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Host-Pathogen Interactions[1]

XXIV. FRAGMENTS ISOLATED FROM SUSPENSION-CULTURED SYCAMORE CELL WALLS INHIBIT THE ABILITY OF THE CELLS TO INCORPORATE [^{14}C]LEUCINE INTO PROTEINS

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NOBORU YAMAZAKI[2], STEPHEN C. FRY, ALAN G. DARVILL, AND PETER ALBERSHEIM[3]
Departments of Chemistry and of Molecular, Cellular, and Developmental Biology (A. G. D., P. A.), University of Colorado, Boulder, Colorado 80309

ABSTRACT

A bioassay to measure the incorporation of [^{14}C]leucine into acid-precipitable polymers of suspension-cultured sycamore (*Acer pseudoplatanus* L.) cells is described. Using this assay, cell wall fragments solubilized from sycamore cell walls by partial acid hydrolysis are shown to contain components that inhibit the incorporation of [^{14}C]leucine into the acid-precipitable polymers. This inhibition was not attributable to a suppression of [^{14}C]leucine uptake. The effectiveness of the wall fragments in inhibiting [^{14}C]leucine incorporation was substantially relieved by plasmolysis of the cells. Fragments released from starch and citrus pectin are shown not to possess such inhibitory activities.

The hypersensitive resistance response is a widely observed mechanism by which plants defend themselves against potential microbial pathogens. This response involves the apparent death of one or more cells of the plant at the site of microbial attack. The ability to kill plant cells has been attributed to a variety of molecules produced by microbes. For example, it has been suggested that cell wall preparations from *Phytophthora infestans* can cause the hypersensitive response in potato tubers (8, 13). Further, it has been suggested that a lipid fraction from *P. infestans* can elicit the hypersensitive response (14). Lipopolysaccharides from the bacterial pathogen *Pseudomonas solanacearum* have been invoked as the inducers of the hypersensitive response in tobacco (24, 27). Glycoproteins isolated from cell walls of the fungal pathogen *Cladosporium fulvum* can cause host cell necrosis in tomatoes (9, 16). Glucans from the walls of *P. infestans* have been reported to agglutinate and to result in the death of protoplasts obtained from potato leaf tissue (18). The physiological significance of these observations remains to be ascertained.

The possibility that hypersensitive death of plant cells is induced by fragments of the plant's own cell walls, that is, that plant cell wall fragments can induce cell necrosis, has not been investigated. However, there is indirect evidence that plant cell wall fragments could be capable of inducing the hypersensitive response. It has been known for many years that purified pectic-degrading enzymes, which cause tissue maceration, can kill plant cells (6, 12, 23). The mechanism by which pectic-degrading enzymes initiate cell death has not been determined, although one possibility is that these enzymes release fragments of the pectic polysaccharides of the host plant's own cell walls and that these cause necrosis of the plant cells.

In a widely observed phenomenon that is related to the ability of pectic-degrading enzymes to induce necrosis of plant cells, plant tissues must be plasmolyzed before treatment with pectic-degrading enzymes to isolate viable cells (3, 4, 5, 20). These results have been interpreted as suggesting that plasmolysis prevents osmotic bursting of the cells (3–6) and that pectic-degrading enzymes release something from the plant cells that causes necrosis of the plant cells (20). In fact, plasmolysis could interfere with the interaction of cell wall fragments with the protoplasts and thereby prevent necrosis.

More recent experiments have shown that fragments of plant cell walls can function as regulatory molecules (1, 2). Pectic fragments of plant cell walls elicit the production of phytoalexins (11). A pectic enzyme secreted by a fungus (18) and another such enzyme secreted by a bacterium (K Davis, G Lyon, AG Darvill, P Albersheim, unpublished) can themselves initiate phytoalexin accumulation by releasing oligosaccharides from covalent linkage in plant cell wall polysaccharides. Pectic fragments of plant cell walls are also known to be able to induce plants to accumulate inhibitors of the proteinases of insects and bacteria (19).

These observations led us to attempt to determine whether fragments isolated from plant cell walls can induce necrosis of plant cells. In our initial investigations, we used microscopic observation of plant cells stained with fluorescein diacetate (26) or Evans blue (10) to determine whether cells remain viable in the presence of wall fragments. We observed that the wall fragments did induce necrosis, many of the cells appearing broken and collapsed, but, because of this cell breakage, we were unable to quantitate accurately the killing effects of the wall fragments. Therefore, we decided to limit our later studies to the more easily analyzed question of whether fragments isolated from plant cell walls can inhibit incorporation of radiolabeled amino acids into acid-precipitable polymers. To accomplish this, we used a radiolabeled leucine incorporation assay (7, 27) that was developed to measure quantitatively the 'vitality' of cultured cells. The results of these experiments are reported in this paper.

MATERIALS AND METHODS

Cell Suspension Culture. Sycamore cells were derived from a strain originally isolated by Lamport (15) from cambial tissue of the angiosperm *Acer pseudoplatanus* L.; the cells have been maintained in this laboratory since 1960. These cells were grown in suspension culture in the dark in M6 medium (21, 22) at 24°C on a gyratory shaker (64 rpm) and subcultured weekly. For all experiments, cells were harvested 6 or 7 d after transfer, when they

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[2] Permanent address: Industrial Products Research Institute, 1-1-4, Yatabe-machi Higashi, Tsukuba-gun, Ibaraki 305, Japan.

[3] To whom correspondence should be addressed.

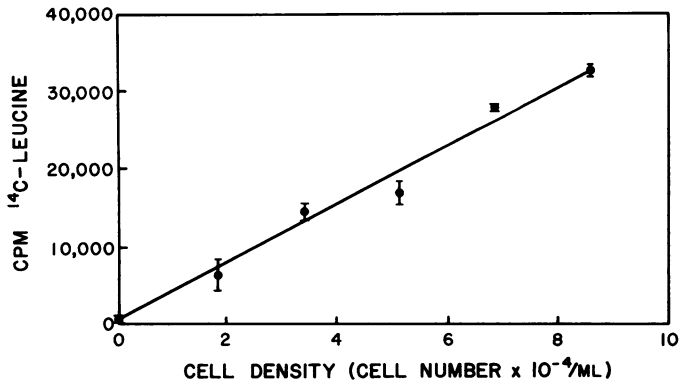

FIG. 1. Effect of cell number on the incorporation of [^{14}C]leucine into the acid-precipitable polymers of sycamore cells in a 2-h incubation.

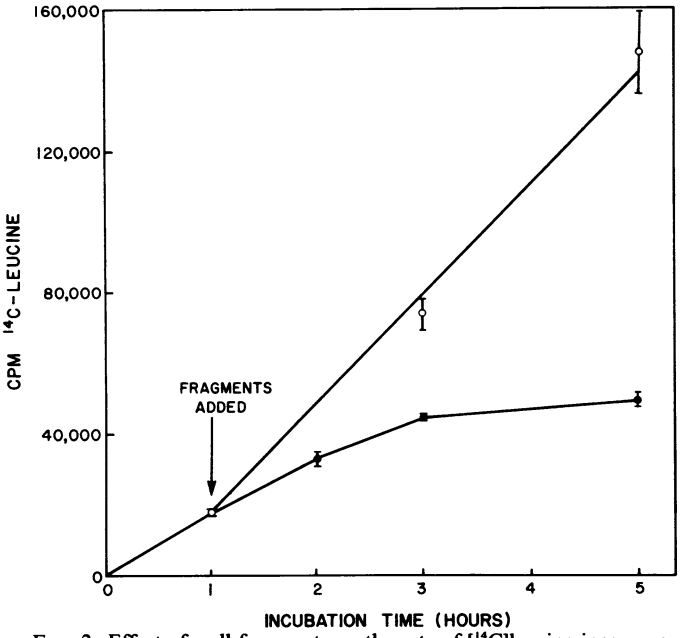

FIG. 2. Effect of wall fragments on the rate of [^{14}C]leucine incorporation into the acid-precipitable polymers of sycamore cells. The cells and [^{14}C]leucine were mixed at zero time. Wall fragments or an equivalent volume of incubation medium were added after 1 h. The reactions were stopped at the times indicated. Each incubation mixture contained 5×10^4 cells in 0.5 ml. (○), Control values (no wall fragments). (●), Values obtained in the presence of 300 μg of wall fragments.

were in the logarithmic phase of growth. The cells were filtered through a cotton mesh (with openings of less than 0.5 mm) to remove large cell aggregates. The cells that passed through the cotton were collected by centrifugation at 100*g* for 2 min, washed once with yeast-extract-free M6 medium, centrifuged at 100*g* for 2 min, and resuspended in yeast-extract-free M6 medium. The final suspension was adjusted to have a packed cell volume equal to approximately 1% of the total volume. The cell density of a 1% suspension is approximately 4×10^4 cells ml^{-1}, as determined by counting in a Neubauer hemocytometer with a 0.2-mm depression. The cells were examined microscopically with the aid of Evans blue staining (10); more than 95% of the cells in the suspension were found to be viable.

Isolation of Cell Walls. Cell walls were prepared from suspension-cultured sycamore cells by the method of Talmadge *et al.* (21).

Preparation of Wall Fragments. Sycamore cell walls treated with α-amylase (Sigma, 1 g) were suspended in 100 ml of 2 N TFA in a Kimax tube, capped with a Teflon-lined screw top, and heated

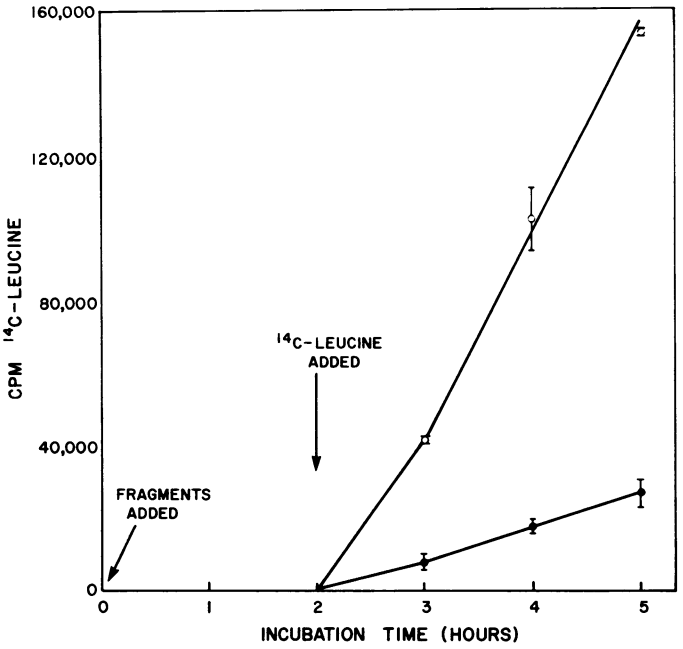

FIG. 3. Effect of preincubation of sycamore cells with wall fragments on the rate of incorporation of [^{14}C]leucine into the acid-precipitable polymers of the cells. Cells and wall fragments or an equivalent volume of culture medium (as a control) were mixed at zero time. [^{14}C]Leucine was added after 2 h and the incubation stopped after a total of 3, 4, or 5 h. The incubation mixture contained 4.3×10^4 cells in a total of 0.5 ml. (○), Control samples. (●), Values obtained in the presence of 300 μg of wall fragments.

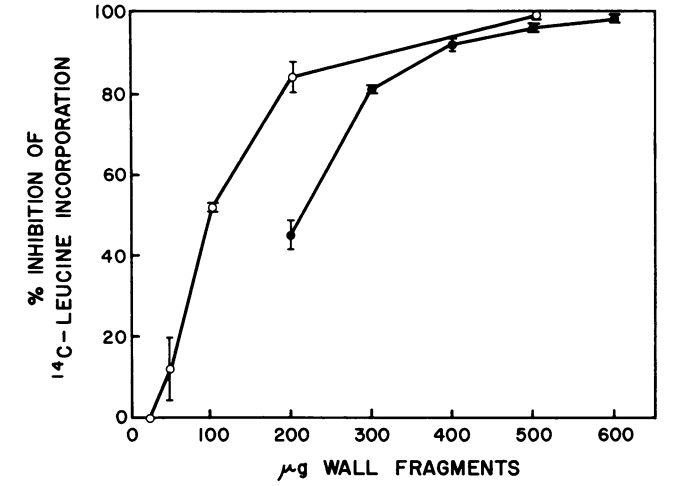

FIG. 4. Effect of the number of sycamore cells on the ability of wall fragments to inhibit [^{14}C]leucine incorporation into the acid-precipitable polymers of the cells. Cells and wall fragments or culture media were mixed at zero time. [^{14}C]Leucine was added after 2 h. The incubation was terminated after a total of 5 h. (○), Values obtained with 2.3×10^4 cells. (●), Values obtained with 5×10^4 cells.

at 85°C for 2 h. The tube was cooled to room temperature, and the insoluble residue removed by filtration through two layers of Whatman GF/A glass-fiber paper. The solubilized material, which contained acid-solubilized wall fragments, was dried under reduced pressure in a rotary evaporator. The dried residue was resuspended in a mixture of methanol:water (1:1 [v/v]), and the solvent evaporated. This last step was repeated twice more. The residue was then resuspended in methanol and evaporated to dryness, and this process was repeated once. The residue was then

Table I. *Comparison of the Effect of Wall Fragments on [^{14}C]Leucine Uptake and Incorporation*

Fragments Used	Uptake Rate[a]	Incorporation Rate[b]
μg/vial	*cpm/h·μl PCV*	*cpm/h·μl PCV*
0	33,100	2,120
100	19,200	67
200	20,600	10
300	17,300	1
400	17,600	0

[a] Measured with 9.5 μl PCV, over a period of 10 min.
[b] Measured with 1.55 μl PCV, over a period of 3 h.

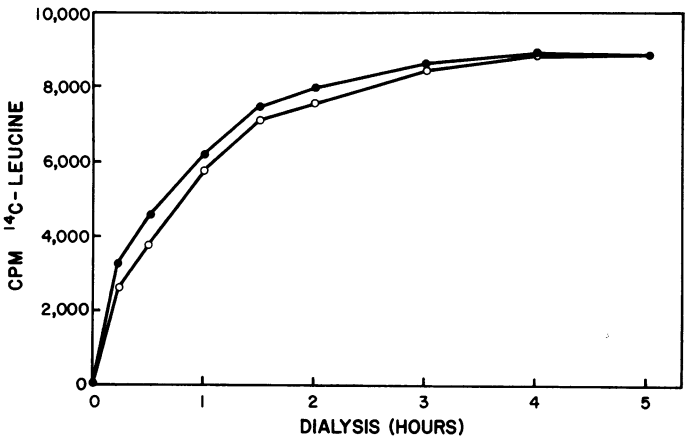

FIG. 5. Rate of dialysis of [^{14}C]leucine in the presence (●) and absence (○) of wall fragments.

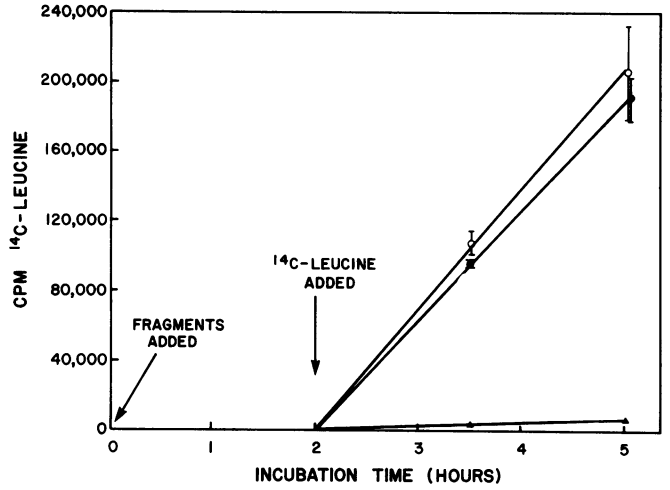

FIG. 6. Effect of starch fragments on the ability of sycamore cells to incorporate [^{14}C]leucine into acid-precipitable polymers. The experimental procedure is as described in Figure 3, except that the incubations were stopped after 3.5 or 5 h. (○), Control values. (●), Values obtained in the presence of 500 μg of starch fragments. (△), Values obtained in the presence of 500 μg sycamore cell wall fragments. The incubation mixtures contained 6.8 × 10^4 cells.

dissolved in a small volume of water. The pH of the solution was adjusted to 6.5 with dropwise addition of 1 N NaOH. A precipitate that formed was removed by centrifugation (2,000*g* for 10 min) and discarded. The supernatant solution was dialyzed in Spectra-Por 6 dialysis tubing (1,000 D cutoff). The dialysis tubing was kept gyrating by vigorous stirring of the solution around the tubing. The 4 L of water outside the tubing were changed three times in 24 h. The dialyzed wall-fragment solution was freeze-

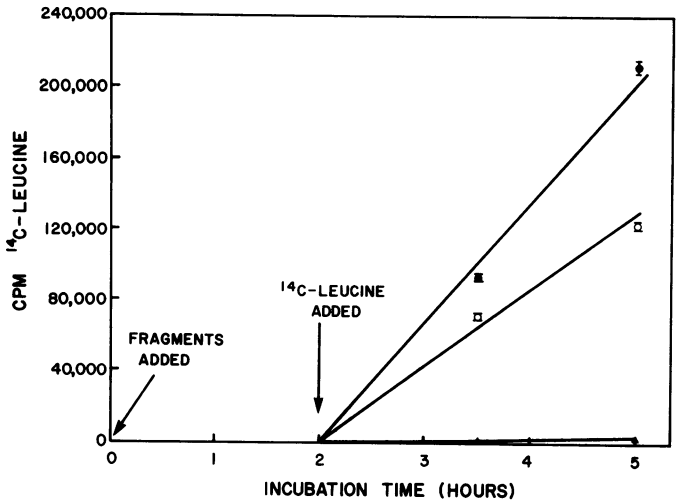

FIG. 7. Effect of citrus pectin fragments on the ability of sycamore cells to incorporate [^{14}C]leucine into acid-precipitable polymers. The experimental procedure is as described in Figure 6. (○), Control values. (●), Values obtained in the presence of 500 μg of citrus pectin fragments. (△), Values obtained in the presence of 500 μg of wall fragments. The incubation mixtures contained 6.8 × 10^4 cells.

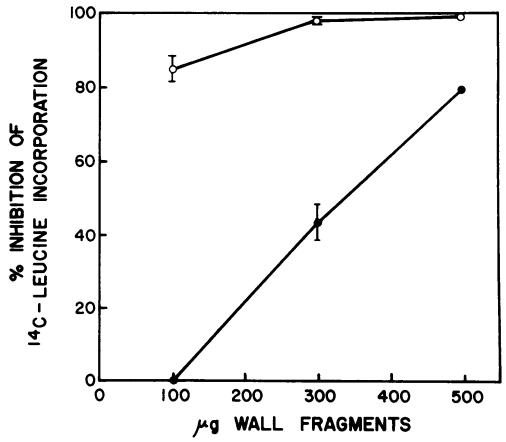

FIG. 8. Effect of acid hydrolysis followed by dialysis on the ability of the wall fragments to inhibit [^{14}C]leucine incorporation into acid-precipitable polymers of sycamore cells. The details of the acid hydrolysis conditions are given in "Materials and Methods." The experimental procedure is as described for the results in Figure 4. (○), Values obtained in the presence of wall fragments exposed to the acid but not heated, and then dialyzed. (●), Values obtained in the presence of wall fragments that were heated in the acid and then dialyzed. The wall fragment concentrations (μg/0.5 ml of incubation mixture) indicated on the horizontal axis are for the wall fragments that were exposed to acid but not heated (control fragments). Residues of the wall fragments after the hot acid hydrolysis and dialysis were dissolved in the same volume of water as the control wall fragments. Incubation mixtures contained 3.3 × 10^4 cells.

dried and then dried in a vacuum oven at 45°C overnight. The dried wall fragments represented 7 to 8% of the isolated cell walls.

Preparation of Starch and Citrus Pectin Fragments. Oligosaccharide fragments were prepared from soluble starch (Merck and Co., Inc.) and from citrus pectin (Sunkist Growers, Inc.). The procedure was the same as that for the preparation of sycamore cell wall fragments. The yield of white material from starch and slightly yellow material from pectin represented, in each case, 6% of the starting material.

Assay for [^{14}C]Leucine Incorporation. In most of the work described here, wall fragments were dissolved in 0.1 ml distilled

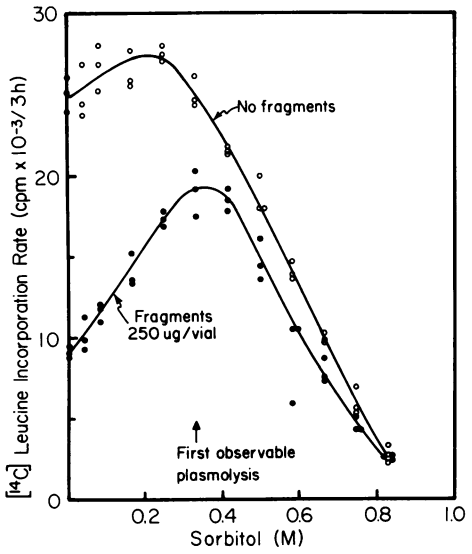

FIG. 9. Effect of plasmolysis on the susceptibility of sycamore cells to wall fragments. The cells were preincubated for 1 h in medium containing the sorbitol concentration shown in the figure, and then treated with 250 μg of wall fragments (●) or an equal volume of water (○) for 2 h. [^{14}C]Leucine incorporation into proteins over the following 3 h was measured.

H_2O in 19- × 48-mm glass vials and 0.1 ml of twice-concentrated incubation medium (yeast-extract-free M6 medium) or, in the absence of wall fragments, 0.2 ml of the regular incubation medium was added. A suspension of cultured sycamore cells (0.3 ml) was then placed in the vials. Five μl containing 0.5 μCi L-[U-^{14}C]leucine (340 mCi/mmol, New England Nuclear) was added and incubated for various time periods on a gyratory shaker (103 rpm) in the dark at 24°C. The pH of the incubations was 5.5. Reactions were terminated by the addition of 5 ml of cold 10% TCA, and the vials were placed in ice for a minimum of 30 min. The samples were then filtered on a Millipore model 1225 sampling manifold with 8.0-μm membrane filters, and the retained precipitate washed five times with 15 ml of cold 5% TCA. The filters were suspended in 7 ml of Biofluor cocktail (New England Nuclear), and, after standing overnight, counted for 10 min in a Beckman LS-250 liquid scintillation counter. All of the assays were performed on duplicate or triplicate samples. The results presented are the mean of samples, and the variation between the measurements is shown. This procedure was varied slightly for the experiments testing the effect of sorbitol on [^{14}C]leucine incorporation; these variations are described later.

Assay for [^{14}C]Leucine Uptake. To test the effect of wall fragments on the cells' ability to absorb [^{14}C]leucine, direct measurements of the uptake rate were undertaken. Cells were treated with wall fragments (100–400 μg) for 2 h, in 0.5 ml of medium buffered at pH 5.5 with 30 mM Mes (Na^+ salt). [^{14}C]Leucine (0.04 μCi) was then added, and the incubation was continued for an additional 10 min. Five ml of fresh nonradioactive medium was added, the suspension was quickly filtered on Whatman GF/C glass fiber paper, and the cells rapidly washed with an additional 2 × 5 ml of fresh medium. The ^{14}C retained on the filter was determined by liquid scintillation counting, and was regarded as [^{14}C]leucine 'uptake.'

Equilibrium-Dialysis Experiment. Aliquots (0.5 ml) of yeast-extract-free M6 incubation medium for test and control solutions were prepared. A solution of the wall fragments (500 μg) was added to one of the samples. The two samples were placed in Spectra-Por 6 dialysis tubing (1,000-D cutoff). Five μl (1.11×10^6 dpm) of L-[U-^{14}C]leucine was added to each dialysis tube, and the tubes placed vertically in 35- × 80-mm beakers containing 50 ml of incubation medium. The medium in the beakers was stirred vigorously and maintained at 24°C. Samples (0.5 ml) of the outer solution were removed at various time intervals. The samples were mixed with 7 ml of Biofluor cocktail and counted for 10 min.

Acid Treatment of the Active Fragments. An aliquot (0.23 ml) of a water suspension containing 2.2 mg of the wall fragments was placed in each of two tubes and freeze-dried. The fluffy residues were dissolved in 0.8 ml of 2 N TFA. The tubes were sealed with Teflon-lined screw tops. One tube was then heated at 121°C for 2 h, and the other tube was kept in an ice bath for 2 h. The solutions were cooled in ice, neutralized by adding 0.8 ml of cold 1 M Na_2CO_3, dialyzed in 1,000-D cutoff Spectra-Por 6 tubing, and freeze-dried. The fluffy residue of the nonheated sample was weighed and then dissolved, producing a solution in which the fragments were at a concentration of 500 μg/0.1 ml water. The residue from the heated sample was dissolved in an equal volume of water. The heated and nonheated fragments were assayed at three different concentrations for their ability to inhibit [^{14}C]leucine incorporation into acid-precipitable polymers.

Sorbitol Protection Experiment. The influence of various concentrations of sorbitol on the ability of wall fragments to inhibit [^{14}C]leucine incorporation was studied. The sycamore cells (about 7 μl PCV[4]) were preincubated for 1 h in 475 μl of medium containing various concentrations of sorbitol. This medium was buffered at pH 5.5 with 30 mM Mes, Na^+ salt. Cell wall fragments (250 μg in 25 μl water, pH 5.5) or water (25 μl) was then added, and the incubation continued for an additional 2 h. Finally, [^{14}C]leucine (0.04 μCi) was added, and incorporation of radioactivity into acid-precipitable material after an additional 3 h was determined.

RESULTS

[^{14}C]Leucine Incorporation Assay. Experiments were performed to establish the rate at which the sycamore cells incorporate [^{14}C]leucine into acid-precipitable polymers. The cells, after a lag of 30 to 60 min, exhibited a linear increase in the amount of [^{14}C]leucine incorporated for a period of at least 4 h. The amount of [^{14}C]leucine incorporated was proportional to the number of cells in the incubation mixture (Fig. 1). These experiments were repeated several times with similar results.

Effect of Cell Wall Fragments on the Ability of the Sycamore Cells To Incorporate [^{14}C]Leucine into Acid-Precipitable Polymers. Addition of acid-solubilized fragments of isolated sycamore cell walls to an incubation mixture containing sycamore cells and [^{14}C]leucine inhibited the leucine incorporation into acid-precipitable polymers. In the experiment summarized in Figure 2, addition of the wall fragments 1 h after the start of the incubation of cells with [^{14}C]leucine resulted in partial inhibition of [^{14}C]leucine incorporation in less than 1 h and about 70% inhibition of [^{14}C]leucine incorporation after 2 h. This experiment was repeated several times with the same result. Addition of wall fragments 2 h before the addition of the [^{14}C]leucine resulted in immediate inhibition of [^{14}C]leucine incorporation. The experiment summarized in Figure 3 resulted in an approximate 85% inhibition of [^{14}C]leucine incorporation assayed 1 h after addition of the [^{14}C] leucine. Similar results were obtained with repeat experiments using the same preparation of fragments as well as the fragments obtained from different preparations of cell walls.

The ability of the acid-solubilized wall fragments to inhibit [^{14}C]leucine incorporation for two different concentrations of sycamore cells is illustrated by the data in Figure 4. At high concentrations of wall fragments, the incorporation of [^{14}C]leucine was inhibited essentially completely regardless of the number of sycamore cells in the test suspension. However, at the lower

[4] Abbreviation: PCV, packed cell volume.

concentrations of the fragments, greater inhibition occurred when fewer cells were present. For example, 100 μg/0.5 ml of the wall fragments was sufficient to cause 50% inhibition of 2.3×10^4 cells in 0.5 ml, but 200 μg/ml of fragments was necessary to cause 50% inhibition of 5×10^4 cells in 0.5 ml.

Effect of Wall Fragments on [^{14}C]Leucine Uptake. The inhibitory effect of cell wall fragments on [^{14}C]leucine incorporation into protein could be due to an inhibition of protein synthesis or an inhibition of amino acid uptake. To test the latter possibility, we estimated the uptake rate over such a short period of time that incorporation into protein was negligible (Table I). The uptake rate in control cultures (measured over 10 min) was 33,100 cpm/h·μl PCV, compared with an incorporation rate (measured over 3 h) of 2120 cpm/h·μl PCV, a 15-fold excess. Furthermore, the wall fragments (100 μg) inhibited the uptake rate by only 42%, whereas in the same experiment, they inhibited [^{14}C]leucine incorporation by 97% (Table I). We concluded that incorporation was not rate limited by uptake, and that the observed effects of fragments on incorporation, described elsewhere in this paper, truly represented an inhibition of the utilization of intracellular [^{14}C]leucine in protein synthesis.

Equilibrium Dialysis of a Mixture of Wall Fragments and [^{14}C]Leucine. We attempted to determine whether the acid-released wall fragments would have an affinity for, that is, bind to, the [^{14}C]leucine. Such an interaction would inhibit the [^{14}C]leucine from reaching the cells, thereby causing an apparent inhibition of [^{14}C]leucine incorporation. An equilibrium-dialysis experiment was designed to assess the affinity of the [^{14}C]leucine for the acid-solubilized cell wall fragments. An equal amount of [^{14}C]leucine in yeast-extract-free incubation medium was put into each of two dialysis membranes; wall fragments were added to one of these solutions. The solutions were then dialyzed against yeast-extract-free incubation medium in separate containers. Figure 5 shows that the rate at which the [^{14}C]leucine was detected in the incubation medium outside the dialysis membranes was the same whether or not the dialysis membranes contained wall fragments. This result indicated that any interaction between the wall fragments and [^{14}C]leucine would not be sufficient to inhibit the rate of uptake of the [^{14}C]leucine into the sycamore cells.

Effect of Starch and Citrus Pectin Fragments on [^{14}C]Leucine Incorporation. Acid-produced fragments of starch and of citrus pectin were prepared, and the ability of these fragments to inhibit [^{14}C]leucine incorporation into the acid-precipitable polymers of sycamore cells was compared to the inhibitory activity of acid-produced cell wall fragments. Starch fragments had little effect, whereas the wall fragments resulted in almost complete inhibition of [^{14}C]leucine incorporation (Fig. 6). A similar experiment using acid-produced fragments of citrus pectin resulted in a stimulation of the rate of [^{14}C]leucine incorporation, while the wall fragments resulted in almost complete inhibition (Fig. 7).

Effect of 'Strong' Acid Hydrolysis Followed by Dialysis on the Ability of the Wall Fragments To Inhibit [^{14}C]Leucine Incorporation into the Acid-Precipitable Polymers of Sycamore Cells. Wall fragments heated in the presence of 2 N TFA for 2 h at 121°C lost most of their inhibitory activity (Fig. 8). At 100 μgm of wall fragments per 0.5 ml of incubation mixture, the unheated fragments inhibited [^{14}C]leucine incorporation by more than 80%, while the equivalent amount of fragments, after acid hydrolysis and dialysis, retained no measurable ability to inhibit [^{14}C]leucine incorporation.

Protection of Sycamore Cells, by Plasmolysis, from Inhibition of [^{14}C]Leucine Incorporation by Wall Fragments. In view of the observation that plasmolysis protects cells against the toxic effects of pectic-degrading enzymes (3–5, 20), the influence of cell wall fragments on [^{14}C]leucine incorporation by plasmolyzed sycamore cells was tested. The results (Fig. 9) clearly showed that sorbitol protected the cells against the inhibitory action of wall fragments. Similar results were obtained in a separate experiment. A sorbitol concentration of 0.45 M was required for about 50% of the cells to show some degree of observable plasmolysis, and at about 0.7 M sorbitol, 50% of the protoplasts were fully rounded and withdrawn from the cell walls. At sorbitol concentrations large enough to cause more than incipient plasmolysis, [^{14}C]leucine incorporation was inhibited in the absence of added wall fragments; nevertheless, the addition of wall fragments to the plasmolyzed cells resulted in only slightly increased inhibition of [^{14}C]leucine incorporation.

DISCUSSION

The initial objective of the experiments described was to determine whether acid-solubilized fragments of sycamore cell walls initiated death of suspension-cultured sycamore cells. The results of our early experiments, using the vital stains fluorescein diacetate (26) and Evans blue (10) indicated that fragments produced by partial acid hydrolysis of sycamore cells did result in the death of a significant proportion of the suspension-cultured sycamore cells. However, it was difficult to quantitate these results. Inability to quantitate cell death with microscopic observation led us to adopt a [^{14}C]leucine incorporation assay as a measure of the ability of the cells to incorporate an amino acid into proteins (7, 27). This assay is not a direct measure of cell death, but the assay has been demonstrated to be a good measure of the phytotoxicity of compounds (7). The assay is reasonably quantitative and rapid.

The results presented in this paper demonstrated that the mixture of fragments produced by partial hydrolysis of sycamore cell walls contained one or more components capable of inhibiting the incorporation of [^{14}C]leucine into the acid-precipitable polymers of suspension-cultured sycamore cells. The activity of the fragments was either destroyed or the active fragments were degraded to fragments that passed through 1,000 D dialysis tubing by hydrolysis conditions that are known to hydrolyze glycosidic linkages (Fig. 8), suggesting that the active fragments solubilized from the insoluble cell walls contained glycosyl residues.

The ability of the sycamore cell wall fragments to inhibit [^{14}C]leucine incorporation was shown to be a property of cell wall fragments but not of fragments released from starch and from citrus pectin. The chemical nature of the active component(s) in the wall fragments remains to be determined. Indeed, the biological significance of the ability of these wall fragments to inhibit [^{14}C]leucine incorporation into acid-precipitable polymers and very probably protein synthesis of plant cells cannot be judged at this time. However, we may speculate that the activity of these wall fragments is related to the phytotoxic effect of pectic-degrading enzymes. According to this hypothesis, such enzymes would release from the cell walls of living plants pectic oligosaccharides that are thereby freed to act on the plant cells, inhibiting protein synthesis and eventually causing death. The demonstrated ability of plasmolysis to protect plant cells against the toxic effects of the wall fragments (Fig. 9), as well as those of pectic-degrading enzymes (3–5, 20), supports this idea. The ability of plant cell wall fragments to inhibit [^{14}C]leucine incorporation in plant cells is another example of the biological activity of cell wall fragments (1, 2, 10, 19).

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