

IONIC EFFECTS ON LIGNIFICATION AND PEROXIDASE IN TISSUE CULTURES

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ABSTRACT

Crown-gall tumor tissue cultures release peroxidase into the medium in response to the concentration of specific ions in the medium. This release is not due to diffusion from cut surfaces or injured cells. Calcium, magnesium, and ammonium were, in that order, most effective in increasing peroxidase release. The enzyme was demonstrated cytochemically on the cell walls and in the cytoplasm. Cell wall fractions, exhaustively washed in buffer, still contained bound peroxidase. This bound peroxidase could be released by treating the wall fractions with certain divalent cations or ammonium. The order of effectiveness for removing the enzyme from the washed cell walls is: $\text{Ca}^{++} \approx \text{Sr}^{++} > \text{Ba}^{++} > \text{Mg}^{++} > \text{NH}_4^+$. These data support the thesis presented that specific ions can control the deposition of lignin on cell walls by affecting the peroxidase levels on these walls.

INTRODUCTION

The deposition of an organic polymer referred to as lignin on the secondary walls of plant cells is one of the more striking examples of cellular differentiation. This process, which occurs in a large variety of plants, has been intensively studied in intact plants, tissue cultures, and model (non-living) systems. These studies, reviewed by Brown (1), and by Siegel (2, 3), have led to the concept that the biosynthesis of lignin in plant cells, as well as the formation of lignin in model systems, is dependent upon the presence of suitable precursors, a matrix such as cellulose upon which the reaction takes place, hydrogen peroxide, and peroxidase. Numerous studies on lignification in intact plants have demonstrated a direct relation between peroxidase levels and lignification (4). In model systems, it is necessary that peroxidase be absorbed on the matrix if lignification is to occur. The absence of a matrix in these model systems prevents the formation of lignin. In living cells, as well as in model systems, there is no evidence that lignin is formed at any site other

than the matrix, which in the living cell is invariably the cell wall.

Studies of lignin deposition in tissue cultures have for the most part been oriented towards elucidating the biochemistry of lignin synthesis. For example, von Wacek and others (5-7) have demonstrated that the addition of certain lignin precursors, such as coniferyl alcohol, to the media upon which tissue cultures are grown, brings about an increase in the amount of lignin deposited on cell walls. Other investigators (8-12) have demonstrated a similar action of eugenol, cinnamic acid, and its derivatives, on the lignification of isolated plant organs.

Recently Lipetz (13) reported that the lignification of sunflower crown-gall tumor tissue, and several tissues derived from healthy plant stems and roots, was markedly influenced by the concentration of calcium in the growth medium. The lignification of these tissues was promoted by low levels, and inhibited by high levels, of calcium in the medium. A similar effect of low calcium levels

on lignification is suggested by earlier reports on the growth of intact plants in calcium-deficient solutions (14-17).

In order to elucidate the mechanism by which calcium controls the lignification of tissue cultures, we explored the distribution of peroxidase in tissues grown on high and low calcium levels.

MATERIALS AND METHODS

TISSUE: Crown-gall tissue of sunflower, clone S8, originally isolated from a primary stem tumor in 1955 by Dr. T. Stonier, was routinely grown on White's medium (18) modified to include iron as a chelate (19).

MEDIA: As mentioned, the composition of the medium was a slightly modified White's. Media containing the major mineral salts $MgSO_4$, $Ca(NO_3)_2$, Na_2SO_4 , KCl , NaH_2PO_4 and KNO_3 at the concentrations given by White are referred to as 1XW; media containing these salts at 10 times this concentration are referred to as 10XW. Cultures were maintained on 10XW media made by diluting the supernatant of a stock solution of 100XW. For experimental work, media were made up by combining the desired quantities of separate stock solutions of each component.

For studies on the effects of specific ions, the desired salts were added to 1XW at the concentrations cited, the pH adjusted to 5.5, 2 per cent sucrose was added, and the medium solidified with 1 per cent agar. In some experiments, the desired salt solution was made up in pH 5.5 Veronal buffer without the addition of any other salts. Salts were all of reagent or analytical reagent grade.

DIFFUSION TESTS: Pieces of tissue approximately $5 \times 5 \times 5$ mm were cut from rapidly growing cultures and aseptically placed on the desired media in 15-mm petri dishes, which were then incubated at $25^\circ C$ for 24 hours. After this time the tissues were removed from the medium, which was then flooded with a saturated (0.025 per cent) aqueous solution of benzidine. Five minutes later 0.1 ml of 3 per cent H_2O_2 , freshly diluted from 30 per cent H_2O_2 , was added to the benzidine. Color was allowed to develop for 10 minutes after which the reaction mixture was poured off and the diameter of the blue-brown halo measured.

For some experiments, tissues a little smaller than usual were allowed to grow on 10XW for 4 to 12 days, then used for diffusion tests. These tissues, referred to as "pre-grown," were not cut before being placed on the diffusion plates. Tests were generally run in quintuplicate and repeated at least twice. Controls were run either by omitting the H_2O_2 , or by pre-treating the plates with $10^{-2} M$ KCN.

HISTOCHEMISTRY: Small pieces of tissue were vacuum infiltrated with O. C. T. compound (Lab-

Tek, Inc., Westmont, Illinois) for about 30 seconds. The tissues were then rapidly frozen and cut at 10μ in a Lab-Tek Cryotome at $-20^\circ C$. Sections were picked up on cold slides, and either directly treated with van Duijn's benzidine reagent (20) or first air dried and then treated with the reagent.

Controls were run by omitting the H_2O_2 from the reaction mixture or alternatively by incubating the sections in $10^{-2} M$ KCN for 30 seconds to 1 minute before treating them with the reagent.

Tests for lignin-like compounds were made with the reagents listed in a previous publication (13).

CELL FRAGMENTATION: Tissue was homogenized in a chilled glass mortar with a motor-driven Teflon pestle for 1 to 1.5 minutes in enough cold pH 6.1 Veronal or sodium hydrogen maleate buffer to cover the tissue. The resulting homogenate was strained through a double layer of cheesecloth, then centrifuged at 1,500 RPM for 15 minutes at $6^\circ C$ in a model L Spinco preparative centrifuge. The precipitate was discarded; the supernatant was examined microscopically to determine whether it was homogeneous and free of intact cells, and was then centrifuged, again at $6^\circ C$, for 20 minutes at 10,000 RPM. The supernatant was assayed for peroxidase activity; the precipitate, termed "wall fraction," was resuspended in buffer and centrifuged at 10,000 RPM. The last two operations were repeated until the supernatant had little or no demonstrable peroxidase activity. Aliquots of these exhaustively washed wall fractions were then suspended in solutions of the various salts tested and centrifuged at 10,000 RPM for 30 minutes. The supernatant and the precipitate were both assayed for peroxidase activity.

The salt solutions were made up in sodium hydrogen maleate buffer adjusted to pH 6.1. To minimize possible variation arising from the use of separate pieces of tissue, each experiment was performed on pooled cell wall fractions prepared in sufficient quantity to test all of the salts tried.

PEROXIDASE DETERMINATIONS: Peroxidase determinations of the supernatants described above were carried out by the method described by Lance (21), in which guaiacol is oxidized to a brown chromogen by peroxidase in the presence of H_2O_2 . The reactions were run in Coleman tubes, the changing optical density was read at 15-second intervals for 2 to 3 minutes, at $470 m\mu$ on a Coleman Jr. spectrophotometer equipped with an electronically regulated power supply.

Enzyme localization on the precipitate referred to as "wall fraction" was performed by mixing a drop of the fraction with a drop of van Duijn's reagent on a glass slide, and observing the reaction under the microscope. Controls were run by adding $10^{-2} M$ KCN to the reaction mixture, or alternatively by omitting the peroxide from the mixture.

TABLE I
Diffusion of Peroxidase from Clone S8 Tissue

Medium	Molarity of added salt	Diameter of benzidine reaction in mm	
		Pregrown 4 days	Fresh cut
1XW		13 ± 1.2	12 ± 0.7
10XW		18 ± 1.3	21 ± 1.4
1XW + 10X KNO ₃	8 × 10 ⁻³	12 ± 1.7	13 ± 0.7
1XW + 10X KCl	9 × 10 ⁻³	13 ± 0.9	12 ± 1.7
1XW + 10X Na ₂ SO ₄	1.4 × 10 ⁻²	12 ± 1.2	14 ± 1.0
1XW + 10X Na ₂ HPO ₄	1.2 × 10 ⁻³	13 ± 0.7	14 ± 2.6
1XW + 10X MgSO ₄	3 × 10 ⁻²	15 ± 1.8	15 ± 2.6
1XW + 10X Ca(NO ₃) ₂	1.2 × 10 ⁻²	18 ± 1.0	18 ± 1.4
Average for all media		14 ± 2.3	15 ± 1.4
		Pregrown 12 days	Fresh cut
1XW		11 ± 1.6	11 ± 1.2
10XW		18 ± 1.2	19 ± 1.3

RESULTS

DIFFUSION TESTS: A blue-brown halo developed on the benzidine-treated plates after the addition of H₂O₂. This benzidine-positive reaction for peroxidase was almost totally inhibited if the plates were treated with 10⁻² M KCN before the benzidine was added. The chromogen was never observed on control plates where H₂O₂ was omitted.

Tissues grown on 10XW released considerably more peroxidase into the medium than comparable tissues grown on 1XW, as shown by the respective sizes of the benzidine-positive halos. The addition of each major mineral of White's medium at 1 or 10 times the concentration in 1XW demonstrated that only Ca(NO₃)₂ or MgSO₄ at 10XW concentration were nearly as effective as 10XW medium in promoting peroxidase loss from the tissues. These data are presented in Table I.

Increasing the concentration of NO₃⁻ or SO₄⁼ by adding NaNO₃ or Na₂SO₄ so that the molar concentrations, or ionic strengths, of these cations were as high as and higher than those found in Ca(NO₃)₂ and MgSO₄ in 10XW did not markedly increase the loss of peroxidase (Table II). Tests using other salts of Ca⁺⁺ and Mg⁺⁺ yielded similar data.

Of the tested ions other than Ca⁺ or Mg⁺⁺,

TABLE II
Diffusion of Peroxidase from Clone S8 Tissue:
Effects of High Ionic Concentrations

Molarity	NaNO ₃	KCl	Diameter of benzidine reaction in mm	
			M	
1.5 × 10 ⁻³			8	8
1.5 × 10 ⁻²			8	10
1.5 × 10 ⁻¹			13	15

TABLE III
Diffusion of Peroxidase from Clone S8 Tissue:
Effect of NH₄⁺

M Concentration	(NH ₄) ₂ SO ₄	Na ₂ SO ₄	Ca(NO ₃) ₂	
			Diameter of benzidine reaction in mm	
1.2 × 10 ⁻³	8	11	11	
1.2 × 10 ⁻²	16	13	15	

only NH₄⁺ at a relatively high concentration appreciably enhanced peroxidase loss from the tissues (Table III). Other salts at similar and higher concentrations (10⁻¹ M) caused only a slight increase in peroxidase loss from the tissues (Table II).

The data in Table I also indicate that there is

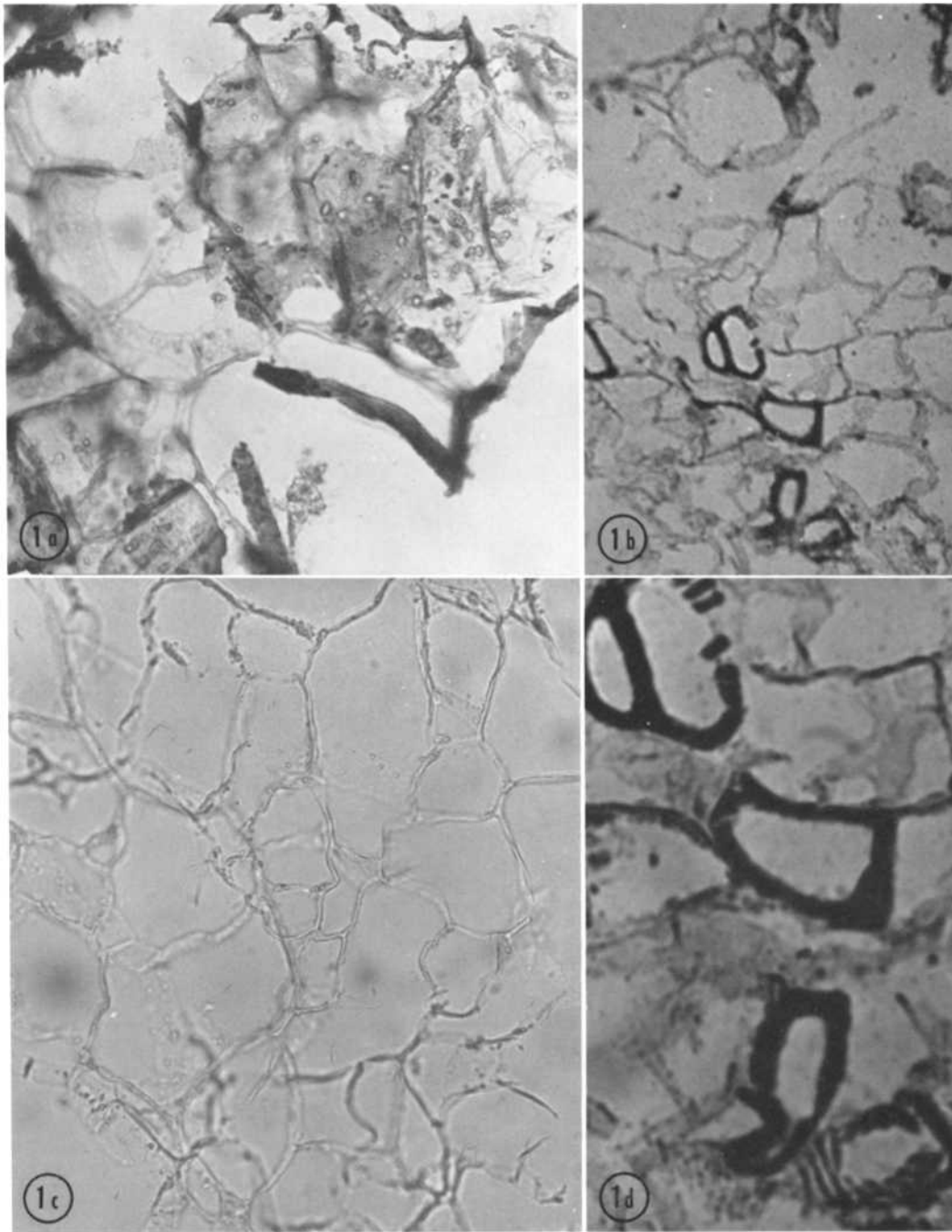


FIGURE 1 *a* to *d* Cold microtome sections ($10\ \mu$) of clone S8 tissue. *a*, Treated with van Duijn's reagent. Note dense reaction on many cell walls, and less dense reaction in cytoplasm. *b* and *d* represent air-dried sections treated with van Duijn's reagent. Note the very strong reaction on the walls and secondary thickenings of the tracheids. *c*, Control treated with 10^{-2} M KCN and van Duijn's reagent. Note only a faint reaction on some of the cell walls. 1 *a*, $\times 500$; 1 *b*, $\times 300$; 1 *c*, $\times 500$; 1 *d*, $\times 750$.

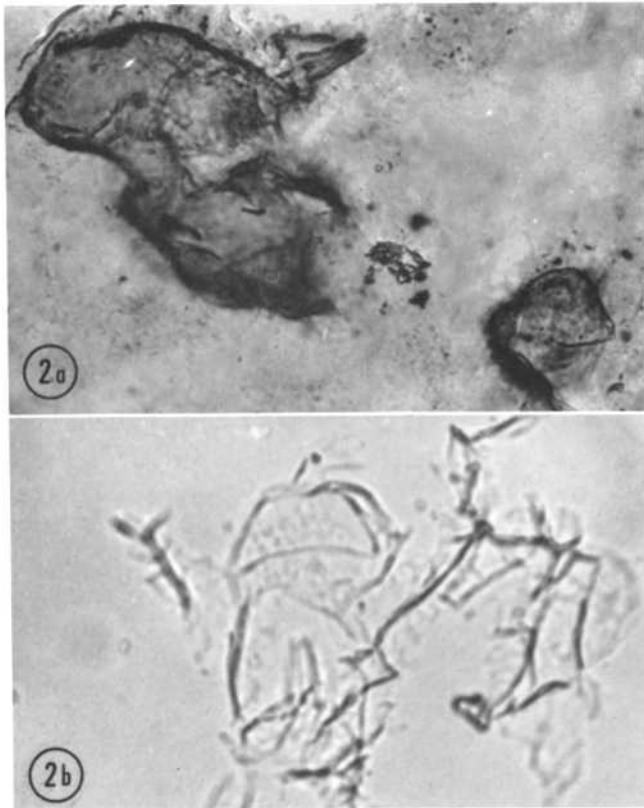


FIGURE 2 *a*, Crude homogenate treated with van Duijn's reagent. Note strong reaction of broken cell walls and of cytoplasmic debris. *b*, Control treated with 10^{-2} M KCN and van Duijn's reagent. Most of the reaction is inhibited. 2 *a*, $\times 338$; 2 *b*, $\times 338$.

no significant difference in the loss of enzyme from freshly cut tissues and from tissues pregrown for as long as 12 days.

In order to test the possibility that the ions might influence the rate of peroxidase diffusion in the agar rather than the loss of enzyme from the tissues, samples of commercially obtained peroxidase preparations were allowed to diffuse for 24 hours from center wells in plates containing the various media tested. The plates were then assayed for the extent of enzyme diffusion. The diameters of the ensuing zymograms were the same in all the media tested.

Peroxidase also diffused from tissues placed on media containing only trace quantities of ions. This "background" effect could not be modified by changing the sucrose concentration of the medium, or by increasing the purity of the water or the agar used.

An inverse relation between peroxidase loss and lignification was observed in tissues grown on media containing various concentrations of ions at 1, 10, or 15 times White's. The addition of

NH_4NO_3 (1.2×10^{-2} M) also inhibited lignification and increased the loss of enzyme from the tissue.

LOCALIZATION OF PEROXIDASE: Sections of rapidly growing tissues gave a positive reaction for peroxidase with van Duijn's reagent. This enzyme was localized in the cytoplasm and either on, or in the cell walls. The walls and secondary thickening of tracheids were also highly reactive. The cytoplasmic sites of activity appeared to be particulate in nature, whereas most walls appeared to stain more or less uniformly (Figs. 1 *a*, *b*, and *d*).

Omission of either the NH_4Cl or EDTA from the reaction mixture led to the formation of a brown rather than a blue reaction product, as reported by van Duijn (20). Furthermore, the brown product was more fugitive than the blue one, and gave a muddy appearance to the preparation. The blue reaction product was quite insoluble; extrasectional areas of the slide showed chromogen only when a large proportion of the cells in the section were broken, and presumably

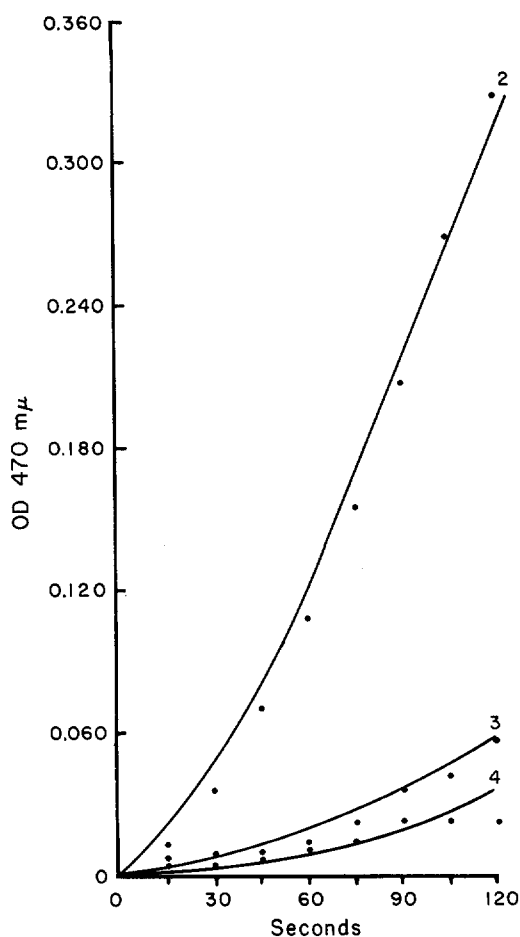


FIGURE 3 Peroxidase activity of successive washings of cell wall fractions with sodium hydrogen maleate buffer. Readings were taken at 15-second intervals. Enzyme concentration in the first washing was too high for inclusion in this figure.

lost peroxidase to the surrounding area. The reaction was almost completely inhibited by prior incubation of the section for 30 seconds in 10^{-2} M KCN or the omission of the H_2O_2 from the reaction mixture (Fig. 1 *c*), thus making it likely that the reaction is specific for peroxidase.

CELL FRACTIONATION: Examination of the crude homogenate revealed that many of the cells were fragmented, but that a fair number of intact cells remained. When treated with van Duijn's reagent, cell fragments and clumps of cytoplasm gave strong positive, cyanide-sensitive reactions (Fig. 2 *a* and *b*). After filtration and centrifugation at low speeds, the ensuing precipitate referred to

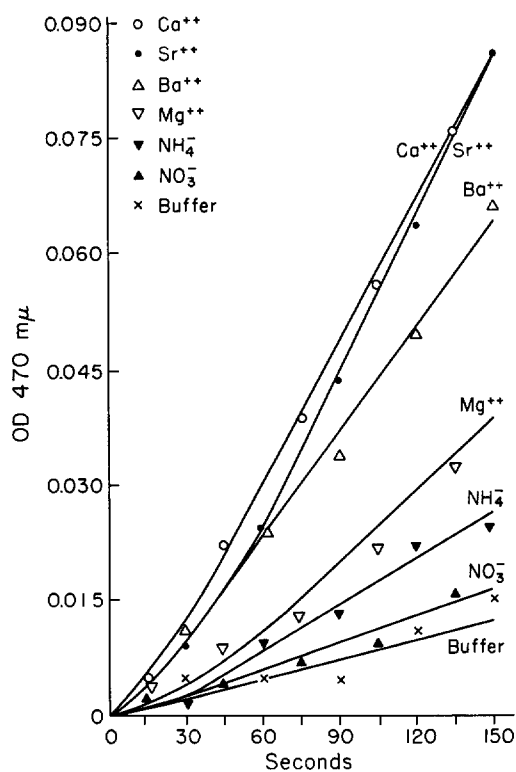


FIGURE 4 Peroxidase activity of the supernatant of exhaustively washed cell wall fractions, extracted with the indicated ions. Readings were taken at 15-second intervals. To simplify the figure, only alternate points are plotted.

as cell wall fraction contained only an occasional intact cell.

The supernatants from successive washings of this fraction with buffer progressively contained less peroxidase per unit volume of buffer, until after 4 or 5 washings the supernatant was essentially free of assayable enzyme (Fig. 3).

When aliquots of exhaustively washed cell wall fractions were resuspended in either $Ca(NO_3)_2$, $Sr(NO_3)_2$, $Ba(NO_3)_2$, $Mg(NO_3)_2$ or NH_4NO_3 and spun down, the supernatants were found to contain peroxidase. Supernatants from similar aliquots extracted with buffer or KNO_3 at a concentration equal to the NO_3^- concentration of the $Ca(NO_3)_2$ showed no appreciable activity. These data are presented in Fig. 4 from which it can also be seen that the order of effectiveness of the ions in releasing peroxidase from exhaustively washed cell wall fractions is $Ca^{++} \approx Sr^{++} > Ba^{++} > Mg^{++} > NH_4^+$. Beryllium was found to be ineffective

in releasing peroxidase from these wall preparations.

The effectiveness of Ca^{++} in releasing peroxidase from washed cell walls is dependent upon its presence in concentrations of at least 2.5×10^{-2} M; lower concentrations were ineffective, and higher concentrations failed to enhance enzyme release.

The addition of van Duijn's reagent to exhaustively washed cell wall fractions treated with buffer or 0.1 M KNO_3 revealed that many of the wall fragments and bits of cytoplasm attached to them gave a strong, cyanide-sensitive, positive reaction. Wall fragments of similar aliquots of "wall fraction" treated with Ca^{++} , Sr^{++} , Ba^{++} , Mg^{++} or NH_4^+ at concentrations of 0.05 M were either devoid of or very low in peroxidase activity. Regrettably, these points cannot be adequately depicted without the use of color photographs.

The addition of salts, at the concentrations tested, to aliquots of commercially purchased horseradish peroxidase or of crude peroxidase obtained from the supernatant of the first washing of homogenized tissue did not have any effect on the rate of the enzyme action.

DISCUSSION

ENZYME LOSS FROM TISSUES: The release of enzymes and other substances from plant tissue cultures has been extensively documented. Among the enzymes released from these tissues are amylase (22), acid phosphatase (23), indole acetic acid oxidase (24) and peroxidase (25). Although variations in the magnitude of such losses exist, it is fairly well agreed that tissue cultures derived from plants or animals are "leaky."

The source of the enzymes released from the tissues has not been clearly established; the possibility of enzyme loss from cut surfaces or from injured cells has not been totally excluded. We believe that our data comparing enzyme loss from freshly cut tissues and "pregrown" tissues establish that intact, undamaged tissues release peroxidase into the medium at about the same rate as injured tissue. This release can be greatly increased by the addition of calcium, magnesium, or ammonium ions at concentrations used in tissue culture media. These ions appear to act by increasing the background leakage of the tissues. This background leakage could not be decreased by using de-ionized water and highly purified agar in the medium. Thus, it would appear that

the clone S8 tissue releases peroxidase even in a very low ionic environment.

The specificity of the action of Ca^{++} , Mg^{++} and NH_4^+ was demonstrated by the fact that equivalent and higher molar concentrations of the anions making up the salts used were not effective in increasing the release of peroxidase by the tissue.

The various media tested were found to have no effect on the diffusion rate of peroxidase, thus making it unlikely that our data represent an effect on the diffusion rate of peroxidase in agar rather than an actual loss from the tissues.

These data are in agreement with the recent findings of Straus and Campbell (23) who demonstrated the release of peroxidase and other enzymes by ten different plant tissue cultures. These investigators reported that 0.05 M CaCl_2 increased the release of peroxidase from tissues soaked in this solution, and also of IAA (indole acetic acid) oxidase and acid phosphatase from cell wall preparations. They did not, however, establish the specificity of the calcium ion for these effects.

LOCALIZATION OF PEROXIDASE IN TISSUES: Sunflower tumor tissues, in common with many other tissues studied (4), contains peroxidase at two sites, the cell wall and the cytoplasm. The strongest reaction was observed at sites at which lignification was in progress, or had already occurred, *i.e.* the walls and secondary thickenings of tracheids. The walls and cytoplasm of other cells gave considerably weaker reactions. These data are in agreement with the view that there is a correlation between sites of peroxidase activity and lignification (4).

CELL FRACTIONATION EXPERIMENTS: The data collected from these experiments support the histochemical evidence that cell walls contain a fraction of the total cellular peroxidase. This fraction is apparently bound to the walls and cannot be removed by repeated washings unless certain specific divalent cations or ammonium are added to the washing solution. The relative effectiveness, on a mole per mole basis, of these ions is $\text{Ca}^{++} \approx \text{Sr}^{++} > \text{Ba}^{++} > \text{Mg}^{++} > \text{NH}_4^+$ which is in agreement with the more limited data obtained in the diffusion studies. The effects of barium and strontium salts on enzyme release from tissues were not studied because the toxicity of these ions would make it difficult to determine whether an effect was due to toxicity or to a calcium-like effect.

Jansen, Jang, and Bonner (26) have demon-

strated that *Avena* cell wall preparations can bind peroxidase, and Straus and Campbell (23) have suggested that plant tissue culture cell wall preparations may bind enzymes released by homogenizing procedures and then released in the presence of CaCl_2 . Our data lead us to believe that at least a part of the peroxidase released from cell wall preparations by divalent cations is peroxidase which is normally found on or in cell walls.

IONIC CONTROL OF LIGNIFICATION: An inverse relationship between peroxidase loss from tissues and lignification was observed for clone S8 tissue. The calcium ion was found to be the most effective in increasing the amount of enzyme released by this tissue, and, as reported earlier (13), was also the most effective inhibitor of lignification in tissue cultures. The inhibitory action of Ca^{++} on lignification was also reported by Siegel (2), who stated that calcium inhibited the formation of lignin from eugenol.

On the basis of our results and the known role of peroxidase in lignification, we suggest a mechanism to account for the effect of calcium and certain other ions on the control of lignification in tissue cultures. At sufficiently high concentrations, Ca^{++} , and, to a lesser extent, Mg^{++} and NH_4^+ , greatly enhance the release of peroxidase from cell wall fragments. It seems reasonable to assume that the effect of these ions on tissue cul-

tures is at the same site. Thus, tissues grown on high levels of these ions would be composed of cells whose walls are low in peroxidase. These walls could not lignify, since one of the essential factors for lignification would be present in sub-optimal concentrations. By the same token, the walls of tissues grown on low levels of these ions would be relatively rich in peroxidase and thus capable of lignifying.

The control of peroxidase levels on, or in, plant cell walls may thus represent a mechanism by which differentiation is controlled by suitable concentrations of specific ions.

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