentalization in the Intact Cell*

NUCLEUS AND CYTOPLASM: The present paper, together with the evidence presented in a previous paper (1), demonstrates that the cell nucleus is intimately involved in the accumulation of water-soluble inorganic phosphate ions. The mean intracellular concentration of inorganic orthophosphate is rather high in both plant and animal cells, as indicated by direct chemical analysis of total cell extracts (9, 10). We have found that in maize root tips the average concentration in the nucleus is roughly five to ten times higher than the mean level per total cell. Therefore, the conclusion seems inescapable that the concentration of inorganic phosphate in the cytoplasm must be much lower than the mean cellular level since the nucleus represent about 10% of the total cell volume.

The nuclear membrane—its inner layer (1)—marks the boundary between a nuclear, phosphate-rich compartment, and the cytoplasmic compartment with a low orthophosphate level. This finding carries biochemical implications for a number of enzymatic reactions which take place in the cytoplasm, particularly those in which the concentration of orthophosphate controls the reaction rate. Indeed it has been suggested already that compartmentation could control the availability of intracellular orthophosphate (11, 12). Our results provide the evidence for the existence of such a regulation process in the living cell. Of course, other mechanisms for the regulation of enzymatic activity are also operational (13).

The importance of inorganic orthophosphate in the economy of the intact cell is clearly evidenced by the well known fact that the concentration of this anion is one of the factors which control the rate of both glycolysis (13, 14) and respiration (15). Some of the other factors are metabolites (AMP, ADP, ATP) which exist in (enzymatic) equilibrium with orthophosphate itself (14, 15). Respiration occurs in mitochondria, while glycolysis apparently takes place in both the cytoplasmic matrix and the nucleus. The distribution of glycolytic enzymes between nucleus and cytoplasm is reported to be similar (16). However, the contribution of the nucleus to the glycolytic ATP formation in the intact cell should be considered also in relation to the availability of both ortho-

phosphate and glucose, among other parameters. In this connection, it is interesting to quote here that diphosphopyridine nucleotide (DPN), a key coenzyme for both glycolysis and respiration, has been definitely proven to be synthesized in the nucleus (16), and is compartmentalized between the nucleus and cytoplasm (17).

The unequal distribution of orthophosphate ions, as reported here, strongly suggests that the nucleus has a regulatory or control function over the phosphate metabolism of the whole cell. We do not have any evidence on the mechanism of entrance of the phosphate ions into the nucleus and whether some type of active transport through the nuclear membrane is involved in building up a concentration gradient. What is evident is that the total cellular inorganic phosphate is not fully available to the cytoplasm. This structural non-availability presumably is a limiting factor in the rate of glycolysis and respiration, and probably of other reactions in which orthophosphate exists in enzymatic equilibrium with organic phosphates in the cytoplasm.

This regulation of the metabolic processes occurring in the cytoplasm could certainly control the phosphate uptake by the intact cell since the translocation of inorganic phosphate ions through the cellular membrane is known to be an active process, i.e., dependent on a functional energy-yielding process such as respiration or glycolysis (11, 14, 15). In this connection, it is very suggestive that anucleate cell fragments obtained experimentally from nonphotosynthetic cells such as *Amoeba* and *Stentor* showed an extraordinary decline in $P^{32}O_4$ uptake, followed by altered levels of ATP and carbohydrate utilization (18, 19).

From the high level of inorganic phosphate found within the nucleus, as reported in this paper, a similarly high concentration of cation(s) as counterions is predictable. Fragmentary reports in the literature strongly suggest that the cell nucleus may, indeed, be involved in the accumulation of other ions too. As early as 1949, Abelson and Duryee (20), using the large frog ovarian oocyte, demonstrated a striking accumulation of $Na^{24}$ in the nucleus. These experiments were confirmed and extended by Naora et al. (21) who showed an accumulation of both sodium and potassium in the nucleus of the oocyte, the respective concentrations in the nucleus being about five and three times higher than in the cytoplasm. In this large specialized cell, a striking nuclear accumulation was also found for inorganic phosphate and sulfate ions as well as for several aminoacids (21). A relationship between the nucleus and iron uptake has also been described in liver (22) and pea root (23) cells.

It is of special interest that all these ions have been traced into the nucleus in the *intact* cell; this fact shows that the nuclear membrane is permeable to these ions and, more significantly, that their (mean) concentration in the nucleus is much higher than that in the cytoplasm. In this connection, it is interesting that the cytoplasmic components and the nucleus have different pH values; the nucleus has a slightly alkaline pH, which is about one unit higher than the pH of the cytoplasmic matrix (24). Rat liver nuclei isolated by the Behrens's nonaqueous procedure were reported to contain also considerable quantities of sodium and potassium (16) as well as inorganic phosphate (25). The ratio nuclei/cytoplasm for sodium and potassium obtained by this technic in rat liver was about 10 and 1.3, respectively (16).

INTRANUCLEAR COMPARTMENTATION: The second point demonstrated is that the distribution of water-soluble inorganic orthophosphate ions within the nucleus is not homogeneous. The condensed chromatin appears relatively free of these anions, whereas they are dispersed through the nucleoplasm and are heavily concentrated in the nucleoli (1). The nucleolus boundary itself clearly delimits the regions between a nucleolar, very rich compartment and the extranucleolar compartment with much lower orthophosphate level (Fig. 1). Particularly striking is the accumulation of phosphate ions inside the nucleolus since this structure does not have a limiting membrane. Evidence for the probable existence of a "binder" in the nucleolus itself has been pointed out before (1). Diffusion artifacts as a source of false location for the nucleolus have been eliminated (1) and will not be referred to here.

The nucleolus has been demonstrated to supply the cell with the two major ribosomal RNA's found in the cytoplasmic ribosomal subunits (26). The inorganic phosphate level in the nucleoli of maize root tips, as determined with the electron microprobe, represents three to five times the amount of the macromolecular organic phosphate (which is mainly in RNA; 6). This is roughly of the order expected for a "local" synthesis of RNA, i.e., starting with the ribonucleotide triphosphates and releasing two phosphate groups (as pyrophosphate). In fact, RNA polymerase (as well as DPN pyrophosphorylase) is concentrated and

firmly bound in the nucleolus (27, 28). In this respect, it is interesting that we have not detected in the nucleolus pyrophosphate which would be expected to accumulate through the action of RNA polymerase. This finding probably points to a more complex enzymatic situation, resulting in hydrolysis to inorganic orthophosphate. Indeed, both biochemical and cytochemical evidence clearly has indicated the presence of ATPase(s) (27, 29, 30), ribonuclease (27), and several phosphatases, including pyrophosphatase, (31) in the nucleolus. Of course, other explanations for the accumulation of orthophosphate are possible since we do not have any evidence on the mechanism of entrance of phosphate ions into the nucleolus. For instance, we have found that about 30%—and up to 50%—of the total cellular orthophosphate is accumulated in the nucleolus of maize root tip cells, and it is quite possible that this particular pool reflects a general situation found in the phosphorus metabolism of the whole cell. In this phosphorus metabolism, although both inorganic ortho- and pyrophosphate would be expected to exist in enzymatic equilibrium with organic phosphates, the high level corresponds only to the orthophosphate. The amount of inorganic pyrophosphate is negligible, and the orthophosphate accounts for more than half of the total soluble phosphates in maize root tips (9, 32). A similar high level for inorganic orthophosphate has been found also in the liver (10).

A second problem that should be considered is the possibility that the inorganic orthophosphate ions of the nucleolus are the immediate precursors of the nucleoside triphosphates. Whether some phosphorylative system (27) or glycolysis (which results in ATP formation) occurs in the nucleolus itself is not known. In fact, it has not been determined whether ATP in the nucleus—which is needed for synthesis of nucleic acid, DPN, or any other reaction—stems from nuclear synthesized ATP or is provided by the cytoplasm (27).

The high ionic strength found in the nucleolus could also affect the latency of several enzymes (e.g., ribonuclease; 27, 33). Craig et al. (34) have found that a high ionic strength suppresses the nonspecific degradation of RNA in isolated nucleoli.

From the unequal distribution of orthophosphate anions within the nucleus, a similar distribution of cation(s) as counterions is expected. Some experimental evidence supports such an expectation. Spicer et al. (35), using the pyroantimonate–osmium tetroxide procedure, found electron-opaque deposits, presumably of sodium pyroantimonate, localized mainly in the nucleus; these precipitates were limited by the nuclear membrane and were heavily deposited in the nucleoli. This pattern of $Na^+$ localization would be consistent with the pattern of localization found for orthophosphate, although some of the counterparts for phosphate could be basic groups of proteins. Regarding this subject, it is interesting to remark that dense electron-opaque inclusions—the "foamy particles"—have been reported in the nucleoli of some cells. Particularly striking are the cases of onion and broad bean root tip cells, in which these "particles" were reported by Lafontaine (36); in later work, he could not find them (37). An examination of his papers reveals that these "particles" were found in those cases in which the plants were grown in tissue medium culture, which contains relatively large amounts of divalent cations (e.g., calcium). It is possible that the "foamy particles" represent a precipitation reaction between the high concentration of endogenous nucleolar inorganic orthophosphate and the divalent cations of the culture medium. Whether this takes place in vivo or occurs during the fixation process deserves further study.

The high level of inorganic orthophosphate anions in the nucleolus (roughly 0.5–0.8 M), together with their cationic counterparts, strongly suggests a role in the solubilization of ribosomal proteins and other types of protein located in the nucleolus which are known to be rather insoluble in dilute aqueous salt solutions, but soluble in salt solutions of high ionic strength (38, 39).

The relatively high intranuclear level of orthophosphate ions certainly could affect the association and dissociation of nucleic acids with proteins. An interesting correlation was suggested (1) with the virtual absence of completed ribosomes in the nucleoli (40–42) or even in the whole nucleus (41) and the dissociating effect of phosphate ions on ribosomes (43). High levels of inorganic phosphate cause also reversible disaggregation of chromatin structures (44). Such a process has been related to activation of repressed chromatin (45). Therefore, regulation of the ionic intranuclear environment may be involved in genetic regulatory mechanisms. It is very suggestive that after partial hepatectomy, in which changes of the regulatory mechanisms seem to be involved, increases in the intracellular

content of inorganic orthophosphate and sodium have been observed that become maximal within 5 or 10 min after hepatectomy (46). In the liver, as well as in a number of other animal tissues, the pattern of inorganic phosphate localization is essentially similar to that in the plant cells (see reference 1).

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