THE LOCALIZATION OF INTRACELLULAR ORTHOPHOSPHATE: THE ROLE OF THE NUCLEOLI

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SUMMARY

Intracellular orthophosphate has been identified as the fraction which precipitates in the nucleoli after Pb acetate fixation. The results are in good agreement with the important role played by the nucleus—and specially the nucleolus—in the phosphorus metabolism of the cell. On the other hand it is not known how much of the total orthophosphate of the whole cell is present in the nucleoli.

INTRODUCTION

Inorganic phosphate has been localized in the nucleolus by a cytochemical method[1-3]. The unexpected result occurs that when lead acetate is employed as a fixing fluid, a precipitate of lead phosphate appears in the nucleoli. It is clear that lead acetate is able to precipitate the phosphate ions inside the cells; this is, to our knowledge, the first case in which a small, diffusible ion has been localized at the cellular level. The following work was carried out in order to examine this phenomenon more closely.

MATERIALS AND METHODS

The materials used consisted of onion, maize and broad bean roots, ungerminated broad bean embryos, the large internodal cells of *Nitella clavata* and the livers of guinea pigs and chickens. The cytochemical method used was essentially the same as previously described[1]. Tissue extracts were prepared with ice cold 5 % TCA, 4 % perchloric acid or 50 % ethanol plus 5 % acetic acid for 30 min. 5 to 10 ml of extracting fluid were used per g of wet weight tissue. The tissues were not homogenized so that the results may be compared more directly with the cytochemical data. Acetic acid–ethanol and TCA extracts (TCA removed with ethyl ether) were employed either immediately or concentrated over $CaCl_2$ for 24 h at room temperature. An ascending chromatogram was obtained with Whatman No. 1 paper washed with 10 % acetic acid and glass bi-distilled water[4] and a solvent consisting of acetone–acetic acid–water (50:15:35)[5,6]. This solvent, reported for the chromatography of phosphorylated compounds, was found to give good separation with phenolic and ninhydrin-positive substances also. The spots were detected by the application of several color reagents using the "dipping" and "multiple dipping" procedures[7,8]. Pb-precipitating substances were detected by drawing the chromatogram through a 5 % sol. and allowing

it to hang for 15 min. Three washings with bidistilled water, 5 to 10 min each time, were found necessary in order to remove the excess of Pb from the paper; after evaporation of the water the chromatogram was suspended in an H_2S atmosphere. Pb-precipitated substances appeared immediately as brown spots. Pb orthophosphate can be shown on the same paper by dipping the paper into the molybdate reagent[5]: the PbS spot is transformed after a few minutes into a blue spot of phosphomolybdate. Pb precipitates, insoluble in acetic acid, were detected by immersing the Pb acetate dipped-chromatogram into a 5 % Pb acetate solution in glacial acetic acid (*cf.*[1]) for 1 h and proceeding as before. Azide-iodine[9] acts as an iodine stain[10]; brown spots were detected in those cases where no color was seen with iodine alone. Ultraviolet absorbing spots were detected by GORDON'S blueprint paper method[11]. Orthophosphate and the total acid-soluble phosphates were measured by the method of KING[12]. Ribonuclease (Sigma, crystalline) was employed at a concentration of 5 mg/ml distilled water.

RESULTS

The chemical nature of the nucleolar precipitate

Table I shows that very probably the Pb-precipitable substances are extracted by cold TCA. After Pb acetate treatment of TCA fixed roots, a PbS stain is developed in the whole root (approximately 1 mm thick) but no precipitate is found in the nucleoli. If treatment with Pb in glacial acetic acid is made after the aqueous Pb

TABLE I

REACTIVITY OF LEAD WITH ACID-SOLUBLE AND ACID-INSOLUBLE FRACTIONS IN ONION AND MAIZE ROOTS AFTER SEVERAL TREATMENTS

Fixation	*Post-treatment*	*Precipitate in the nucleoli*	*PbS stain in the whole root*
Pb acetate		+	+
Pb acetate	Pb in acetic acid	+	+
Cold TCA	Pb acetate	—	+
Cold TCA	Pb acetate, then Pb in acetic acid	—	—
Pb acetate	Cold TCA, then Pb acetate, then Pb in acetic acid	—	—
Liquid N_2	Pb acetate, then Pb in acetic acid	— (some + diffusion figures)	+

(+) or (—) indicate whether the Pb-precipitate is present or absent from the nucleoli and, in the case of the PbS stain, whether the roots are stained black or remain unstained.

acetate the roots remain completely unstained. This shows (a) that all the acid-insoluble compounds which show affinity for Pb are extracted by the Pb-acetic acid and (b) that very probably the nucleolar substance does not remain in the tissue in a diffused condition, *i.e.*, it must be present in the TCA extract. Immersion in liquid nitrogen is not as effective as Pb fixation: after Pb acetate treatment the phosphate is preserved in a diffused condition in the tissue. The freezing-substitution procedure of RUSSELL *et al.*[13] in which basic Pb acetate is added to the chilled ethanol, is also

TABLE

COLOR REACTIONS APPLIED TO THE CHROMATOGRAM

Spots numbered according

Spot No.	*Pb ac.*	*Pb ac.-acetic acid*	*Uranyl ac.*	*Molybdate*	*u.v. absorbing*	*$FeCl_3$*	*Diazotized sulfanilic acid*	*Diazotized p-nitroaniline*	*Gibbs*
10	+				+	violet	red–orange	violet	blue
9	+				+	violet	red–brown	violet	yellow
8									
7, 7′					+?	yellow	light red	yellow	yellow
6									
5	+	+	+	+					
4									
3							pink	violet	
2, 2′							pink	violet	
1							pink	violet	

ineffective in precipitating the phosphate at the cellular level. Fig. 1 shows a diagram of a one-dimensional ascendent chromatogram of acid-soluble substances in maize roots (var. Colorado Klein). At least 12 substances can be detected by the application of several color reactions, but only 3 give a visible precipitate with Pb acetate; of these only one, orthophosphate, is insoluble in Pb-acetic acid. The same can be said in the cases of onion and broad bean roots. Spots No. 9 and 10, which form Pb precipitates soluble in acetic acid, are flavonoid compounds[14] for they give typical reactions for phenolic substances and a positive reaction for ketones with 2,4-dinitrophenyl hydrazine. *o*-diphenols are known to precipitate with neutral Pb acetate and to

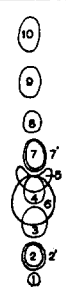

Fig. 1. Pattern of acid-soluble substances in maize roots (cold TCA or acetic–ethanol extracts). Whatman No. 1, ascending for 24 h; solvent, acetone–acetic acid–water (50:15:35); see Table II for detection of the spots; only spots No. 5 (orthophosphate), 9 and 10 (flavonoid compounds) are present in appreciable enough amounts to give a precipitate with Pb acetate; of these, only orthophosphate forms a Pb precipitate insoluble in a 5% Pb acetate solution in glacial acetic acid.

II

OF ACID-SOLUBLE SUBSTANCES IN MAIZE ROOTS

to diagram in Fig. 1.

x-nitroso β-naphtol	*Iodine*	*Azide-iodine*	*Alkaline $AgNO_3$*	*2,4-dinitro phenyl hydrazine*	*Ninhydrin*	*Isatin*	*2,6-dichlorc phenol-indophenol*	*Bromocresol green*	*Basic Pb ac.*
red	+	+	brown	+			purple (bleaches)		light yellow
yellow	+	+	brown	+			purple (bleaches)		light yellow
		+?			blue				
red	+	+		+?	yellow	blue	blue	blue	
			black						
		+			blue				
		+			blue		purple		
		+			yellow		purple		
		+			blue				

strongly reduce the alkaline silver reagent[15]. This is not the present case since only a brown spot is obtained with the silver reagent; the precipitating property is rather due to the relative positions of the OH-groups and CO-groups[16]. They are easily detected as strongly u.v. absorbing spots or as violet and light yellow spots with $FeCl_3$ and basic lead acetate[17] respectively; they give different colors with diazotized sulfanilic acid. On the basis of the typical blue color given by Gibbs reagent (2,6-dibromoquinonechloroimide) and the red spot obtained with α-nitroso-β-naphthol it is concluded that only spot No. 10 has unsubstituted *para* and *ortho* positions. In onion and broad bean roots this fraction is found in a position just above the orthophosphate spot; they also give a Pb precipitate soluble in acetic acid and a positive ketone reaction, but a blue color with Gibbs reagent and a violet spot with $FeCl_3$ is given by the broad bean only. Substances with these characteristics have not been found in liver extracts. The same chromatographic pattern is obtained when the tissues are submitted to extraction with ethanol–acetic acid or when Pb acetate-fixed roots are extracted with cold TCA; in the last case a drop of a concentrated solution of ethylenediamine (EDTA) is added to prevent precipitation of lead as the TCA is gradually removed with ethyl ether. Spots Nos. 1, 2, 3, 4, 7 and 8 are ninhydrin-reacting substances; spots Nos. 2 and 7 give a yellow color instead of the blue color characteristic of the great majority of amino acids. A parallel run with asparagine showed that it has the same R_F as spot No. 2 and that spot No. 7 is an isatin-reacting substance. On this basis, and because they have been reported in the maize anther[18], spots Nos. 2 and 7 were identified as asparagine and proline respectively. Neither of these ninhydrin-reacting substances is tryptophan or arginine since no reaction can be detected on the paper chromatogram with the Ehrlich or Sakaguchi reagents. Asparagine was also detected in broad bean and onion roots. Another substance with

the same R_F as asparagine is present in position 2 (spot No. 2'); it gives an intense pink color with diazotized sulfanilic acid and a violet spot with diazotized nitroaniline. Another substance is also present in position 7 (spot No. 7'); it is a basic compound for it gives blue spots with the pH indicator reagents. That we have here two substances is also indicated by the fact that in onion and broad bean roots, as well as in the liver, spot No. 7 (proline) is absent while the basic compound (spot No. 7') is present. The large spot No. 6 is due to sugars. Phosphate analysis on cold 5 % TCA and 4 % perchloric acid of maize and onion roots showed that orthophosphate accounts for 60–65 % of the total acid-soluble phosphates; there is almost twice as much orthophosphate per g of wet weight tissue in onion as in maize roots. Organic phosphates are present in amounts too small for detection on the paper chromatogram by chemical means (*cf.*[19]). Similar high values for orthophosphate have been found in barley roots and seedlings[20,21]. It is concluded that orthophosphate is the only substance present in the tissue in appreciable enough amounts to give a visible precipitate at the cellular level (Pb salt insoluble in Pb-acetic acid). In the liver, where large quantities of extracellular phosphate are present, the cytological image shows a granular precipitate in the intercellular spaces and specially around the central veins. With perfused liver practically all the "granular" precipitate is absent from the tissue while the dense nucleolar precipitate persists (extracellular phosphate washed out by perfusion with physiological saline). It is concluded that the fraction precipitated by Pb acetate within the nuclei is intracellular orthophosphate.

The nucleolar precipitate in modified cells

Pb acetate fixation of ribonuclease-treated (*in vivo*, 8 h) onion roots shows that phosphate precipitates in the nucleoli even when nucleolar basophilia is lost (staining for RNA with toluidine blue after Carnoy fixation). This is in agreement with the finding that ribonuclease neither markedly affects the inorganic phosphate content[22] nor the uptake of ^{32}P in onion roots[23]. In the large intact cell of *Nitella* the lead precipitate appears specifically located in the nucleoli; more than 100 nuclei can be seen in one large cell and each nucleus contains approximately 20 to 50 Pb-positive nucleoli (Fig. 2). There is no indication of Pb-precipitates in the nuclear membrane, cytoplasm or in the large central vacuole; during nuclear division by amitosis[24] the nucleoli remain Pb-positive. In cut cells most of the vacuolar sap flows out but the protoplasm remains within the cell wall; the results are usually the same as in the intact cell. Since the integrity of the cell membrane is destroyed this suggests that the membrane is not significantly involved in the retention of orthophosphate and that it is "bound" intracellularly. Moreover, the membrane cannot act as a barrier to lead penetration; it is interesting that, according to OSTERHOUT[25,26] 0.01 *M* Pb acetate is not toxic to *Nitella* (it stops protoplasmic motion and contracts the chloroplasts but these effects can be reversed by washing with distilled water). Although the cell's metabolic activity is a complicating factor, it is possible that Pb acetate applied at a sufficiently high concentration enters the cell by physical processes only; this certainly applies to physiologically dormant cells. Excised ungerminated broad bean embryos show the characteristic Pb phosphate precipitate in the nucleoli after Pb fixation (either ice cold or at room temperature). On the other hand, no precipitate is formed in dividing cells at those stages of mitosis where the nucleolus is absent; this result has a bearing on the strongly decreased uptake of ^{32}P in dividing cells[27].

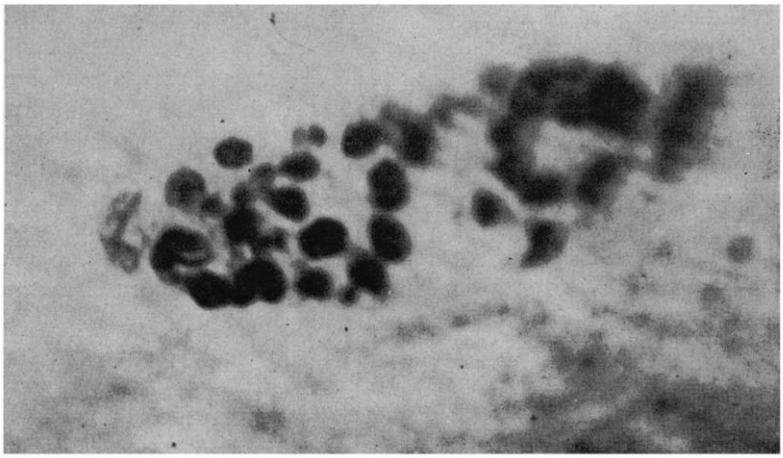

Fig. 2. A nucleus of the multinucleate internodal cell of the fresh-water alga *Nitella clavata* showing the nucleolar localization of orthophosphate; there are more nucleoli in other planes which are not seen in the photograph. Cut cells show the same pattern of localization as in the intact living cell. Fixation in 5% Pb acetate, followed by treatment with 5% Pb acetate in glacial acetic acid, washing in distilled water and staining with H_2S; total mount. The photograph represents magnification 2430 ×.

DISCUSSION

The ability to precipitate orthophosphate inside the cell is a characteristic of the nucleolus itself, since nothing is precipitated at meta- and anaphase when this structure is absent. Since the phosphate precipitate is found specifically in the nucleoli it is difficult to avoid the conclusion—and it is the simplest explanation—that orthophosphate is present in the nucleolus. Of course, a negative result does not mean that inorganic phosphate is absent from the cytoplasm since the degree of dispersion can affect its visualization very markedly. This means that if the number of orthophosphate ions per unit volume (concentration) is greater in the nucleolus than in the cytoplasm, "cytochemically detectable amounts" will be found only in the nucleolus even though it might be only a small fraction of the total orthophosphate of the whole cell. A large number of investigations have established that the nucleus—and specially the nucleolus—plays an important role in the phosphorus metabolism of the cell; it is very likely that the presence of orthophosphate in appreciable amounts in the nucleolus (nuclear orthophosphate) is connected with: (a) high metabolic and specific activities of the nucleolar RNA[28–35], (b) the dependence of the nucleus on the net synthesis of cytoplasmic RNA[36–38], (c) the synthesis of other nucleotides besides RNA[39,40], (d) the specificity of localization of DNA in the nucleus and (e) the essentially anaerobic metabolism of the nucleus[28,41]. It might well be that the nucleolar orthophosphate is the precursor of RNA in the cell; the synthesis of RNA is made more efficient by the coexistence in the nucleoli of both orthophosphate and a very active RNA fraction (*cf.* ^{32}P uptake and RNA synthesis in *Amoeba* cell fragments[38,42,43]). The experiments presented here suggest that the final concentration of orthophosphate is greater in the nucleolus than in the cytoplasm. The question of how much of the total orthophosphate of the whole cell is present in the nucleoli—we may say within

the nucleus—must be tested experimentally in several organisms and under different conditions. In *Acetabularia* quantitative determinations showed that not only RNA is concentrated in the nucleated fragment (rhizoide) but also that the total phosphorus is about twice as high as that of the non-nucleated stem[43,44]. It would be interesting to investigate the distribution of orthophosphate in this large unicellular alga.

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