43° C and the hole was sealed with beeswax. When placed in the dye solution 90 % of these seeds took up liquid through the strophiole. In the remaining 10 % the liquid entered through the seed coat generally. In no case was it primarily through the hilum. Examination of these seeds after the preliminary soaking revealed cracks in the strophiole of most of the seeds. This observation is in accord with that of Kuhn (5). Of the thousands of seeds observed, only 1 seed not treated as above had liquid entry through the strophiole.

Summary

Examination of the path of liquid entry into impermeable seeds of *Lupinus angustifolius* (a hard-seeded sweet blue strain) after they were scarified in sulfuric acid indicates that entry occurs through either the hilar fissure or pits eroded through the testa. The impermeable layer of the testa is located at the outer edge of the palisade epidermis. Entry was permitted through the hilar fissure due to partial hydrolysis of the counter palisades which normally cause the hilum to close when the moisture outside the seed exceeds that on the inside.

The only time that the strophiole is important in uptake of liquid is after the seeds have taken up water and been redried. This process causes a cleft to be formed in the strophiole.

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SOME FACTORS WHICH AFFECT THE SYNTHESIS OF CHLOROGENIC ACID IN DISKS OF POTATO TUBER[1, 2]

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The work of Neish and his colleagues (9, 10) on the synthesis of aromatic compounds in buckwheat indicates that shikimic acid is a precursor to phenolic constituents such as caffeic acid. Chlorogenic acid, a phenol present in a wide variety of plants, is an ester of caffeic acid and quinic acid, the latter being quite similar to shikimic acid in structure. Unfortunately little is known of the mechanism of biosynthesis of this interesting phenol. The recent observations of Johnson and Schaal (3, 4) and of Kúc, et al (6) have shown that, in contrast to the outer periderm, the pulp of the potato tuber is almost devoid of chlorogenic acid. Kúc (5) has further shown that disks of pulp tissue, nevertheless, are capable of producing this compound. Consequently, tissue from the inner core of the potato tuber appears to offer a relatively simple system for study of the pathways by which chlorogenic acid is synthesized.

The purpose of the work reported in this paper, then, is to investigate the effects of environmental factors on the synthesis of chlorogenic acid. Data are also given which offer an explanation for the difference in distribution of this substance between periderm and pulp.

Materials and Methods

Chlorogenic acid synthesis was studied in disks, 12.5 mm in diameter and 1 mm thick, sliced with a hand microtome from cylindrical plugs cut out of Kennebec potato tubers. The tubers were kept at 7° C until used. Disks were sliced from the inner part of the tuber only, the outer 5 to 8 mm of tissue and skin being discarded. Each experimental treatment consisted of a sample of 10 disks (weighing about 1.5 g) which were withdrawn at random from the washed slices, blotted lightly, and placed in 5 ml of the appropriate solution contained in a 9 cm Petri dish. The covered dish was held at room temperature (23 to 25° C), in the dark, until the disks were ready for assay. All culture solutions contained 25 ppm of Neomycin sulfate to control bacterial contamination. This addition had no effect on synthesis of chlorogenic acid. The data are presented as the total chlorogenic acid content of each sample. Treatments were run in

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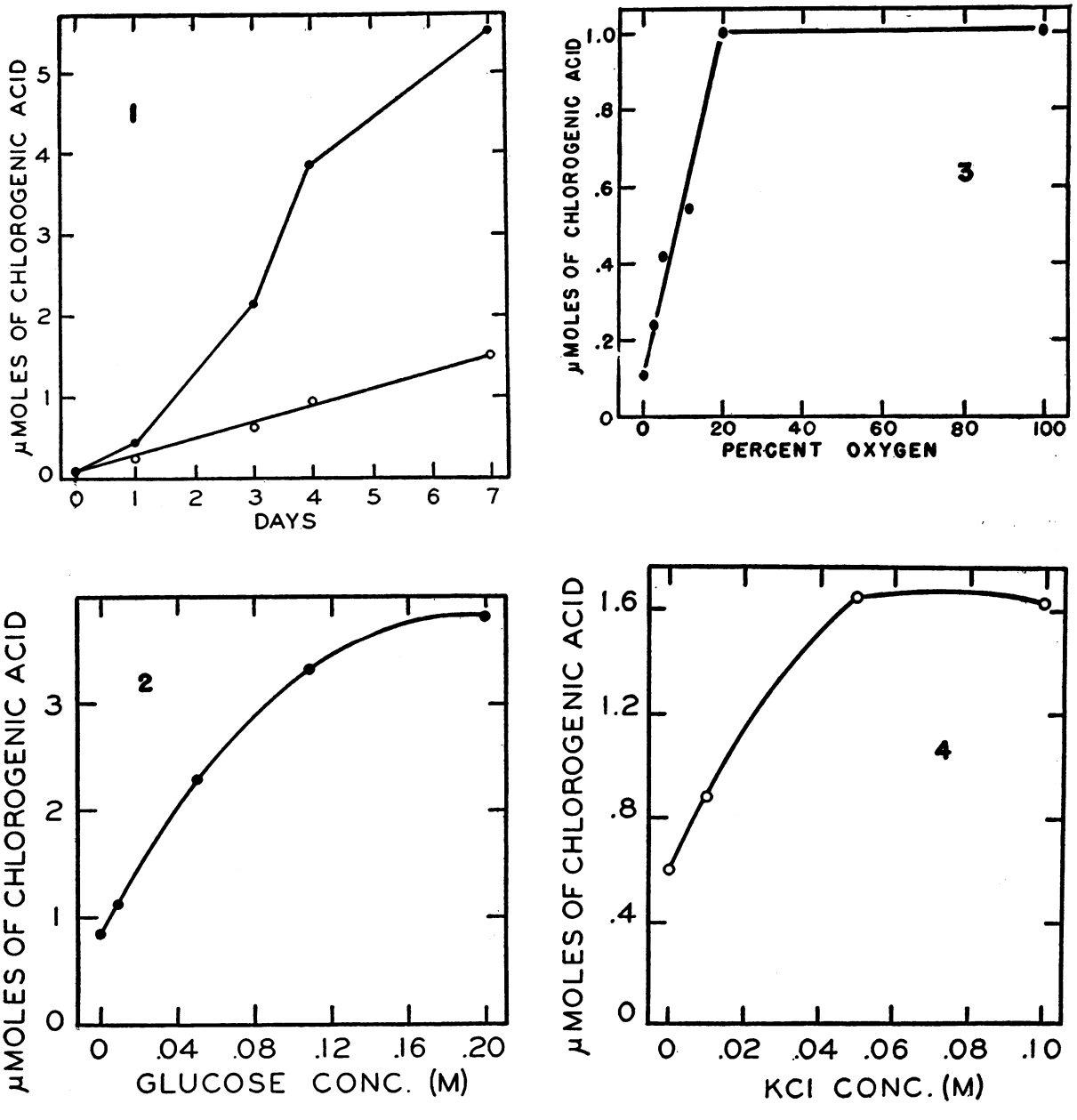

FIG. 1. Time course of synthesis of chlorogenic acid in potato tuber discs. ●, culture solution 0.1 M glucose and 25 ppm of Neomycin sulfate. ○, distilled water containing 25 ppm of Neomycin sulfate.

FIG. 2. The effect of different concentrations of glucose on synthesis of chlorogenic acid in potato tuber disks. Determinations made at end of 72 hours. All culture solutions contained 25 ppm of Neomycin sulfate.

FIG. 3. Effect of oxygen pressure on synthesis of chlorogenic acid. Disks on moist filter paper in sealed flasks were assayed after 72 hours of culture. Each flask was flushed daily with the appropriate mixture of air and oxygen or nitrogen.

FIG. 4. The effect of different KCl concentrations on synthesis of chlorogenic acid in potato tuber disks at the end of 72 hours. All culture solutions also contained 25 ppm of Neomycin sulfate.

duplicate, and the difference between duplicate samples was usually less than 10 % of the mean.

Chlorogenic acid was extracted by grinding the disks in 15 ml of 95 % ethanol with a Ten Broeck glass homogenizer. The suspension in a 25 ml volumetric flask was brought to volume with the ethanol washings of the homogenizer and filtered. Either 2.5 or 5 ml of the clear, filtered extract were placed on an alumina column and assayed for chlorogenic acid by the modified Hoepfner nitrous acid reaction as previously described (11).

The absorption spectrum of the initial alcoholic filtrate from cultured disks was similar to that of chlorogenic acid, indictating that this substance is a major ultra-violet absorbing component of the extract. Ascending paper chromatograms of evaporated alcoholic extracts verified this observation. These chromatograms were run on Whatman no. 1 paper with acetic acid : HCl : water (6 : 1 : 3), 1-butanol : acetic acid : water (6 : 2 : 2), the organic phase of 1-butanol : 2 *N* HCl (1 : 1), and the aqueous phase of *n*-butyl acetate : acetic acid : water (5 : 10 : 75) as solvents. In all solvents, the most intensely fluorescent band had an R_f the same as that of authentic chlorogenic acid. As many as 5 fluorescent bands appeared with the butanol : HCl and butanol : acetic acid solvents. All gave a positive Folin phenol test (1). As anticipated, the eluate of the band the R_f of which was the same as that of chlorogenic acid also had the absorption spectrum of this substance. When chromatograms containing all 5 bands were sprayed with the Hoepfner reagents (2, 7), the band believed to be chlorogenic acid turned bright yellow while several of the other bands became orange. All of these bands turned red with alkali. To determine how much of the material reactive in the chlorogenic acid assay was actually located in the chlorogenic acid band, a butanol : HCl chromatogram was cut into strips, and the eluates of the strips were assayed for chlorogenic acid. About 80 % of the reactive material came from the chlorogenic acid band. Consequently, a correction factor based on this figure was used to calculate the number of micromoles of chlorogenic acid present. Interestingly enough, all treatments which gave rise to a synthesis of chlorogenic acid also appeared to stimulate the production of the other phenolic substances.

Results

In confirmation of previous work (3, 6), disks removed from the inner tissue of the potato tuber contained almost no chlorogenic acid, i.e., less than 0.2 micromoles per gram of fresh weight. However, as shown in figure 1, they began to synthesize chlorogenic acid when immersed in a shallow layer of water, and maintained a steady rate of synthesis for at least 7 days. It can also be seen that glucose markedly affected the synthesis of chlorogenic acid stimulating the rate 3- to 4-fold. The optimal concentration of glucose for synthesis was approximately 0.1 M (fig 2). Sucrose had a similar stimulatory effect.

The low concentration of chlorogenic acid in freshly cut disks suggested that some factor necessary for synthesis was limiting or was absent within the tuber. The cold temperature of storage (7° C) was not responsible for the lack of synthesis since tubers taken from storage and placed at room temperature showed no increase in chlorogenic acid concentration.

In order to test the possibility that a diminished availability of oxygen to the tissue prevented synthesis, disks were placed in different depths of culture solution. Table I shows that limiting the oxygen supply by such treatment had a marked effect on the synthesis of chlorogenic acid. Completely submerged disks synthesized no chlorogenic acid whatsoever. Even the thin film of solution covering the disks under standard conditions (5 ml of solution) decreased the amount of chlorogenic acid formed when comparisons were made with disks completely exposed to the atmosphere. The lack of oxygen in the totally submerged disks was evidenced by the fact that these disks remained white, whereas the surface of disks from the other treatments turned light brown.

Table I

Effect of the Depth of the Culture Fluid on Synthesis of Chlorogenic Acid

Depth of culture fluid	μM chlorogenic acid
Initial content	0.15
8 to 10 mm (disks completely submerged)	0.14
0.8 to 1 mm (disks covered by a thin film of water; standard condition)	0.63
0 (disks not submerged)	1.14

Completely submerged disks were bathed in 50 ml of distilled water (containing 25 ppm Neomycin). Disks not submerged were placed on filter paper moistened with 2 ml of solution.

Samples were assayed after 72 hours of culture.

The importance of an adequate supply of oxygen for the synthesis of chlorogenic acid was demonstrated directly by varying the partial pressure of oxygen to which the disks were exposed. Figure 3 indicates that maximal synthesis was not obtained until a concentration of 20 % oxygen was reached.

Further evidence that lack of oxygen limited synthesis in tuber tissue was provided by an experiment in which the thickness of disks was changed. Table II

Table II

Effect of Disk Thickness on Synthesis of Chlorogenic Acid

Thickness of disks mm	μM chlorogenic acid
0.5	0.92
1.0	0.73
2.0	0.63

Samples of 20 disks 0.5 mm thick, 10 disks 1.0 mm thick, and 5 disks 2.0 mm thick were assayed after 72 hours of culture in distilled water containing Neomycin.

shows that increasing the thickness from 0.5 mm to 2 mm decreased the amount of chlorogenic acid synthesized per unit weight by one third. This result indicates that diffusion of oxygen through 2 mm of tissue is sufficiently hindered to curtail chlorogenic acid synthesis. The amount of chlorogenic acid produced by the standard 1 mm disks was intermediate between that of the two extremes.

In these experiments equal weights of tissue were assayed. That is, 5 disks each 2 mm thick were used in the one treatment while 20 disks 0.5 mm thick were used in the other. Consequently, the area of wound surface present in one sample was 4 times that of the other. Since no such corresponding increase in synthesis of chlorogenic acid occurred, wound reactions or "wound respiration" is apparently not involved.

Potassium chloride is known to stimulate utilization of sugar and respiration in potato disks (8). Figure 4 shows that KCl also increases production of chlorogenic acid. A maximal effect was obtained with 0.05 M KCl. At plasmolytic concentrations above 0.1 M, the tissue became brown and the chlorogenic acid content decreased just as in disks treated with high concentrations of glucose. The maximal KCl stimulation, although varying from experiment to experiment, was always considerably less than the corresponding stimulation of chlorogenic acid synthesis by glucose. Addition of KCl to an optimal concentration of glucose (0.1 M and above) produced no further stimulation of synthesis, but rather inhibited the production of chlorogenic acid (table III). Only

TABLE III

EFFECT OF GLUCOSE AND KCl ON SYNTHESIS OF CHLOROGENIC ACID

GLUCOSE CONC (M)	μM CHLOROGENIC ACID −KCl	μM CHLOROGENIC ACID +KCl*
0	0.86	2.03
1×10^{-2}	1.12	2.24
5×10^{-2}	2.29	2.40
1×10^{-1}	3.33	3.14
2×10^{-1}	3.82	2.16

All samples were assayed after 72 hours of culture. The initial chlorogenic acid content was .16 μM per sample (see fig 2 also).

* The KCl concentration was 0.05 M.

when glucose concentrations were sub-optimal did KCl enhance the glucose effect.

The salt effect was not limited to KCl alone (see table IV). The lack of stimulation by potassium phosphate was unexpected but reproducible, and no explanation can be offered at present. The small effect of NH_4Cl is in part attributable to the fact that disks in this salt, although they remained turgid, were noticeably darker brown than those exposed to any other treatment. This suggests that some of the chlorogenic acid synthesized had been oxidized to the brown pigments observed.

TABLE IV

EFFECT OF SALTS ON SYNTHESIS OF CHLOROGENIC ACID

SALT*	μM CHLOROGENIC ACID
Water	0.70
Potassium chloride	1.83
Potassium nitrate	1.82
Potassium phosphate buffer, pH 6.8	0.70
Calcium chloride	1.49
Ammonium chloride	1.04

All samples were assayed after 72 hours of culture. The initial chlorogenic acid content was 0.15 μM per sample.

* Each salt was present at a concentration of 0.05 M.

Because of the inter-relationships mentioned above (see Introduction) between shikimic acid and quinic and caffeic acids, these compounds were tested for their ability to stimulate synthesis of chlorogenic acid. Table V shows that at half the concentration, quinic acid was as effective as glucose, while shikimic acid produced only a slight stimulation. Caffeic acid proved to be much too toxic to test in this manner.

TABLE V

EFFECT OF QUINIC AND SHIKIMIC ACIDS ON SYNTHESIS OF CHLOROGENIC ACID

CULTURE SOLUTION	μM CHLOROGENIC ACID
Water	0.74
0.1 M glucose	2.02
0.05 M potassium quinate, pH 4.5	1.98
0.05 M " shikimate, "	0.98

Samples were assayed after 72 hours of culture. The initial chlorogenic acid content was 0.16 μM per sample.

DISCUSSION

The present data indicate that synthesis of chlorogenic acid requires a relatively high oxygen tension. The sensitivity to the supply of oxygen may explain why the inner tissue contains little chlorogenic acid even though this phenol can be produced from endogenous substrates. Indeed, the availability of oxygen can account for the biochemical differentiation of the potato tuber, at least in respect to the production of chlorogenic acid. The slow rate of diffusion of oxygen through the skin into the potato establishes an environmental gradient which limits synthesis to the outer layer of tissue.

Both glucose and quinic acid stimulate the synthesis of chlorogenic acid markedly, whereas shikimic acid appears to have no effect other than a salt stimulation. If glucose and quinic acid were only acting indirectly by stimulating general metabolic processes, then shikimic acid, which resembles quinic acid closely in structure, might be expected to produce similar

effects. Since this has not been observed, the data suggest that glucose and quinic acid exert their effects by giving rise to precursors of chlorogenic acid.

Summary

Chlorogenic acid formation has been shown to occur in disks of potato tuber tissue which are initially almost devoid of this compound. Glucose, quinic acid, and, to a lesser extent, salts such as KCl stimulate the synthesis of chlorogenic acid. Availability of oxygen is an important factor in the synthesis of the acid since, under reduced oxygen concentrations, little or no formation of chlorogenic acid could be detected. The sensitivity to oxygen may well explain the presence of chlorogenic acid in the outer few mm of the potato tuber in contrast to its absence in the pulp.

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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DINITROPHENOL ON THE UPTAKE AND METABOLISM OF EXOGENOUS SUBSTRATES BY CORN ROOTS [1, 2, 3]

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There are many similarities between 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dinitrophenol (DNP) in their action on plant metabolism. Both of these compounds may inhibit salt accumulation (10, 12), increase respiration (1, 6, 8, 13), inhibit growth (3, 6) and uncouple oxidative phosphorylation in mitochondrial preparations (5, 14). Whether these similarities are superficial or whether they result from a similar mode of action of the two compounds is still an open question (4).

This paper reports the results of an investigation on the effect of DNP on the uptake and metabolism of exogenous substrates by root tips from 2,4-D-treated and buffer-treated corn seedlings. It is concluded that, although both 2,4-D and DNP promote catabolism, they differ in their effect on uptake of the substrates by the root tips.

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Materials and Methods

Corn seedlings (*Zea mays* L., var. Funks G-50) approximately 60-hours-old were used as plant material. The seedlings were placed with their roots in 10^{-2} M potassium phosphate buffer, pH 5.3, with or without 10^{-3} M 2,4-D. During the 12 hour treatment period the seedlings were placed in the dark at 21 to 22° C. After treatment, root tips (2 cm long) were cut from the seedlings and placed in Warburg vessels with 10^{-2} M potassium phosphate buffer, pH 5.3. Each vessel contained 7 root tips (about 180 mg fresh weight). To measure $C^{14}O_2$ production, 0.1 ml of C^{14}-substrate solution was added to each vessel. The vessels were shaken in the dark for 3.5 hours at 25° C, and the $C^{14}O_2$ released in respiration was collected in the KOH of the center well. Detailed experimental procedure is given in a previous communication (7).

For the measurement of substrate uptake, groups