THE LABELING OF CULTURED CELLS OF *ACER* WITH [^{14}C]PROLINE AND ITS SIGNIFICANCE

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ABSTRACT

The distribution of the radioactivity from [^{14}C]proline that is bound in cultured cells of *Acer* has been determined by electron microscope autoradiography. In this way proline may be related to the cell wall as a morphological entity rather than as a fraction in a biochemical separation of a heterogeneous crop of cells. The cells in culture may vary greatly. Some are active growing, turgid cells, with thin protoplasts tightly pressed against their walls; in others the protoplasts may spontaneously withdraw from the wall; in still others the protoplasts disorganize, and walls thicken and become sculptured as the cells differentiate and even senesce. Different culturing practices may affect the status of the cells, and this, in turn, affects the distribution of radioactivity from proline in the cells. Cells which are actively growing, turgid, and nucleated have the highest grain density in their protoplasts and nuclei; as the protoplasts of such cells withdraw from their walls, they retain the bulk of the radioactivity. On the other hand, in cells which have thickened walls and sparse protoplast contents, the radioactivity is accumulated in their walls. A high content of proline and hydroxyproline-rich protein is, therefore, not a necessary or invariable feature of the cell walls of cultured *Acer* cells but depends on the state of development of these cells.

INTRODUCTION

The distribution of bound [^{3}H]proline (confirmed by use of [^{14}C]proline) in the cells of rapidly growing, aseptically cultured carrot (*Daucus carota*) explants was comprehensively investigated by Israel et al. (1968) using the combined techniques of electron microscopy and high-resolution autoradiography. This was done because proline forms a hydroxyproline-rich non-metabolized moiety in these cells (Pollard and Steward, 1959; Steward and Thompson, 1954; Steward et al., 1958), and it was necessary to supplement the biochemical procedures to determine its distribution within the cells. The study in question showed clearly that the radioactive label, representing proline and the hydroxyproline derived from it, was not in the walls of the living cells but in their protoplasts. Whereas at first the label was concentrated in nuclei, even in nucleoli, it subsequently spread through the ground cytoplasm and was also concentrated in plastids. Although cultured carrot cells did not accumulate radioactivity from proline in their cell walls, they did so freely from other substrates (Steward and Israel, 1972).

Vesicles of *Valonia ventricosa* also provided a clear case (Steward et al., 1970) in which the cell wall was easily and sharply separated from the

living cytoplasmic content of the vesicle. By these means, it was shown that the cellulose wall was not a site of conspicuous accumulation of bound [^{14}C]proline or hydroxyproline, or even of protein in general; whereas the peripheral cytoplasm of the vesicle did incorporate ^{14}C from proline.

Other workers have studied the hydroxyproline-containing complexes in suspension cultures of cells of *Acer pseudoplatanus*. From work on the crops of these cells, Lamport and Northcote (1960) concluded that the bound hydroxyproline exists predominantly, if not wholly, in the cell walls in the form of a special "cell wall protein." The name "extensin" was given by Lamport (1963) to this "wall protein" although the substances which contained the hydroxyproline when isolated were arabinose-containing glycopeptides (Lamport, 1967; 1969). However, in these studies the term "cell wall" was used in an operational sense, for it related to a fraction in a biochemical separation of the crop of cells harvested in bulk. By contrast, in the work on carrot cultures and on *Valonia*, the term "cell wall" referred to a cellulose wall as a morphological entity in the cells or vesicles.

Much plant physiological work which deals with the presence, location, and significance of combined hydroxyproline in plant cells has developed from the events recorded above. For this reason, some time ago, the cells of *Acer* were examined by the methods used in the work on cultures from carrot (Israel et al., 1968). The distribution of ^{14}C from proline in cells that were demonstrably viable was consistent with the results obtained on carrot, and their publication seemed then unnecessary. However, Roberts and Northcote (1972) have now published work on the autoradiographic localization of incorporated proline in cultures of *Acer* which they state "refutes the findings of Israel et al. (1968)." This being so, it is appropriate now to publish our data on *Acer* in order to set in perspective the problem of combined proline in cultured cells.

CULTURING THE *Acer* CELLS

In 1968 a stock *Acer* culture on agar medium was obtained from Dr. D. T. A. Lamport[1] together with a sample of the nutrients then being commercially formulated for its cultivation. This culture was first grown in this prepared liquid medium and then lightly inoculated (as is our custom with carrot cultures) for growth on horizontally shaken cultures, following the practice of Lamport. The first growth obtained in this way was very sparse, and it became evident that to grow dense cultures in liquid required a relatively heavy inoculum, as indeed was then being practiced in Lamport's laboratory. Even so, the crop of cells so grown, when examined under both light and electron microscopes, contained many empty, obviously senescent cells and very few cells with abundant streaming cytoplasm, active nuclei, and visible evidence of division.

At this point, parallel cultures were prepared using the commercially prepared medium, referred to above, and another basal medium (B_W), based on that of White, both being supplemented with 10% coconut milk (CM) and 2,4-dichlorophenoxacetic acid (2,4-D) at 1 ppm (Steward and Caplin, 1951). (For the analysis of the medium B_W with and without CM, reference may be made to Mott and Steward, 1972.) These media in the standard, slowly rotated "nipple" flasks (Fig. 1) in use in this laboratory (Steward and Shantz, 1956) were lightly inoculated from a suspension culture of the *Acer* strain obtained from Dr. Lamport. Fig. 1 (right) shows that after an appropriate period, the culture grown in the freshly prepared medium (B_W + CM + 2,4-D) produced a heavy crop of cells, and the cells so produced (Figs. 4 and 5) were more frequently living with conspicuous cell contents (nuclei, plastids, cytoplasmic strands, etc.). By contrast (cf. Figs. 2 and 3), the smaller cells in the parallel culture on the alternative medium were predominantly devoid of organized protoplasts and had walls which were locally thickened or sculptured. Therefore, the crops of cells used for the autoradiographic study were obtained from cultures pregrown in flasks as shown in Fig. 1 (right) and in the medium (B_W + CM 10% + 2,4-D 1 ppm).

Acer CELLS LABELED WITH RADIOACTIVE PROLINE

From the stock culture grown as described, portions (10 ml) were removed to tubes, and uniformly labeled [^{14}C]proline was added to give an activity in the medium of 5 μCi/ml. Even though ^{14}C gives somewhat lower autoradiographic resolution (Salpeter and Salpeter, 1971), [^{14}C]proline was here used because the products so labeled with ^{14}C may be less labile than those labeled with ^{3}H. The culture tubes were slowly rotated as in our

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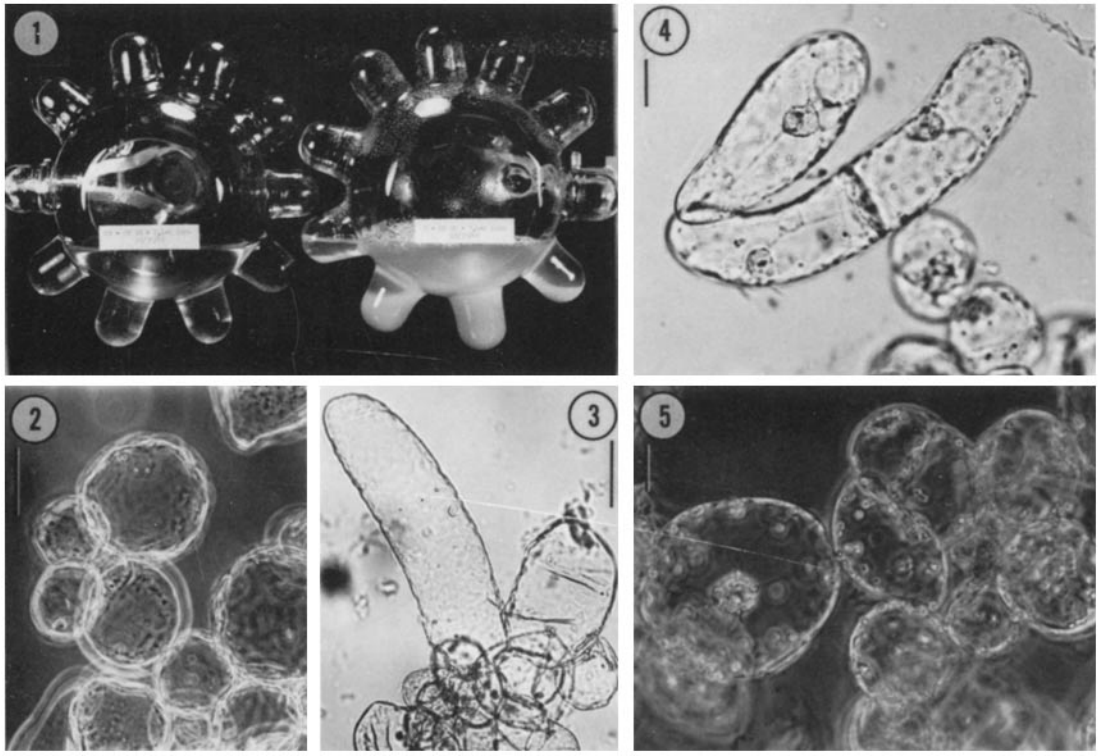


FIGURE 1 Crops of *Acer* cells grown during 3 weeks at constant diffuse light and 21°C in lightly inoculated liquid media (250 ml) in slowly rotated flasks. × 0.2. Left: prepared Lamport basal medium with CM + 2,4-D. Right: freshly prepared basal medium after White (B_w) + CM + 2,4-D.

FIGURES 2 and 3 Cells with markedly sculptured walls as grown in flask at Fig. 1 (left) and as seen under phase (Fig. 2) and in bright field (Fig. 3). Calibrations 20 μm. × 450.

FIGURES 4 and 5 Cells, larger than those in Figs. 2 and 3, with distinct protoplasts, nuclei, cytoplasmic strands, and organelles as grown in flask in Fig. 1 (right) and as seen under bright field (Fig. 4) and under phase (Fig. 5). Calibrations 20 μm. × 260.

normal practice (Caplin and Steward, 1949; Steward et al., 1952).

Crops of cells were sampled after 1 or 6 days of continuous contact with the labeled proline or after 6 days in labeled proline followed by an additional 10 days in unlabeled medium. (All workers agree that *Acer* cells treated in this way, i.e. growing in suspension culture, are consistently rich in hydroxyproline, as reviewed by Albersheim et al., 1973.) The samples were then centrifuged at low speed and washed with fresh aseptic unlabeled medium. The washed centrifuged preparation was fixed in glutaraldeyhde-osmium tetroxide, rinsed, and resuspended in bovine serum albumin which was then coagulated with glutaraldehyde. The resultant block was subdivided, dehydrated, embedded, and prepared for electron microscopy in the usual way. From each sample a total of 24 ribbons was cut from several blocks, and the autoradiographic technique was carried out, using the Ilford L4 emulsion (Ilford Ltd., Essex, England) and the methods previously described (Israel et al., 1968). The fields examined (each about 200 μm^2) were chance selections under the microscope, and those actually recorded in Table I were representative of the very much larger total area observed on the sample obtained at each of the three time periods.

DISTRIBUTION OF ^{14}C FROM [^{14}C]PROLINE IN CULTURED CELLS OF *Acer*

Cells in the crop harvested were examined in three states. The first consisted of cells which when fixed

TABLE I

*Distribution of Radioactivity from [^{14}C]Proline in Cells of Acer Cultured in a Liquid Medium**

	Status of cells					
	Living‡		Flaccid§		Senescing‖	
No. of fields¶	65		27		13	
Total grains	1,210		425		295	
Cellular region	% Total grains	Grains/100 μm^2	% Total grains	Grains/100 μm^2	% Total grains	Grains/100 μm^2
Interstitial space**	13.3	3.6§§	4.9	2.4§§	13.6	4.3§§
Cell wall	15.1	13.2	4.2	3.9§§	74.2	27.3
Vacuole	15.1	3.1§§	6.4	4.1§§	—	—
Lumen‡‡	—	—	21.7	3.1§§	12.2	3.1
Protoplast	56.5	25.9	62.8	28.8	—	—

* Data are pooled from sampling periods since the frequencies of occurrence did not differ significantly.

‡ Cells which when fixed had normal protoplasm in close contact with the cell wall.

§ Flaccid cells with protoplasm withdrawn from the cell wall.

‖ Representative senescing cells with intact cell walls but no organized protoplasts.

¶ Fields represent areas of 200 μm^2 selected by chance.

** Extracellular space in close proximity to, but not part of, the cell wall.

‡‡ Unorganized free space enclosed by cell wall.

§§ Values not significantly different by χ^2 (P between 0.9 and 0.5).

were turgid living cells with organized protoplasts clearly pressed against the cell wall (Figs. 6–8). Secondly, there were cells which when fixed were flaccid because the protoplasm had *spontaneously* withdrawn from the wall, leaving a space (lumen) between protoplast and cell wall (Figs. 9 and 10). Finally, there were cells that lacked any protoplasmic organization whatsoever; portions of these senescent cells with adjacent interstitial material are shown in Figs. 11 and 12. These cells often showed the sculptured walls that Roberts and Northcote (1972) refer to as "wall warts." As the experiment progressed, the frequency of the flaccid and senescent cells increased.

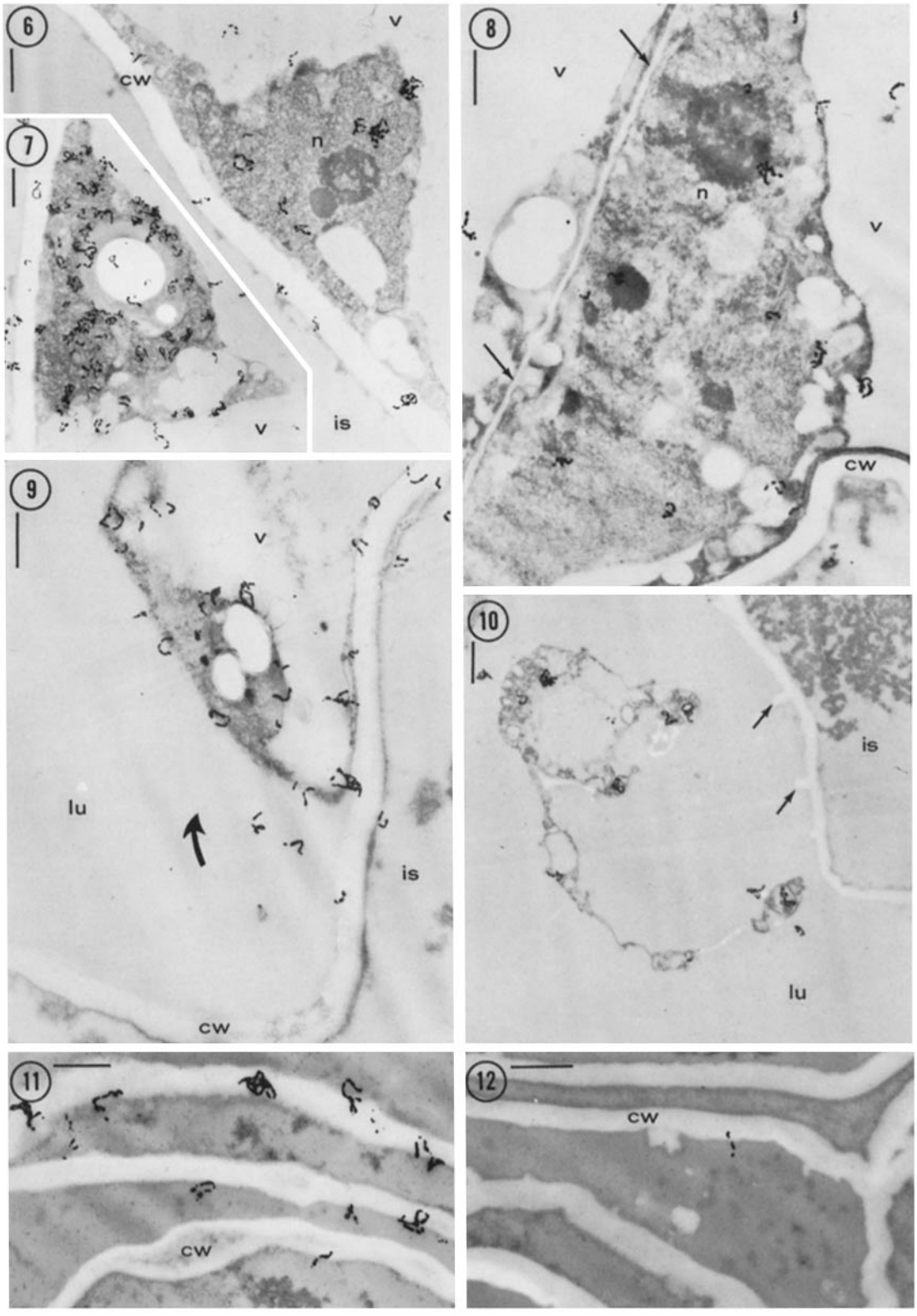
The distribution of radioactivity in cells in each of the states identified as turgid, flaccid, and senescent was determined for each of the time periods described. Grains were counted and entered into a table to show the distribution of radioactivity in different cellular regions for each field examined. The data were presented as percent of total grains and as grains per unit area of

FIGURES 6–12 Autoradiographs of *Acer* cells cultured in the medium as in Fig. 1 (right) and after 6 days of contact with [^{14}C]proline. (*cw*, cell wall; *is*, interstitial space; *n*, nucleus; *v*, vacuole; *lu*, lumen; all calibrations 1 μm).

FIGURES 6–8 Representative turgid, living cells (cf. Figs. 4 and 5) with radioactivity predominantly in their protoplasts (→ designates newly formed wall as in Fig. 8). Fig. 6, × 7,000; Fig. 7, × 6,300; Fig. 8, × 8,700.

FIGURES 9 and 10 Flaccid cells with protoplasts spontaneously withdrawn (curved arrow) from their cell walls and with the label associated with the protoplasts. Fig. 10 shows what may be the start of (→) sculptured cell wall (cf. Fig. 2). Fig. 9, × 8,700; Fig. 10, × 6,700.

FIGURES 11 and 12 The labeling of sculptured cell walls and interstitial spaces of representative senescent cells; label may occur in some walls (Fig. 11) or be absent from others (Fig. 12). Fig. 11, × 9,100; Fig. 12, × 10,000.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tissue region, i.e., grain density. Because the grain distributions were essentially the same for the three incubation times, the data were pooled as shown in Table I. The grains were referred either to whole cells, to interstitial space, cell wall, protoplast, and vacuole, or to the lumen when there was unorganized free space enclosed by the cell walls. Grains were assigned to a given region from the location of the grain centers. The area occupied by each identified region was obtained by superimposing a grid (calibrated as to intersections per square micrometer) over the micrographs and by counting all the intersections over each cell region.

DISCUSSION

At the outset it should be recognized that the crux of the problem is a botanical one. Thus, precise morphological information on the status of the cultures and of their cells is crucial to an interpretation of the distribution of compounds between cell walls or protoplasts. This is the point of this paper.

Many of the techniques that produce suspension cultures in bulk use heavily inoculated cultures which produce dense crops with relatively few actively growing cells. Street and Henshaw (1963) have stressed that both "conditioned medium" and many cells in "relatively massive" inocula are often transferred to make the cultures succeed. Since only a small proportion of the free cells divide, there is then a gradual accumulation of dead cells which, they indicate, "has the effect that with each succeeding culture passage the aliquot used for subculture contains an increasing proportion of dead free cells." It should be noted that the Roberts and Northcote (1970) experimental cultures represent dilutions (1–6) of a pregrown, dense suspension in which it is clear that only a small fraction (order of 6%) of the cells remained able to divide. Thus, a crop so derived and treated after 48 h of contact with [^{3}H]proline must be composed predominantly of mature or senescent cells, many lacking organized protoplasts and comparable to those shown in Figs. 2 and 3. This is the type of cell illustrated by Roberts and Northcote (1972, Figs. 2 and 3) to be heavily labeled from [^{3}H]proline.

However, in an *Acer* culture in which most of the cells are dividing and with their intact organized protoplasts clearly in contact with the cell wall, the greatest concentration of radioactive sites is in protoplasts, even in the cell nucleus.

Due to the geometry of the specimen and the degree of autoradiographic resolution with ^{14}C a considerable fraction of the grains from ^{14}C which overlap the cell wall of turgid living cells will be due to radiation from the relatively more heavily labeled, outermost layer of protoplasm (Israel et al., 1968; Salpeter et al., 1969). Thus no firm conclusion can be drawn regarding the extent of radioactivity truly in the walls of these turgid cells. The conclusion that the labeling is essentially in the protoplast is obtained, however, from those cells (termed flaccid) in which the protoplast, with its radioactivity, spontaneously withdrew from the wall, and the grain density over the cell wall is thereby considerably reduced.

Salpeter and Salpeter (1971) have shown that the grain density distribution around a ^{14}C source is characterized by "a long tail." This produces "a general tissue background" which is significantly above "off-section background." The grain densities over biologically inactive compartments such as the interstitial spaces, the vacuoles, and the unorganized free spaces enclosed by cell walls represent this "general tissue background." When the grain density over the cell walls of flaccid cells was compared with that over these unlabeled compartments, it was found not to be significantly different (χ^2 tests gave P values between 0.9 and 0.1). Thus the cell walls of flaccid cells are not specifically labeled, and the grains over them are due to radiation spread from the nearby labeled protoplasts.

Only when the cell walls have become sculptured and somewhat thickened, as in mature and senescing cells, is their accumulation of ^{14}C increased. Then, the grain density over the walls of these senescent cells (Table I) is significantly different ($P < 0.01$) from that over the walls of the flaccid cells. Interestingly, the grain density over the walls in the senescent cells is of the same high order as that over the protoplasts of the turgid and flaccid cells. The cells which accumulated ^{14}C from proline in their walls had obviously embarked upon a trend of development similar to that of xylem elements *in situ* when (as in vessels, tracheids, or fibers) the wall is thickened and its composition is changed.

Essentially, these conclusions are in accord with the recent findings of Sadava et al. (1973) who reported "that there is an increased synthesis and accumulation of cell wall hydroxyproline coincident with the cessation of elongation growth in pea epicotyls," and that "during elongation

and its cessation in pea epicotyls, differentiation occurs."

Thus, this study helps to resolve the apparent contradiction in the literature concerning the localization of incorporated proline into cell walls by showing that the degree of incorporation is relative to the state of development of the cells in question. The unthickened, unlignified, unsuberized, predominantly cellulose cell wall of unspecialized living parenchymatous plant cells is *not* a primary site of accumulation of proline. The latter concentrates, however, in walls of cells which have already embarked upon a course of differentiation and wall thickening (or senescence) in which the disorganization of the living protoplast is a first or eventual step.

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