173. *Microspectrophotometry of Calcium in the Nucleus of Allium Cepa*^{*)}

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Microspectrophotometry has been used extensively for the cytochemical detection of the nucleic acid. Recently, some components of tissue-cells were investigated by means of the microspectrophotometry (Deitch 1966, Bahr 1966).

Generally, living cells possess several types of calcium: soluble form, ionized or ionizable forms, masked calcium and form that are insoluble (Pearse 1953, Lison 1960). Early workers used alizarin red sulfonate to detect cytochemically the ionized or ionizable calcium (Dawson 1926, Cameron 1930). More recently, the same dye has been employed for the cytochemical detection of calcium (Dahl 1952, McGee-Russell 1958).

It was previously shown with the use of alizarin red sulfonate and microspectrophotometry that calcium is localized in the motor cell of *Mimosa pudica* (Toriyama, Setty and Jaffe 1970, Toriyama and Jaffe, in press). The same principle and methods were applied to detect the localization of calcium in the nucleus of root tips of *Allium Cepa*, and to follow its behaviour during the course of nuclear division.

For the microscopic observation of localization of calcium in the nucleus, root tips were fixed with Lillie's neutral buffered formalin (commercial formalin, 100 ml; distilled water, 900 ml; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.4 g; Na_2HPO_4 , 6.5 g) at room temperature for 24 hours. They were then thoroughly washed in running water for several hours to remove any trace of the fixatives. Water was removed with a dehydration series of tertiary butanol (Johansen 1937), and the tissue was embedded in paraffin. Fifteen micra thick sections were cut and held to a slide with Meyers albumin adhesive. We have modified Dahl's method (Dahl 1952), for cytochemical detection of calcium in the root tip cells. A one per cent alizarin red sulfonate^{**)} (ARS) solution was made with distilled water, and its pH-value was brought

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^{**)} Alizarin S, K&K Laboratories, Inc., Plainview, New York, U. S. A.

exactly to 5.5 with a 2 per cent aqueous solution of NH_4OH . It is worth stressing that the effect of this staining reaction for calcium is delicately dependent upon the pH-value of the ARS solution (Toriyama and Jaffe, in press). The ARS solution thus prepared will be stable for 2 to 3 months, if kept in the refrigerator. After staining for 2 hours, the sections were washed in distilled water for 60 seconds, and then soaked in acetone for 60 seconds. They were then placed in a jar of acetone-xylol (1:1) for about 60 seconds, soaked in xylol for 60 seconds and imbedded in xylol-balsam.

The combining application of Lillie's neutral buffered formalin and ARS, gave excellent results in the fixation of the nucleus and cytoplasm as well as in the detection of calcium. In the preparations, which were made by the use of this technique, the red color reaction so-called "*lake*" (Lison 1960), due to the complex of calcium and ARS, was clearly observable.

On the other hand, we could make red colored complex (*lake*), *in vitro*, when 10 ml of 10 mM CaCl_2 was mixed with 1 ml of 0.2 per cent ARS, and its pH-value was exactly 5.5 with phosphate buffer. Those complexes were prepared on the glass slide with ringed and cover glass. The stained nucleus in a preparation as well as the complex of calcium-ARS, *in vitro*, were quantitatively analyzed with a OLYMPUS double-beam microspectrophotometer, model DMSP-II.

Using our methods, a typical *lake* of calcium-ARS was clearly observable. Examples of the results are shown in Fig. 1. There are obvious changes in the site of the color reaction in the nuclei of the root cells. Under these experimental conditions, the cell wall and cytoplasm could be scarcely stained with ARS. In the young cells and in the elongated parenchyma cells, the ARS reaction is found

Fig. 1 a, b, c. Cytochemical detection of calcium in parenchyma cells of the root tip of *Allium Cepa*. Arrows indicate the nucleus and double arrows indicate the metaphase of nuclear division. The above three sections originated from the material fixed with Lillie's neutral buffered formalin and stained with alizarin red S.

Fig. 2. Absorption spectra of the nucleus in the parenchyma cell of the root tip of *Allium Cepa*. A: stained with alizarin red S, B: non-stained, A-B: difference spectrum between A and B, Ca^{2+} +ARS, lake of calcium and alizarin red S, *in vitro*.

in the resting nucleus (indicated with arrows in Fig. 1a and b). In some areas of the tissue, the resting nucleus assumed a pink color (Fig. 1c), but in the cytoplasm and cell wall the red color did not appear. Furthermore a small number of the nuclei showed an orange color reaction. It is worth mentioning that the dividing nuclei were stained neither red nor pink, as indicated with double arrows in Fig. 1a.

The data obtained by the microspectrophotometry, showed the relative quantity of calcium in the nucleus. The results of absorption spectrum survey of the stained nucleus are shown in Fig. 2. This spectrum had its maxima at 290 nm*** and 510 nm (A). In the non-stained material (B), however, the absorption maximum of the nucleus was found to be 290 nm. Through the observation of these absorption spectra, we may compute the difference of value between

***) nm, nanometer=millimicron

Fig. 3. Scanning data through four nuclei of *Allium Cepa* root tip with 510 nm light beam of which diameter is 3μ . The above four cells originated from the material fixed with Lillie's neutral buffered formalin and stained with alizarin red S.

the spectrum of A and B (Fig. 2, A-B). The absorption maxima of the calcium-ARS, *in vitro*, was found to be 320 nm and 510 nm (Fig. 2). This is reinforced by the fact that both the absorption spectrum of a calcium-ARS as well as the difference spectrum, showed the absorption maxima at 320 nm and 510 nm. We believe that these phenomena clearly show the nucleus contains calcium in it.

Next, we could get the absorption curve through four cells by means of the scanning with 510 nm light beam of 3 micra in diameter (Fig. 3). Thus, we found by the integration of curve A, B, C and D that each nucleus contains the same amount of calcium (Fig. 3). Furthermore, we compared the amount of calcium in the resting nuclei by means of microspectrophotometry; some ones were red colored (Fig. 4a) and, some others were pink (Fig. 4b). We estimated that a small amount of calcium was localized in the dividing nucleus (Fig. 4c). From these data we can conclude that most of calcium left the nucleus of the dividing cells and moved to the proto-

Fig. 4. Scanning data of the nucleus and the nuclear division of *Allium Cepa* root tip with 510 nm light beam, of which diameter is 3μ . a: resting nucleus stained a reddish color, b: resting nucleus stained in pink color, c: metaphase of nuclear division. The above four nuclei originated from the material fixed with Lillie's neutral buffered formalin and stained with alizarin red S.

plast of the neighbor cells. We know this because the large amount of calcium in the resting nuclei in these neighbor cells can be observed (Fig. 1a).

A series of experiments on the effects of calcium-complexing reagents were carried out using procedures similar to those in the foregoing experiments. The root tips were fixed with Lillie's neutral buffered formalin for 24 hours. The preparation procedure was carried out as usual, except that after the tap water wash, the sections on the slide glass were soaked in aqueous solutions of Ca-complexing reagents, GEDTA and EDTA at 20°C , for 20 to 120 minutes. After this procedure, the sections were washed in distilled water thoroughly, and stained with 1 per cent ARS as in the foregoing experiments.

GEDTA, EDTA, acetic acid and nitric acid had a remarkable effect on the ARS color reaction in the nucleus. After this treatment, the nucleus in the root tip, did not react to ARS. It is probable that the calcium in the nucleus was removed by these Ca-complexing reagents or rendered unable to react with ARS. This means also that in the preparations stained with ARS, the red color reactions are due to the localization of calcium. The behaviour of the calcium during the whole process of nuclear division, and the nature of

nucleus, stained in orange color, are subjects for future research.

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