THE CYTOCHEMICAL LOCALIZATION OF ASCORBIC ACID IN ROOT TIP CELLS

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Plate 13

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Ascorbic acid is one of those compounds that excites interest, for while its role in metabolism is unclear, it is known to be necessary for the existence of the cell. In efforts to understand the function of ascorbic acid, its cytological localization has been attempted in both plant and animal cells. This has not been an easy task in plant tissues and the site of ascorbic acid has been variously reported to be the cell wall (Reid, 1941), nuclei and chromosomes (Chayen, 1953), or mitochondria (Chayen, 1953).

In the course of developing a freeze-dry apparatus using a stream of moving air (Jensen, 1954), the localization of ascorbic acid was used to test the effect of air at −30°C. on an easily oxidizable compound. As a result of these experiments it became clear that when carefully handled frozen-dried material was used, the ascorbic acid was located on submicroscopic particles, the majority of which were closely associated with the cell wall.

Materials and Methods

The root tips of *Vicia faba* var. broad Windsor and *Allium cepa* var. white globe were used. The bean seeds were soaked overnight and germinated in moist vermiculite. The tip of the primary root was used. Sets of the onion were placed in small bottles so that the base of each bulb was submerged in water. Adventitious roots 2 to 4 days old were used.

All tissue was frozen-dried. Isopentane cooled with liquid nitrogen was used for freezing. Drying was accomplished in a moving air type apparatus at −30—−40°C. Nitrogen (extra high purity, Linde Air Products Co.) was used in most runs in place of air. The dryness of the nitrogen permitted removal of the condenser in the system normally used to dry the incoming air. Dehydration normally required 12 to 18 hours.

When dry the tissue was infiltrated with and imbedded in 56°C. paraffin. The tissue was sectioned at 10 μ. This relatively thick section was used in order to minimize diffusion of the ascorbic acid from the sections and to decrease the compression of the section during cutting. The sections were mounted by pressing them onto a slide, coated lightly with Haupt's adhesive, with a rolling motion of the finger. They were left on a warming table at 40°C. overnight before use.

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The silver nitrate method was used for the localization of ascorbic acid. This test is based on the fact that while many compounds in the cell will reduce silver nitrate at a high pH, only a few will do so at a low pH. Ascorbic acid is the only compound normally found in plant cells capable of reducing ionic silver in acetic acid. A complete review and critique of the method is given by Chayen (1953), whose recommendations have been followed.

The tissue, still in paraffin, was placed in a 10 per cent silver nitrate solution in 3 per cent acetic acid. The sections were usually left in this solution overnight although shorter periods of times were used. The sections were then rapidly washed and dehydrated in 95 per cent and 100 per cent ethyl alcohol. The paraffin was removed with xylene, and coverslips were mounted with permount. This entire procedure was carried out in the dark using only a weak red safety light. The finished slides were also stored in the dark. Viewing the sections with green filtered light helped to define the silver grains.

When various procedures were used to identify the site of ascorbic acid, it was frequently found that the ascorbic acid had been oxidized to dehydroascorbic acid and had not been removed from the tissue. To achieve visualization it was necessary to reduce the dehydroascorbic acid to ascorbic acid by placing the sections in an atmosphere of H_2S. After approximately 15 minutes the H_2S was removed and purified nitrogen passed over the tissue for another 15 minutes according to the procedure of Bourne (1936). Localization of ascorbic acid was then demonstrated as above.

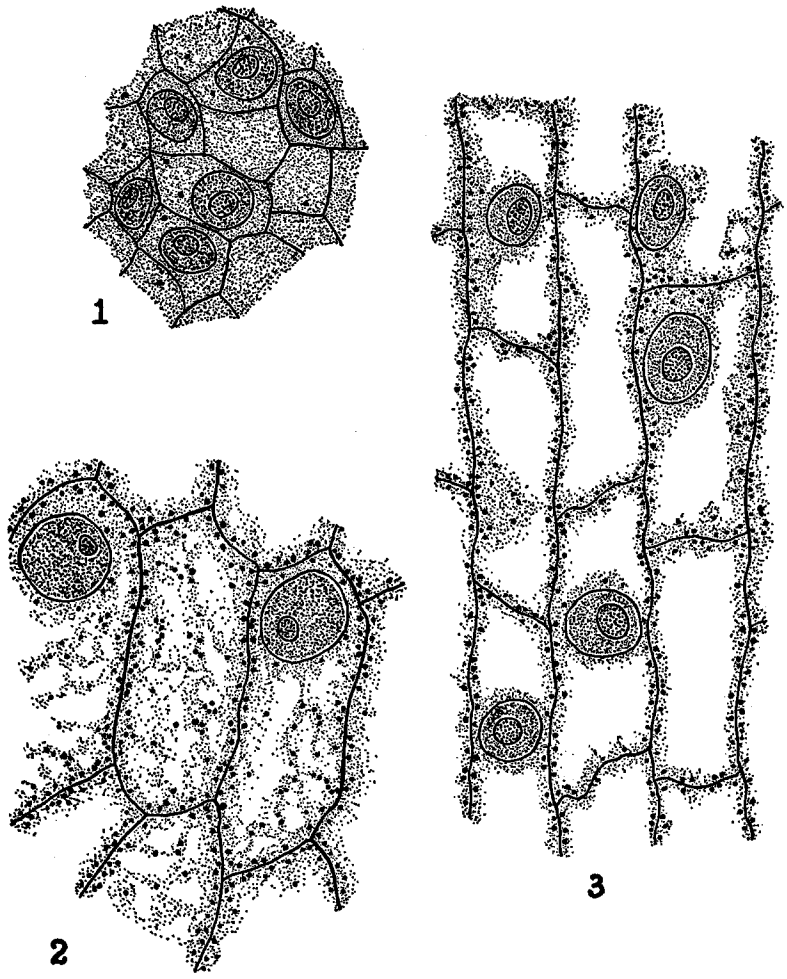
For controls in all procedures, slides were placed in 3 per cent acetic acid minus silver nitrate or given a short exposure to copper sulfate which acted as an oxidizing agent. In all cases morphological controls were run with the treated sections. The controls were handled in the same manner but were stained with azure B or Heidenhain's hematoxylin.

RESULTS

The results of the ascorbic acid localization are shown in Figs. 1 to 4 and Text-figs. 1 to 3. Fig. 1 shows the morphological control stained with Heidenhain's hematoxylin. The cells are well preserved, mitochondria and other cytoplasmic particles are present, nuclei are distinct and homogeneous although deliberately overstained, and the cytoplasm is clear and shows no evidence of shrinkage. Fig. 2 shows the localization control which had been in 3 per cent acetic acid overnight. It is clear that there is no reaction with the acetic acid alone. Similar results are obtained if the section is first placed in $CuSO_4$ for 10 to 15 minutes and then placed in the $AgNO_3$–acetic acid mixture.

The distribution of ascorbic acid is shown in Figs. 3 and 4. The silver has been deposited in very small spheres throughout the cytoplasm with a marked concentration in the region directly against the cell wall. The size of the spheres range from 0.6 μ downward to the limit of resolving power of the microscope (0.2 μ). In no case were the deposits rod-shaped or filamentous.

Histologically, the root cap and all cells that had begun elongation, showed high amounts of ascorbic acid. The cells in the general meristem and stages of development prior to elongation appeared to have very small amounts. In these cells, moreover, the sites were scattered throughout the cytoplasm rather than associated with the cell wall. This is shown in Text-figs. 1 to 3. The developing epidermal cells first showed ascorbic acid associated almost solely with the periclinal walls but later, during elongation, also with the radial walls.



TEXT-FIG. 1. Ascorbic acid localization—*V. faba.* Cross section of meristem cells indicating sparcity of ascorbic acid and lack of association with cell surface. × 1600.

TEXT-FIG. 2. Ascorbic acid localization—*V. faba.* Root cap cells, longitudinal section indicating large amounts of ascorbic acid distributed particularly throughout the cytoplasm and associated with the cell wall. Size of silver deposits slightly larger than in the actual preparation. × 1600.

TEXT-FIG. 3. Ascorbic acid localization—*V. faba.* Longitudinal section of elongating cortical parenchyma showing the ascorbic acid primarily associated with the cell surface. × 1600.

To characterize more clearly the nature of the site of ascorbic acid the sections were pretreated with distilled water, ribonuclease (RNAase 1 mg./1 ml.), or 80 per cent alcohol for varying periods of time. During these treatments the paraffin was present in the tissue and all sections were subsequently treated

with H_2S. The results are shown in Table I. These results indicate that the ascorbic acid is probably associated with sites rich in ribose nucleic acid content and possibly poor in lipide content. The increase in ascorbic acid in the alcohol-treated material may be the result of the precipitation of an associated protein thus decreasing the diffusion of ascorbic acid in water.

TABLE I

The Effect of Various Treatments on the Amount of Ascorbic Acid Present and the Morphological Appearance of the Cells of the Root Tips of Onion and Bean

Pretreatment	Ascorbic acid present	Morphological appearance
None	++++	
H_2O		
½ hr.	+++	Nuclei well preserved, mitochondria and other cytoplasmic
1 hr.	+++	particles present, cytoplasm clear
2 hrs.	++	
Ribonuclease		
½ hr.	++	Nuclei gone, general cytoplasmic staining much decreased,
1 hr.	+	mitochondria present, some decrease in apparent number
2 hrs.	?	of particles, cytoplasm clear
Alcohol 80 per cent		
½ hr.	++++	Nuclei granular; mitochondria still evident after ½ hr., then
1 hr.	++++	becoming indistinct. Clumping of cytoplasm makes identi-
2 hrs.	++++	fication of particles difficult

DISCUSSION

The distribution, size, and shape of the silver deposits eliminate the nuclei, chromosomes, and plastids as sites of ascorbic acid in the root tip. The distribution, particularly in the early stages of cell development, also eliminates the cell surface as the only site. The fact that ascorbic acid can be removed by RNAase treatment further suggests that it is not in a simple relation with the cell wall. This same observation also eliminates the possibility that mitochondria are the main site.

On the basis of the evidence presented here it would seem possible to conclude that ascorbic acid is closely associated with a submicroscopic structure and definitely, but not solely, associated with the cell surface. Since the whole question of submicroscopic particles and structures in the cytoplasm of the plant cell is at present so poorly understood, a more definite conclusion is not possible.

The results presented here confirm the cellular distribution of ascorbic acid predicted by Reid (1941) from indirect evidence. She concluded that ascorbic

acid was associated with the cell surface of the elongating cells and that there was little ascorbic acid in the meristematic regions. Chayen (1953) using cytochemical techniques concluded that ascorbic acid was associated with the mitochondria and not found in the nuclei, chromosomes, cytoplasm, or cell wall. The present results only partially agree with this conclusion. The localization described by Chayen is very similar to the distribution found in the present case in the stages of cell development prior to elongation.

The root tip of *Vicia faba* has been analyzed for a number of compounds and enzymes by Jensen (1955). The oxygen uptake on a per cell basis very closely parallels the distribution of ascorbic acid. Both are very high in the root cap and rise proportionally with cell elongation. There is, however, no basis at present for believing there is any direct connection between the two.

Since ascorbic acid is the substrate for the enzyme ascorbic acid oxidase, it is of interest to compare the intracellular localization of the two entities. By using differential centrifugation techniques Newcomb (1951) and Honda (1955) conclude that the enzyme is associated with the cell wall. The treatment given the cells during homogenation is so gentle that it is quite possible that a certain amount of peripheral cytoplasm was still attached to the cell wall fragments. This would then make the distribution of the ascorbic acid and the enzyme identical with the exception that some of the ascorbic acid is found definitely in the cytoplasm.

SUMMARY

The intracellular distribution of ascorbic acid was studied in frozen-dried root tips of *Allium cepa* and *Vicia faba* by the silver nitrate procedure. The sites of the ascorbic acid as indicated by the deposited silver appear as spherical (0.2 to 0.6 μ in diameter) cytoplasmic particles. The site appears to have small amounts of lipides and to be rich in ribonucleic acid. These particles are concluded to be submicroscopic in size and associated, in the elongating cell, with the cell surface. In the meristematic cells they appear fewer in number and are distributed throughout the cytoplasm.

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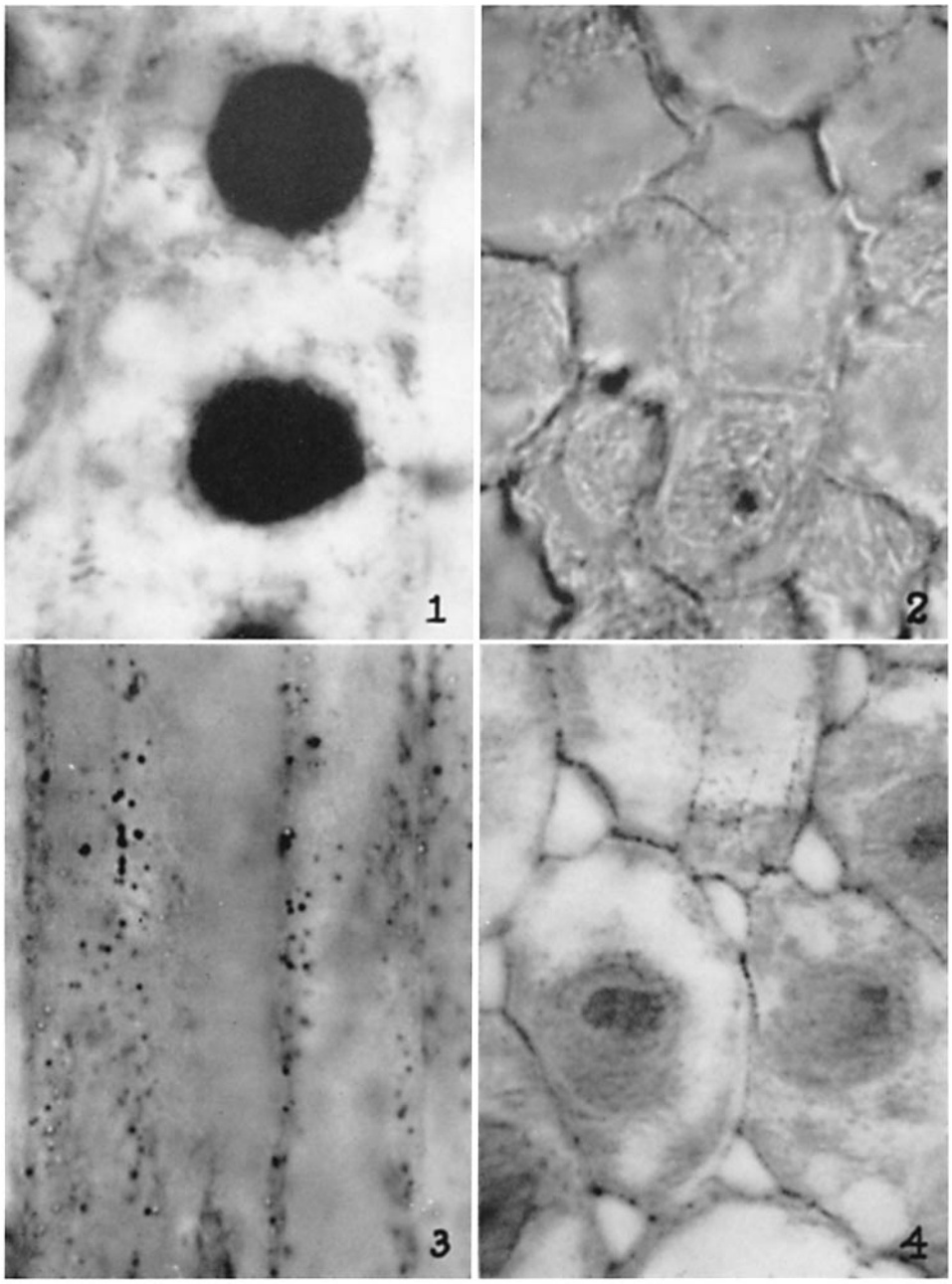
EXPLANATION OF PLATE 13

FIG. 1. Morphological control—*Vicia faba*. Stained with Heidenhain's hematoxylin and orange G. The nuclei are overstained to bring out the cytoplasmic detail. × 2000.

FIG. 2. Localization control—*V. faba*. Tissue treated as in localization procedure but not incubated in silver nitrate solution. The same result was obtained when the *tissue* was first placed in $CuSO_4$ and then in the silver nitrate solution. × 2000.

FIG. 3. Ascorbic acid localization—*V. faba*. Longitudinal view of elongating parenchyma cells showing distribution of deposited silver in small spheres through the cytoplasm and against the cell wall. × 2000.

FIG. 4. Ascorbic acid localization—*V. faba*. Cross section of elongating parenchyma cells. The ascorbic acid is clearly associated with the cell surface but also present in the cytoplasm. The nuclei are somewhat darkened by reduction of absorbed silver nitrate in the light. On immediate observation following localization the nuclei are completely clear. × 1250.



(Jensen and Kavaljian: Ascorbic acid in root tip cells)