ACCUMULATION OF PHENOLIC SUBSTANCES AND ASCORBIC ACID IN POTATO TUBER TISSUE UPON INJURY AND THEIR POSSIBLE ROLE IN DISEASE RESISTANCE[1]

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INTRODUCTION

In a previous publication the authors (3) reported that chlorogenic acid tends to accumulate around a tissue injury in potato tubers. Politis (8) made similar observations. He showed that the acid not only tends to localize adjacent to suberized membranes but that following cuts in plant tissues, which do not normally contain the acid, it will appear after several days in cells adjacent to the wound.

The purpose of this investigation was to follow the rate of accumulation of phenolic substances, particularly chlorogenic acid and other o-dihydric-phenols, in potato tuber slices held at room temperature in a closed high humidity chamber. The effects of various treatments, such as the addition of tyrosinase inhibitors and lowering of the temperature, on the rate of accumulation of phenolic substances are reported. The changes in ascorbic acid content of the slices were also followed.

MATERIALS AND METHODS

Russet Burbank, Triumph and Kennebec varieties were used in this investigation. The tubers used had been stored at 35-38° F. for 6 to 7 months. The tubers were selected for uniformity in size so that uniform slices could be obtained. The tubers were peeled by hand with a "Nee-Action" vegetable peeler. They were then sliced into uniformly thick slices, 3 mm. in thickness, by a Hobart mechanical meat slicer. Only the slices from the center part of the tuber were used. The Triumph slices were slightly larger in diameter than the Burbank slices.

For the experiments to determine the rate of accumulation of phenolic substance in the slices held at room temperature (75° F) sufficient samples were prepared to obtain four one-hundred gram samples of slices. One sample was immediately analyzed for total phenols by means of the Folin-Denis phenolic reagent. The method used was essentially that of Rosenblatt and Peluso (9). The sample was also analyzed for total o-dihydricphenols by the Arnow's (1) colorimetric procedure.

Each of the three remaining one-hundred gram samples was placed on an 8-mesh stainless steel screen in a 9 x 9 in. stainless steel baking pan and then covered with aluminum foil. The slices were placed on the screen in such a manner that they did not touch each other. Moistened paper towels were placed under the screen to maintain high humidity. One of the samples was stored for 2 days at room temperature (75° F) and then

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analyzed. The second sample was stored for four days and the third sample was held for six days. The slices did not lose weight during the storage holding periods.

The normal Kennebec tubers and those infected with aster yellows were only analyzed for phenolic substances immediately after slicing.

The 100 gram sample of slices was extracted with 300 grams of 95 per cent ethanol in a Waring Blender for 5 minutes. The total volume of the ethanol extract after making correction for the volume of the pulp was found to be 454 milliliters. Approximately 200 milliliters of the extract was filtered through a Whatman No. 12 fluted filter into a 250 milliliter Erlenmeyer flask.

Determination of Total Phenols by Folin-Denis Reagent

One ml of the ethanol extract was pipetted into a 100 ml volumetric flask containing 70 ml of distilled water. Two ml of Folin-Denis reagent were added. After 2 to 3 min. of standing, 20 ml of 10 per cent Na_2CO_3 solution were added and the solution made to volume with distilled water. The blue color was allowed to develop for 1.5 hours and was read in an Evelyn Colorimeter using a 660 millimicron filter. In the blank sample for adjusting colorimeter to 100 per cent transmission, two ml of distilled water were substituted for the Folin-Denis reagent in the reaction mixture. Chlorogenic acid was used as a standard for calculating the total phenols. The optical density value of 0.132 for 0.1 mg chlorogenic acid per 100 ml was used.

The Folin-Denis reagent also measures tyrosine which was found to give the same amount of color with the reagent as chlorogenic acid. In the calculation of total phenols correction was made for ascorbic acid which also gives a blue color with Folin-Denis reagent.

Determination of Ascorbic Acid

The ethanol extracts were analyzed for ascorbic acid by the indophenol dye method at the same time they were analyzed for total phenols by Folin-Denis reagent. The purpose of this analysis was to determine the ascorbic acid correction to be made in the determination of total phenols. Ascorbic acid (0.1 mg/100 ml) gave an optical density of 0.162 with the Folin-Denis reagent.

The above ascorbic acid analyses indicated that ascorbic acid also accumulates in tuber slices held at room temperature. For this reason the slices were analyzed each day during a four day storage period. The method used was essentially that of Loeffler and Ponting (6). A 0.25 per cent oxalic acid solution was used for the extraction of ascorbic acid.

Analysis of o-Dihydricphenols

The total o-dihydricphenols were determined colorimetrically using the Arnow reagent (1) which is quite specific for these compounds. This reagent does not give a color with tyrosine.

One ml of the ethanol extract was pipetted into an Evelyn Colorimeter tube. The following reagents were then added: 1 ml of 0.5 N HCl, 1 ml of nitrate-molybdate reagent (10 gm of $NaNO_2$ and 10 gm of Na_2MoO_4

in 100 ml distilled water), 10 ml of distilled water and 2 ml 1 N NaOH. Thirty seconds after the addition of the alkali, the pink color produced was read in an Evelyn Colorimeter equipped with a 515 millimicron filter. In the blank sample used for the adjustment of the instrument reading to 100 per cent transmission, the Arnow's reagent was replaced by 1 ml of H_2O.

Optical density of 0.1 mg chlorogenic acid per 15 ml of the reaction mixture (optical density = 0.248) was used in the calculation of o-dihydricphenols.

The ethanol extracts of the slices held two, four and six days were diluted to twice their volume before sampling.

Separation of Phenolic Substances by Paper Chromatography

A thirty ml aliquot of the ethanol extract was concentrated under reduced pressure to approximately 3 ml. The concentrate was quantitatively transferred by washing it into a 15 ml graduated centrifuge tube. The extract was made to a volume of 5 ml by the addition of 80 per cent ethanol. Any slight amount of insoluble residue was removed by centrifugation. Twelve μl of concentrated extract were used for the separation of the phenolic substances by paper chromatography and were applied by using six 2μl aliquots.

Both one and two dimensional paper chromatography were used. n-Butanol-acetic acid-water (40-10-20) was used as the developing solvent for one-dimensional and also as the first solvent followed by 5 per cent acetic acid in water as the second solvent for two-dimensional paper chromatography.

After air-drying the chromatograms were sprayed with Arnow reagent diluted with 1 part water and two parts ethanol to detect the o-dihydricphenols. The one-dimensional chromatograms were also sprayed with Folin-Denis reagent as described by Johnson and Schaal (3).

Treatment of Whole and Sliced Tubebs

The influence of various treatments on the rate of accumulation of the o-dihydricphenols in tuber slices were also investigated. The treatments included (1) 24 hour immersion of tubers in water before slicing, (2) dipping of slices for two minutes in sodium bisulfite-sodium chloride solution (500 ppm SO_2 and 1 per cent NaCl), (3) dipping slices in 0.25 per cent resorcinol solution.

Results and Discussion

The rate of accumulation of phenolic substances in Russet Burbank and Triumph tuber slices held at room temperature (75° F) are shown in figures 1 and 2. The injury incurred upon cutting the tuber tissue stimulated the formation of phenolic substances in the slices. The ferric chloride spot test showed that these substances accumulate in cells adjacent to the wound. The data represented in figures 1 and 2 show that o-dihydricphenols, as indicated by curve #1, are the principal types of phenolic substances accumulating. Curve #2 shows the rate of accumulation of total phenols as measured by the Folin-Denis reagent. This reagent measures tyrosine as well as the o-dihydricphenols. Since chlorogenic acid

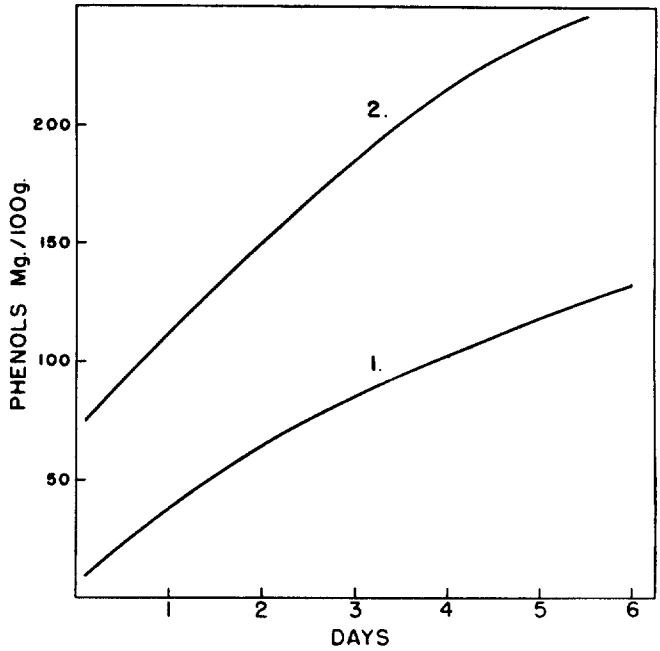

FIGURE 1.—Rate of accumulation of phenolic substances in Russet Burbank tuber slices, (1) Arnow reagent, (2) Folin-Denis reagent.

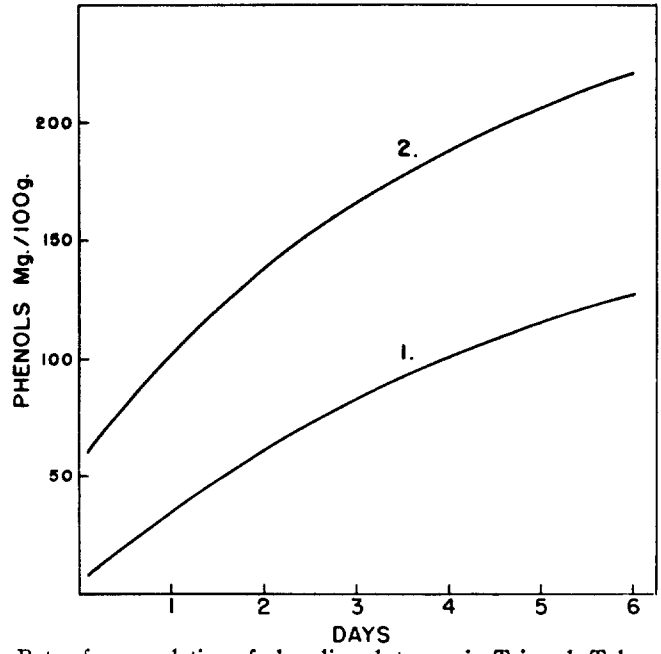

FIGURE 2.—Rate of accumulation of phenolic substances in Triumph Tuber slices, (1) Arnow reagent, (2) Folin-Denis reagent.

and tyrosine produce the same amount of color with the reagent, the difference between the amounts of phenolic substances determined by the two reagents is a fairly good measure of tyrosine. Tryptophan would also be included with tyrosine but since it gives much less color with the Folin-Denis reagent and is present in low concentration, the error caused by the presence of tryptopan would be small.

The fact that there was somewhat greater rate of accumulation of the phenolic substances measured by the Folin-Denis reagent would indicate some accumulation of tyrosine.

Since chlorogenic acid is the principal o-dihydricphenol in potato tubers, one would expect it to accumulate. Figure 3 represents two one-dimensional paper chromatograms of the concentrated extracts prepared from Russet Burbank slices held for 2, 4 and 6 days at room temperature (75° F). Chromatograms A and B which were sprayed with Arnow and Folin-Denis reagents, respectively, indicated that chlorogenic acid is the principal polyphenol accumulating. However, the two-dimensional chromatograms (Figure 4) of extract from Triumph and Russet Burbank slices showed not only the accumulation of chlorogenic acid but the accumulation of three other polyphenols, particularly the substance represented by spot 4. Spot 1 is chlorogenic acid. The nature of the polyphenols other than chlorogenic acid was not investigated except to determine that they were o-dihydricphenols.

Decreasing the temperature at which the slices were held greatly reduced the rate of accumulation of the polyphenolic substances. The comparative results for room temperature (75° F) and refrigerator temperature (35° F) are shown in figure 5. The lower rate at the lower temperature was apparently caused by the lower respiration rate. Other treatments which resulted in low rates of accumulation of phenolic substances in the tuber slices included (1) dipping of slices for two minutes in a 0.25 per cent resorcinol solution, (2) dipping slices in 0.03 per cent sodium bisulfite (500 ppm SO_2) — 1 per cent salt (NaCl) solution and (3) immersion of tubers in water (75° F) for 24 hours before slicing. The results of these treatments are shown in table 1.

The above treatments, and holding the slices at lower temperature, apparently have their effect by decreasing the rate of respiration. It would be logical to assume that respiration is necessary to furnish energy for the synthesis of the phenolic substances. Any treatment which decreases respiration rate would then affect the rate of accumulation of the phenolic substances.

Kennebec tubers showing pronounced net necrosis caused by aster yellows virus infection were found to give intense green color in the necrotic area upon testing with ferric chloride. This test indicated accumulations of orthodihyricphenols. The normal and diseased tubers were analyzed for total phenols (Folin-Denis) and o-dihydricphenols (Arnow reagent). The normal tuber contained 62 mg of total phenols and 4.9 mg of o-dihydricphenols per 100 grams. The diseased tuber contained 132 mg of total phenols per 100 grams and 52 milligrams of o-dihydricphenols per 100 grams. The concentrated extracts were also chromatographed. Although there was a definite increase in chlorogenic acid, the main increase was in the substance represented by spot No. 3, figure 4.

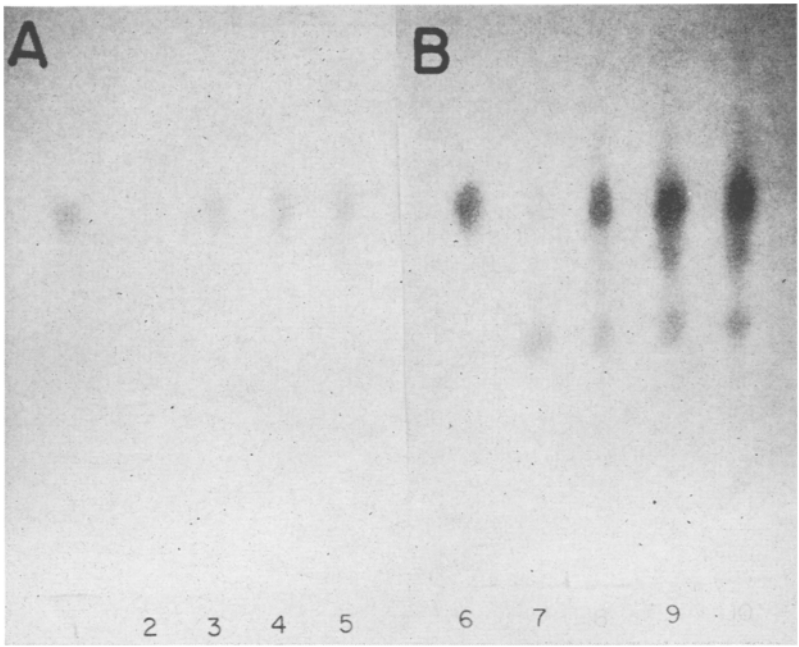

FIGURE 3.—Two one-dimensional ascending paper chromatograms of extracts from Russet Burbank tuber slices held at room temperature (75° F) 0, 2, 4 and 6 days. Chromatogram A sprayed with Arnow reagent and B sprayed with Folin-Denis reagent. Spots 1 and 6, chlorogenic acid; spots 2 and 7, 0 days; spots 3 and 8, 2 days; spots 4 and 9, 4 days; spots 5 and 10, 6 days.

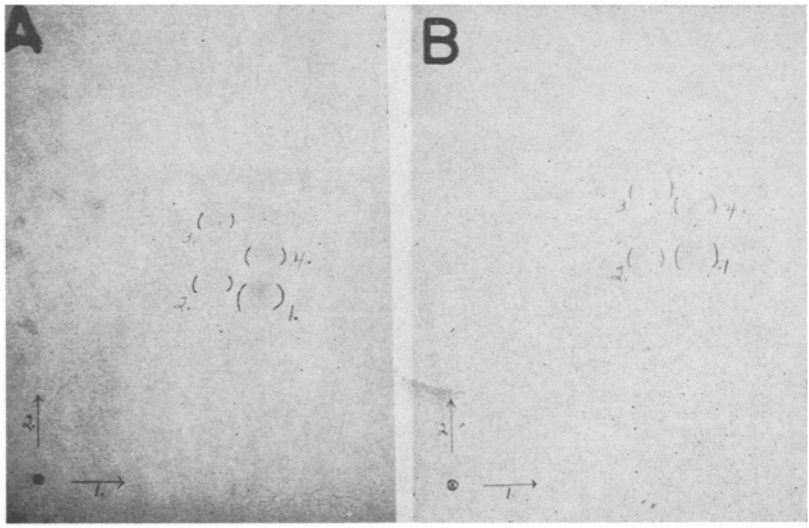

FIGURE 4.—Two dimensional paper chromatograms of extracts from tuber slices. A. Triumph, B. Russet Burbank, held for 4 days at room temperature (75° F). Chromatographed in (1) Butanol-acetic acid-water (40-10-20) and (2) 5 per cent acetic acid in water. Chromatograms were sprayed with Arnow reagent.

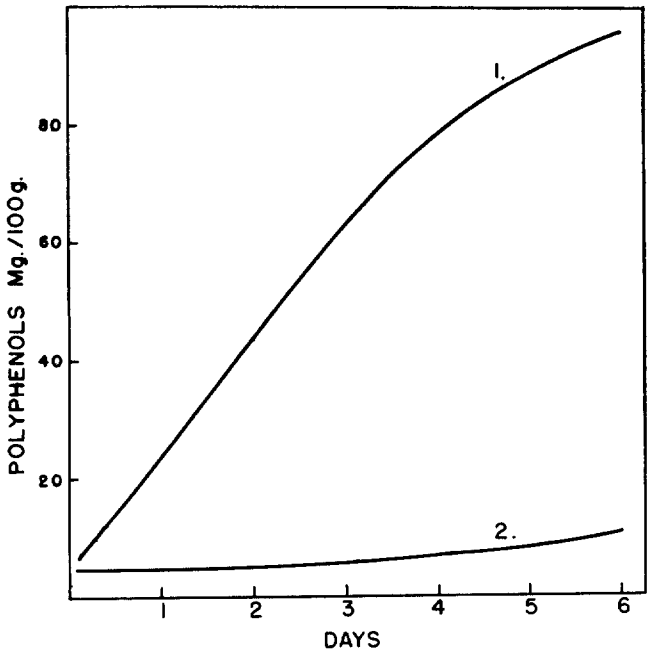

FIGURE 5.—Rate of accumulation of o-dihydricphenols in Russet Burbank tuber slices at (1) 75° F., (2) 35° F.

TABLE 1.—*Inhibitory effects of Resorcinol, Sodium Bisulfite and Immersion in water for 24 hours on the rate of accumulation of o-Dihydricphenols in Russet Burbank potato slices.*

Days Held at Room Temperature	Resorcinol		Sodium Bisulfite		Immersion in Water—24 Hours	
	Control mg/100 g	Treated mg/100 g	Control mg/100 g	Treated mg/100 g	Control mg/100 g	Treated mg/100 g
0	8.6	8.4	5.3	5.1	4.9	4.0
1					27.3	17.0
2	54.0	24.0	69.6	48.8	50.5	41.4
3						
4			118.0	85.5		
6						

which is a chromatogram of Triumph and Burbank extracts. Only chlorogenic acid could be detected in the chromatogram of the extract from the normal Kennebec tuber and that only by fluorescence of the spot under ultraviolet light. The results obtained showed that the same phenolic substances accumulate upon pathological as upon mechanical injury to the tuber by cutting, although the relative amounts of each substance

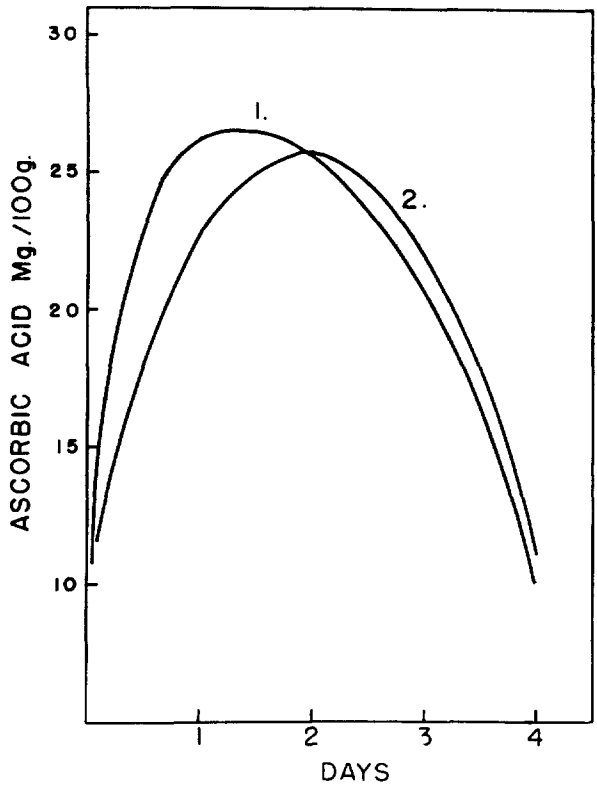

FIGURE 6.—Rate of accumulation of ascorbic acid in (1) Triumph, (2) Russet Burbank tuber slices.

may differ. In Russet Burbank and Bliss Triumph tubers infected with aster yellows, spot No. 4 appeared to accumulate more than spot No. 3. It should be emphasized that phenolic substances other than chlorogenic acid can accumulate in injured tissue.

Kuc, *et al,* (4) reported greater increases in chlorogenic acid and caffeic acid in potato slices inoculated with spore suspension of *Helminthosporium carbonum* than in slices not inoculated but incubated for some length of time at 22° C for 72 hours. Unlike chlorogenic acid, caffeic acid does not accumulate in cells adjacent to the cut surface. The increase in caffeic acid in tuber slices upon inoculation with *H. carbonum* as reported by Kuc (4) might be explained by the fact that it was probably formed by the enzymatic hydrolysis of chlorogenic acid by an esterase secreted by the organism.

RELATION OF ACCUMULATION OF PHENOLIC SUBSTANCES TO DISEASE RESISTANCE

Since there appears to be very little difference between the rate of accumulation of phenolic substances upon injury in the scab-susceptible Triumph and the scab-resistant Russet Burbank varieties, indications are

that this phenomenon is not so important as the relative amounts of chlorogenic acid present in the periderm. However, the accumulation of polyphenols adjacent to the injury may be very important from the standpoint of other types of resistance as indicated by the work of Uritani (10) and Kuc, *et al* (4).

Uritani postulated that the accumulation of chlorogenic acid, caffeic acid, methyl caffeate, umbelliferone, scopoletin, ascorbic acid and ipomeamarone in sound sweet potato tissue adjacent to that rotted by *Ceratostomella fimbriata* was associated with resistance of sweet potato to the attack by that organism. Uritani also showed that respiratory enzymes of the above organism were inactivated during the oxidation of sweet potato polyphenols.

Since there is evidence that the accumulation of phenolic substances is involved in disease resistance, then any predisposing factor which would reduce the rate of synthesis of phenolic substances upon injury by reducing the rate of respiration would then increase the susceptibility of the tuber to certain diseases. Apparently suffocation such as that obtained upon immersion of tubers in water for 24 hours or longer can be a factor which could influence the rate of accumulation of phenolic substances in tubers and therefore make it more vulnerable to attack by certain organisms. Livingston (5) studying mechanisms of action of various predisposing agents on the bacterial soft rot susceptibility of potato tubers has shown that immersion of potato tubers in water by long periods will irreversibly interfere with the natural soft rot resistance mechanism of the tuber.

It is becoming more evident that phenolic compounds can be associated with plant disease resistance and immunity in at least three ways. (1) They can be present in the plant tissue prior to infection. Recently Barnes and Gerber (2) attributed the remarkable resistance to decay of osage orange wood to the presence of 2,3', 4,5' tetrahydroxystilbene. (2) They can be present in the plant tissue prior to infection but oxidation to quinones being necessary for inhibiting growth of the organism and/or stimulating suberization as in the case of scab resistance of potatoes. (3) They can accumulate in sound tissue adjacent to a mechanical or disease induced injury. In the case of the latter this could be an expression of a resistance mechanism on the part of the host in a host-parasite interaction. The oxidation state may be specific or non-specific for the disease organism.

Accumulation of Ascorbic Acid

Ascorbic acid analysis of the ethanol extract was made to correct the error due to ascorbic acid in the analysis for total phenols by the Folin-Denis reagent. The results obtained indicated that ascorbic acid accumulated in the sound part of the tuber tissue, although it is actually oxidized in the damaged cells on the cut surface. The rate of accumulation of ascorbic acid was followed and the results are shown in figure 6. There was a rapid increase in ascorbic acid for the first two days. This increase was followed by a rapid decrease. Just why ascorbic acid accumulates is not known. It might possibly protect the sound tissue from quinones diffusing from the injured cells.

Upon the rapid decrease of the accumulated ascorbic acid, there is good reason to believe that dehydroascorbic acid and other oxidative break-down products of ascorbic acid will accumulate. No attempt was

made to determine these substances. However, autoxidized ascorbic acid was shown by Myrvik and Volk (7) to have certain antibacterial properties. They performed short term growth experiments which indicate that the enediol group had no antibacterial properties but that the oxidized enediol (diketone) produced immediate bacteriostatis. Their work indicates that if oxidized products of ascorbic acid can accumulate in injured plant tissue, there is a definite possibility that these substances can function in the resistance of plants to certain diseases.

SUMMARY

Chlorogenic acid and other o-dihydricphenols were found to accumulate rapidly in the area adjacent to the cut surface of potato tuber slices held at room temperature in a moist chamber.

The rate of accumulation was found to be decreased by: (1) holding the slices at lower temperature; (2) dipping the slices in resorcinol solution; (3) dipping slices in sodium bisulfite-sodium chloride solution; (4) immersion of whole tubers in water (room temperature) for 24 hours prior to slicing.

The same phenolic substances which accumulated in tuber slices were found to accumulate in necrotic areas of tubers infected with aster yellows.

Ascorbic acid was found to accumulate in tuber slices but rapidly decreased after the second day of holding at room temperature.

Several ways in which phenolic substances can function in the mechanism of disease resistance are discussed.

The possibility that the accumulation of oxidized products of ascorbic acid adjacent to infected tissue may play a role in disease resistance is also discussed.

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