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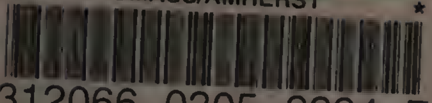
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ABSORPTION OF WATER SOLUBLE COMPOUNDS INTO LEAVES OF MCINTOSH APPLE (*MALUS DOMESTICA*, BORK.)

By

William G. Lord

A THESIS

Submitted to
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ABSORPTION OF WATER SOLUBLE COMPOUNDS INTO LEAVES OF MCINTOSH APPLE (*MALUS DOMESTICA*, BORK.)

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LITERATURE REVIEW

The cuticle is a continuous lipoidal "membrane" covering all living cells of the plant exposed to the air (van Overbeek, 1956). It is generally considered the most important factor limiting the foliar penetration of exogenously supplied substances (Hull, 1964). The main structural component of the cuticle is the cutin matrix, composed of long chain fatty acids and alcohols polymerized by ester linkages (Crafts and Foy, 1962; Matic, 1956; Roelofsen and Houwink, 1951). The cutin matrix is separated from the cellulosic cell wall beneath by an ill-defined layer of pectic substances (Norris and Bukovac, 1968) which extends into the cutin matrix (Roberts, Southwick, and Palmiter, 1948). Within the cutin itself are areas of highly oriented embedded waxes (Martin and Juniper, 1970; Norris and Bukovac, 1968). A layer of waxes cover the outermost surface of the cuticle. These waxes vary greatly from species to species in chemical composition, physical form, thickness, and continuity (Eglington and Hamilton, 1967).

The cuticle is generally thicker on the adaxial surface than on the abaxial surface (Lee and Priestly, 1924) although in some plants such as pear, the opposite is true (Norris and Bukovac, 1968). Older leaves generally have thicker cuticles, yet, this feature is not necessarily associated with decreased

foliar penetration (Schieferston and Loomis, 1959). Cuticle thickness may be affected by a variety of environmental factors. Low humidity and high wind speeds can cause development of thicker, waxier cuticles (Martin and Juniper, 1970). Maximum cuticle development may be dependent upon the ultraviolet portion of sunlight which is filtered out by glass (Hull, 1958).

Cuticle Composition

Pectin

The pectic substances are highly hydrophilic constituents of the cuticle which may be important in the foliar penetration of water-soluble compounds (Roberts *et al*., 1948). These pectins occur as a visibly indistinct band separating the cell wall from the cuticle. Some pectin intrusion into the cuticle of leaves of *Pyrus communis* has been found (Norris and Bukovac, 1968). Roberts *et al*. (1948) reported the presence of a continuous path of pectic substances from the cell wall through the cuticle to the surface of the McIntosh apple leaf.

Pectins are basically composed of long chain polygalacturonic acid molecules with side carboxyl groups (Foy, 1964). Glucuronic acid and arabinose may also be present (Muhlethaler, 1961). Pectins are hydrophilic due to the presence of free carboxyl groups. Pectins appear structureless under the electron microscope indicating that the

constituent molecules of pectin probably occur at random (Muhlethaler, 1961).

Cutin

A cutin matrix forms the basic structural component of the cuticle. Its formation is controlled in part by the enzyme lipoxidase which catalyzes the oxidation of fatty acids such as linolenic and linoleic to hydroperoxides (Linskins, Heinen, and Stoffers, 1965; Siddiqi and Tappel, 1956). These migrate through the cell wall to the cell wall-cutin interface and polymerize through ester, peroxide, and ether linkages (Muhlethaler, 1961). Cutin is deposited continuously until the leaf is fully expanded (Skoss, 1956). Hydrolysis of cutin yields a mixture of saturated aliphatic monocarboxylic, hydroxy-monocarboxylic, dicarboxylic, and hydroxy-dicarboxylic acids of chain length C_{14} through C_{22} (Baker, Batt, Silva Fernandes, and Martin, 1964), although C_{16} and C_{18} chain lengths predominate (Martin and Juniper, 1970). Hydroxy-octadecanoic and hexadecanoic acids containing 1 to 3 hydroxyl groups are the primary cutin constituents in *Agave americana* (Matic, 1956) and *Malus* (Baker and Martin, 1963). The hydroxylated fatty acids make up over 80% of the cutin complex (Baker, Batt, and Martin, 1964). Inner-esterification of these fatty and hydroxy-fatty acids produces the cross-linked polyester, cutin (Matic, 1956). Frey-Wyssling (1948) states that the lipophilic groups are

oriented towards the surface of the leaf whereas the hydrophilic groups are oriented towards the cell wall. The lipophilic nature of the cutin imparts some water-repellency to the cuticle whereas the hydrophilic nature of some groups allows the cuticle to absorb water and swell (Frey-Wyssling, 1948). This swelling permits transpiration and allows the absorption of water soluble substances (Hull, 1970). Cutin has a pK at about pH 5. At a pH below 5 the acids are undissociated and the cutin tends to attract anions. At a pH above 5, the dissociated acids tend to repel anion (Hull, 1964).

Cutin poses no real barrier to the penetration of water soluble compounds (Juniper and Martin, 1970). However, the presence of a wax layer on the surface of the cutin and waxes embedded in the cutin can restrict penetration (Skoss, 1955).

Waxes

The nature of the surface and embedded waxes may contribute greatly to inhibiting foliar penetration. The thin surface layer of waxes is generally continuous and is responsible for much of the water-repellent nature of the cuticle (Hull, 1964). The waxes embedded in the cutin occur in the form of flat platelets arranged parallel to the surface (van Overbeek, 1956). Embedded waxes may decrease the amount of cuticular transpiration (Muellar, Carr and Loomis, 1954; Schieferstein and Loomis, 1956, 1959). These

subsurface wax deposits may also be important in restricting the penetration of water-soluble compounds (Martin and Juniper, 1970) although Bukovac and Norris (1967) have shown little effect on sorption of NAA into isolated cuticle when embedded waxes were removed.

An important factor in foliar penetration is the structure of the surface layer of waxes. Surfaces may either be rough due to projecting rods or leaf-like platelets or smooth because of flat platelets (Muellar, Carr, and Loomis, 1954; Schieferstein and Loomis, 1956; Silva Fernandes, 1965). This physical configuration governs the degree of contact of a spray droplet with the cuticle proper (Silva Fernandes, 1965). These wax projections may cause many droplets to dry out without ever coming in contact with the cuticle proper (Holly, 1964). Removing this surface wax by brushing increases wettability and cuticular transpiration (Hall and Jones, 1961). Brushing peach leaves allowed increased absorption of 3-chlorophenoxy- -propionic acid, presumable due to this effect on surface waxes (Bukovac, 1965). Chemical removal of surface waxes netted similar results with NAA absorption (Bukovac and Norris, 1967).

Chemical composition of the surface waxes may influence the extent of foliar penetration. The waxes are a heterogeneous group including: long chain hydrocarbons (alkanes and alkenes), alcohols, ketones, fatty and hydroxy-fatty

acids, and esters (Martin and Juniper, 1970). Odd numbered n-paraffins (n-alkanes) and n-primary alcohols are the major constituents (Chibnall et al., 1934; Martin and Juniper, 1970; Waldron et al., 1961). Secondary alcohols, diols, ketols, aldehydes, and estolides are minor wax constituents whose presence is species dependent (Eglington et al., 1962; Eglington and Hamilton, 1967; Murray and Schoenfeld, 1955a, 1955b). Phenolic substances migrate to the leaf surface and mingle with the waxy covering (Richmond and Martin, 1959). Triterpenoid compounds such as ursolic acid have been demonstrated in apple leaf cuticle (Silva Fernandes, 1965b). Surface wax constituents may influence wettability of the leaf surface. Ursolic acid, paraffins, and ketones make wetting more difficult and perhaps partially dictate the surface structure (Silva Fernandes, 1965b). A high percentage of esters and low paraffin content were associated with easily wetted surfaces (Silvar Fernandes, 1965b). Work by Silva Fernandes, Baker, and Martin (1964) showed differences in ursolic acid, paraffins, and esters between ventral and dorsal surfaces of apple leaves which could account for the differences in wettability.

Richmond and Martin (1959) quantitatively studied the formation of cuticles in apple leaves and found that the waxy materials remained fairly constant at 20 ug/cm^2. A slight lag in wax development occurred during rapid leaf expansion

suggesting an inability of wax production to keep up with leaf expansion. Bystrom *et al.* (1968) and Martin (1960) noted a continual extrusion and deposition ceased when leaf expansion was completed.

The mechanisms by which wax or wax precursors migrate to the leaf surface are not clear. Schieferstein and Loomis (1956, 1969) could find no evidence for wax canals and concluded that wax was extruded at random through the newly formed cuticle of young leaves. Wax canals could not be demonstrated in either *Eragrostis curvula* (Leigh and Matthews, 1963) or several other plant species (O'Brian, 1967).

There is, however, evidence that such canals do exist in certain plants. Hall and Donaldson (1962) observed that in *Trifolium repens* L. there was a pore present beneath each particle of surface wax. Where there were no pores there were no particles, and vice versa; hence, these pores were considered the source of the secreted waxes. These workers also observed pores associated with wax particles in *Brassica oleracea* L. and *Poa colensoi* Hook f.. Hundreds of small pores were associated with each wax particle in *Brassica*. A modified freeze-etch technique has been used to provide further evidence for the existence of pores (Hall, 1970b). The possibility exists that such pores could be pathways of penetration of non-polar compounds (Linskens *et al.*, 1965).

Cellulose

Another constituent of the cuticle which may be of importance is cellulose, a B 1,4-glucose polymer. Cellulose is arranged in micelles, 10 Å apart, or enough to allow penetration of water and halogens (Frey-Wyssling, 1948). Micelles are further arranged into microfibrils, spaced about 100 Å apart, or large enough to allow penetration of larger organic molecules (Frey-Wyssling, 1948). Recent work by Franke (1971) suggests that ectodesmata (non-cytoplasmic extensions through the cell wall believed important in foliar penetration) are groups of cellulose microfibrils surrounded by pockets of reducing substances.

Portals of Entry

The cuticle is generally considered the major barrier to foliar penetration. However, numerous pesticides, plant growth regulators, and nutrients are foliar applied and do penetrate the leaf. Various preferential pathways of penetration into the leaf have been suggested: areas over anticlinal walls, leaf hairs, areas over veins, guard cells, and stomata.

Anticlinal walls

Chemicals are absorbed preferentially over anticlinal cell walls (Dybing and Currier, 1961), even though the cuticle may be thicker in this region (Baker and Martin, 1963; Norris and Bukovac, 1968). Eglington and Hamilton (1967) demonstrated

more surface wax present over periclinal than over anticlinal walls. Presumably the extrusion of waxes from the cell to the surface is easier over the periclinal walls. Bystrom *et al.* (1968) showed the surface over anticlinal walls in *Beta vulgaris* leaves to be nearly devoid of wax. Norris and Bukovac (1968) reported a decrease in the birefringency of the cuticle over anticlinal walls, suggesting that lesser amounts of embedded waxes were present or that orientation is less regular. Both conditions would facilitate the penetration of water soluble compounds. Much more intrusion of pectic substances into the cutin matrix has been shown over anticlinal than periclinal walls (Norris and Bukovac, 1968). Increased amounts of pectins would make the cuticle more hydrophilic in that area. Franke (1964a) demonstrated the presence of increased numbers of ectodesmata over the anticlinal walls. This may in part explain the preferential absorption of water soluble compounds there.

Leaf hairs

The physical and structural composition of trichomes may provide the shortest pathway of solute entry. Persistent leaf hairs are protoplasmic structures (Esau, 1953) having a thinner cuticle with fewer waxes (Bystrom *et al.*, 1968). Trichomes rise above the surface of the leaf, and hence, above the hydrophobic surface waxes. Basically, then, they appear well suited as sites of foliar penetration. Hull (1964)

using fluorescent dyes has shown preferential absorption into hairs occurred when no surfactant was used whereas, there was a total lack of cuticular and stomatal penetration (Dybing and Currier, 1959). Visible absorption of dyes into trichomes occurred within 20 seconds (Hull, 1964).

Many plant species such as *Pistia* and *Salvinia* have rod-like hydrophobic hairs. These hold the droplet above the surface in much the same way as crystalline wax masses do (Martin and Juniper, 1970). The trapping of air between these projections and the failure of small droplets of sprayed solutions to displace this air may cause high contact angles and lower wettability (Martin and Juniper, 1970).

Veins

The preferential absorption of a wide range of compounds over veins has been reported by Dybing and Currier (1961). Hull (1964), and van Overbeek (1956). Several anatomical distinctions may account for this preferential absorption. The cells present under the epidermis over veinal areas are the bundle sheath cells (van Overbeek, 1956) which are thin wall parenchyma cells surrounding veins (Esau, 1953). The cuticle over veins in pear leaves is actually thicker than the cuticle over non-veinal areas. However, the birefringent layer is interrupted and less intense, suggesting a discontinuous and less oriented layer of embedded waxes (Norris and Bukovac, 1968). Franke (1964a) demonstrated increased numbers

of ectodesmata arranged in rows above veinal areas. Roberts *et al*. (1948) showed the vertical extension of pectic layers through the cutin lamellae of the cuticle. The pectic materials thus form a continuous pathway for water-soluble compound movement from the leaf surface to the vein extensions of bundle sheaths.

Guard cells

The guard cells are unique epidermal cells both morphologically and physiologically. The primary function of the guard cells is to regulate the stomatal aperture, and thus transpiration (Esau, 1953). The guard cells themselves transpire (Franke, 1967), especially through areas around the cuticular ledges (Maercher, 1965). They are the only epidermal cells containing chloroplasts (Ketellaper, 1963; Zucker, 1963). Various exogenouly supplied compounds have been found preferentially absorbed by guard cells. Jyung, *et al*. (1965) found that stomatal frequency and rate of absorption of rubidium by bean and tobacco leaves were closely correlated. Since this occurred independent of the degree of stomatal opening, the guard cells were deemed the important factors. Dybing and Currier (1961) showed that entry of fluorochrome into *Zebrina* leaves occurred preferentially into guard cells. Penetration of 2,4-D into leaves of *Phaseolus vulgaris* and *Coleus blumei* was closely related to stomatal density indicating that the guard cells may be the preferential

site of entry (Sargent and Blackman, 1962). Franke (1964b) treated Spinacea oleracea and Viola tricolor with radioactive sucrose and developed microautoradiograms. These showed high densities of silver grains throughout the guard cells, but especially over the anticlinal walls and the cuticular ledges of the guard cells. Yamada et al. (1966) showed that binding sites of urea and calcium and chloride ions to isolated cuticle surfaces occurred in the same areas, especially near cuticular ledges. Franke (1961, 1964b) demonstrated ectodesmata in these same locations and concluded (1969) that the binding sites in the cuticle lie over ectodesmata in the cell wall and together form a pathway of penetration.

Pores, Cracks, and Fissures

The demonstration of wax-extruding pores by Hall (1970b) opens the possibility of movement of liophilic compounds through these canals. Crafts (1961) suggested that the cuticle is perforated by micropores filled with an aqueous phase which function in the penetration of polar substances. Gaff, Chambers, and Markus (1964) demonstrated the presence of such canals, 30 nm in diameter, along which particles as large as colloidal gold might pass. Obviously, further substantiation of both types of pores and demonstration of their importance on foliar penetration is necessary. There is little doubt that insect punctures, cracks, and other forms

of mechanical injury to the cuticle allow mass flow of foliar applied substances into the leaf (Currier and Dybing, 1959; Orgell, 1955).

Ectodesmata

Ectodesmata have been suggested as possible portals of entry of foliar applied substances into leaves since increased numbers of ectodesmata coincide with preferential sites of absorption: guard cells, anticlinal walls, veinal areas, and trichomes (Franke, 1964b, 1967). The similarities in distribution patterns of ectodesmata and silver grains in autoradiograms of labeled sucrose penetration over guard cells, veins, and anticlinal walls bears this out (Franke, 1964a, 1964b). Further, Franke (1969) showed a similar distribution of ectodesmata within the epidermal walls and binding sites for inorganic ions in isolated cuticle.

The exact structure of ectodesmata is not clearly understood. In cross-section they appear as thread or ribbonlike forms extending from the cell protoplast to the cuticle (Franke, 1964a). They are non-protoplasmic structures and do not originate from the cytoplasm (Clowes and Juniper, 1968). Basically, ectodesmata are thought to be "bundles of interfibrillar spaces within the cellulosic walls distinguished probably by specific physiochemical features" (Franke, 1971). They extend as free space conjunctions between areas of the cuticle permeable to aqueous solutions and the plasma

membranes of the protoplasts (Franke, 1971). Ectodesmata never extend to the outside of the plant (Clowes and Juniper, 1968). The free spaces are almost always filled with aqueous liquid (Franke, 1971) probably containing reducing substances such as ascorbic acid which reduce Gilson fixative to Hg_2Cl_2 (Schonherr and Bukovac, 1970b). Finally, Schonherr and Bukovac (1970a) conclude that ectodesmata are not definable cell wall structures, and further, that the distribution pattern of ectodesmata in the cell wall is a function of cuticle permeability (development of mercury percipitates under areas of the cuticle permeable to the Gilson fixative). Ectodesmata may not be preferred paths of entry, but just preferred regions for Hg_2Cl_2 crystallization (Martin and Juniper, 1970).

Stomatal penetration

The importance of stomata in the penetration of water soluble substances is open to debate. Stomata are generally present in large numbers on the lower surface and absent on the upper surface, although the upper surface generally receives the bulk of sprayed materials. At first sight, stomata appear to be obvious routes of penetration; however, aqueous solutions without added surfactants are unable to penetrate through the stomata (Fogg, 1948; Dybing and Currier, 1961; Weaver and DeRose, 1946). The occurrence of stomatal penetration in the presence of surfactants has yet to be clearly answered.

Stomatal penetration is defined, herein, as the movement of a solution from the surface of the leaf, through the stomatal pore, into the substomatal chamber. Substances in the substomatal chamber must traverse an internal cuticle which lines the substomatal chamber of many plants (Norris and Bukovac, 1968; Yamada *et al*., 1966), before entering the protoplasm of the plant. This internal cuticle is thinner and composed principally of cutin (Franke, 1967).

There is much evidence refuting the existence of stomatal penetration. Westwood *et al*. (1960) showed a major portion of dinitro-o-creosol applied to apple leaves being absorbed after the spray had dried and thus ruled out stomatal penetration. Middleton and Sanderson (1965) dismissed stomatal penetration of inorganic ions for several reasons including the observation that absorption was linear with time and stomatal penetration was characterized by initial rapid uptake rates. Rodney (1952) showed that the entrance of urea and other nitrogenous compounds into apple leaves was the same whether applied to the astomatous upper or stomatous lower surface and thus concluded stomata unimportant. Wallihan and Heymann-Hershberg (1956) found similar results with foliar applied Zn^{65} in citrus and also concluded that stomatal penetration was unimportant. Similar results have been obtained by Brian (1967) who looked at the absorption of Diquat and Paraquat into tomato and sugar beet leaves;

Muzik *et al*. (1954) who studied absorption of CMU; and Swets and Addicott (1955) who studied the effect of placement on the leaf on the action of the defoliant, NH_4SCN.

Other reports have shown that stomatal penetration is important. Cook and Boynton (1952) showed a rapid initial rate of urea absorption by the lower surface of apple leaves, followed by a steady slower rate over 48 hours. Currier *et al*. (1964) found agreement between the degree of stomatal opening and 2,4-D penetration. Similar results were obtained with NAA (Harley *et al*., 1957) and Co^{60} (Gustafson, 1956). Eddings and Brown (1967) found foliar penetration of ferric ions primarily stomatal under their total immersion system. Foy (1962) showed that stomatal penetration of Dalapon into leaves of *Tradescantia fluminensis* was extremely erratic. In many of the above cases, the possibility of preferential absorption into guard cells rather than stomatal penetration cannot be overlooked.

Certainly, there must be reasons why there is controversy in the literature regarding the existence and importance of stomatal penetration. An obvious reason is stomatal opening. Various environmental factors are known to influence stomatal opening and closing: light, carbon dioxide concentration, temperature, water stress, and levels of potassium ions in the leaf. Stomata are generally closed in darkness, and open when exposed to light (Brun, 1961). Higher light

intensities up to a maximum cause greater final openings (Virgin, 1956; Zelitch, 1961). Light quality also affects stomatal opening. The action spectrum of stomatal opening resembles that for photosynthesis (Ketellaper, 1963). Carbon dioxide concentration has a marked effect on stomatal opening; lower CO_2 levels induce opening and higher CO_2 levels cause closure (Fischer, 1968). Temperatures within the range of 10 to 25°C have little effect on stomatal aperture; however, temperatures over 30-35°C can cause closure (Rees, 1961). Changes in water deficit will effect stomatal movement with closure coming when the water deficit reaches a critical value (Stelfelt, 1955). A requirement for potassium ions in the process of stomatal opening has been demonstrated (Fischer, 1971; Humble and Raschke, 1971; Sawhney and Zelitch, 1969). Potassium deficiencies under field conditions caused closure in corn (Peaslee and Moss, 1966). The problem involved in controlling stomatal opening, then, is a complex one and an inability to control opening consistently has added to the wide range of results obtained.

Differences in pore size (geometry) may contribute to the wide range of results obtained. Pore size varies according to plant species (Esau, 1953) and may be influenced by environmental conditions during development. Foy and Smith (1965) and Dybing and Currier (1961) noted the ability of surfactants to lower the surface tension of aqueous solutions

and found stomatal penetration dependent upon the concentration of the surfactant. Schonherr and Bukovac (1972) attempted to assess the contributions of leaf wettability, solution surface tension, and stomatal morphology to stomatal penetration and reached several conclusions: (1) there is a critical surface tension (25-30 dyne cm^{-1} for Zebrina) below which contact angle is zero and spontaneous infiltration occurs; (2) generally, spontaneous infiltration occurs only when the contact angle is smaller than the wall angle of the capillary; (3) the degree of stomatal opening is of little importance; and (4) cuticular ledges present at either end of the capillary caused near zero wall angles, and thus played a major role in exclusing water from the substomatal chamber.

Methods of Application

Substances have been applied to leaves for penetration studies by various means. Foliar sprays were used by Westwood and Batjer (1958), leaf immersion by Eddings and Brown (1967), and the leaf disc method by Sargent and Blackman (1962) and Greene (1969). The leaf disc method entails affixing glass cylinders to excised leaf discs and incubating these in Petri dishes. Sargent (1965) cites the various advantages of this method: (a) this method prevents runoff and forces the solution to adhere to the surface, (b) the solution is prevented from drying out thereby maintaining concentration and allowing for maximum uptake, (c) translocation away from the

penetrating area is prevented by the use of leaf discs, though the importance of this is doubtful, and (d) possible influences by surrounding tissues or organs are eliminated. However, in the selection of a method, one must not ignore the potential problems involved in using excised tissue.

Factors Influencing Foliar Penetration

Environmental factors

The effect of environment on cuticle development may be manifested through such factors as leaf wettability (via surface waxes) and cuticle thickness, both of which have a decided effect on foliar penetration of applied substances. Hull (1958) has demonstrated that a positive correlation exists between the melting point of waxes, as well as the total wax content in *Prosopis* seedling leaves grown under different temperatures. No microscopic differences among the various cuticles were discernable. Skoss (1955) has demonstrated that leaves from the same plant species grown under different conditions may vary widely in the amounts of cutin and waxes and the ratio of wax to cutin. Certain plants, e.g. *Nicotiana glauca*, under continuous water stress, develop a cuticle almost twice as thick as similar plants under normal conditions (Skoss, 1955). Donoho *et al*. (1961) have shown that both cool air temperatures and high humidity during the development of peach and apple leaves favored the absorption

of ^{14}C-NAA. Presumably the temperature effect was on total cuticle development and wax production, but high humidity favors the development of cuticle (Martin and Juniper, 1970). The effect of high humidity might be to decrease the amount of waxes formed. Leaf maturity may influence foliar penetration. Bukovac (1965), Greene (1971), and Sargent and Blackman (1962) showed that absorption of 3-CP, NAAm, and 2,4-D, respectively was greater in immature leaves than in mature leaves. High light intensity has been shown by Orgell (1955) to enhance cuticle thickness. In *Hedera helix*, more wax and cutin are developed in sun leaves than shade leaves (Martin and Juniper, 1970). Plants grown under direct sunlight are less easily wetted than leaves grown under partially shaded conditions (Skoss, 1955). Hull (1964) has suggested that plants grown under greenhouse glass, since ultraviolet light is filtered out, do not develop cuticles as thick as plants grown in direct sunlight. This may explain observed increased wettability of wheat leaves grown in the greenhouse compared with those grown outside (Troughton and Hall, 1967). Martin and Juniper (1970) state that high wind speeds result in the development of a denser surface wax layer than that of plants grown in still air conditions.

Light

The role of light in influencing foliar penetration is,

at best, a complex one. Currier and Dybing (1959) suggest that light may directly promote penetration by promoting stomatal opening, presumably through photosynthesis. Light is one variable affecting stomatal opening under field conditions. Sargent and Blackman (1962) found that light enhanced the penetration of 2,4-D into bean and Coleus leaves. Light pretreatment enhanced absorption in the light but had no effect in the dark. It has been suggested by Kamimura and Goodman (1964c) that increased absorption of labeled streptomycin and leucine with increasing light intensity was probably due to a buildup of photosynthetic reserve. They based their conclusions on the following: (a) increasing light duration resulted in increased foliar absorption, (b) those wavelengths of light most favorable to photosynthesis also favored higher levels of absorption, (c) uptake in the dark increased when apple leaves were fed sucrose exogenously, and (d) the process of foliar absorption was inhibited by 2,4-dinitrophenol, iodoacetate, and arsenate. In contrast, Ahlgren and Sudia (1967) suggested that light-dependent foliar penetration requires energy from photophosphorylation. Actual uptake into the epidermis may not be energy requiring but rather a diffusion process whereas translocation away from the epidermis may be energy dependent (Sargent and Blackman, 1962, 1965; Gustafson and Schlessinger, 1956). Penetration in the dark can indeed occur. Brian (1967) showed that uptake

of diquat and paraquat increased in the dark. Light stimulation of penetration occurred in the upper surface of pear leaves when greenhouse grown, but not when grown outside (Greene, 1969). When light stimulation did occur, the presence or absence of light had an immediate effect on penetration (Greene, 1969).

Temperature

Generally, within a temperature range in which the plant is functioning normally, there is a direct correlation between temperature and foliar penetration (Hull, 1964). Greene (1969) found that penetration of NAA and NAAm into pear leaves increased with increasing temperature between 25 and 35°C. Sargent and Blackman (1962) found this same positive correlation between temperature and uptake into leaves of *Coleus blumei*.

High temperature coefficients are believed to indicate a dependence on metabolic energy. Goodman and Goldberg (1960) showed Q_{10} values of 2.0 and 2.4 for bean and apple leaves respectively and concluded that metabolic energy was necessary for absorption. Similar conclusions were reached by Sargent and Blackman (1962). Hull (1964) views absorption as a biphasic process. The first phase is temperature independent whereas the second phase has a Q_{10} of 2.0 or more, suggesting at least partial mediation by respiratory energy. Using isolated pear leaf cuticle, Norris and Bukovac (1969) found

a temperature coefficient of about 5.6 between 15 and 25°C. Van Overbeek (1956) had suggested that temperature might be important in influencing the permeability of the embedded waxes. At low temperatures these are nearly solid and have low permeability; however, higher temperatures cause a loss of viscosity and higher permeability. Norris and Bukovac (1969) concluded that their high Q_{10} value was related to temperature induced changes in lipids in the cuticle. A high energy of activation was believed necessary to enter or leave the cuticle. Therefore, high Q_{10} values for foliar applied compounds do not necessarily mean that metabolic energy is required for movement into the leaf.

The effects of temperature on foliar penetration are not related only to cuticular penetration since temperature has an effect on stomatal opening also (Hofstra and Hesketh, 1969). Basically, normal temperatures have little effect, but temperatures over 30 to 35°C may cause closure (Rees, 1961).

pH

Low pH of a treating solution is generally associated with increased foliar penetration (Currier and Dybing, 1959). Sargent and Blackman (1962) found that pH values below 6.0 increased the amount of 2,4-D entering bean and *Coleus blumei* leaves compared with pH values above 6.0. Weak acids generally penetrate the lipoidal portions of the cuticle best if they are in an undissociated state (van Overbeek, 1956),

a condition induced by low pH values. Norris and Bukovac (1972) studied the effects of pH on absorption of NAA and NAAm. NAA penetration was maximum at pH's below the pK (4.2 for NAA). NAAm penetration was not affected by pH, presumably due to its high pK (14.0). Control of dissociation, and hence polarity, played a major role in penetration. Sorption of NAA into the cuticle, believed an initial step in the process of penetration, was also favored by lower pH's. Bukovac *et al*. (1971) also assert that penetration of weak organic acids is favored in the undissociated state but places the emphasis on the lipid solubility of the compound, rather than the dissociation state.

The cuticle itself is affected by changes in pH. Van Overbeek (1956) demonstrated a pK of over 5 for cutin, compared to a pK of about 3 for the surface waxes of pear cuticle (Norris and Bukovac, 1966). Since cutin contains a large number of free carboxyl groups, the pH of the applied solution may effect the charge in the cuticle. At pH values below 5, these carboxyl groups would be largely undissociated and anions would be attracted and penetrate easily. At higher pH values, more carboxyl groups would be dissociated, the cuticle would be negatively charged, and anions would be repelled (Hull, 1964). Of course, the penetration of all substances is not pH dependent (Crafts, 1961). Absorption rates as affected by pH may vary under different conditions and for different

species (Hull, 1964).

Concentration

There is general agreement that the concentration of an applied solution is linearly correlated with the amount of a substance entering the leaf. This direct correlation was found for the absorption of 2,4-D (Sargent and Blackman, 1962) and NAA and NAAm (Greene, 1969; Norris and Bukovac, 1969; Westwood and Batjer, 1958), as well as the uptake of various salts (Middleton and Sanderson, 1968). Deviations from this relationship were seldom observed. However, rubidium uptake at high concentrations was not found to be linear by Jyung and Wittwer (1964). Also, Greene (1969) showed that NAA penetration into pear leaves was linear except at high concentrations at the lower surface where uptake began to plateau. Presumably this was due to saturation of the leaf disc.

Time-course

A method which can be successful in determining preferential uptake patterns is the use of time-course studies. Various investigators have found absorption linear with time; Greene (1969) with NAA and NAAm, Jyung and Wittwer (1964) with phosphate and rubidium, and Sargent and Blackman (1962) with 2,4-D. Penetration is not always linear with time. A two-phase pattern of uptake with the first phase more rapid would suggest initial stomatal penetration followed by slower, linear cuticular uptake. Such were the findings of Cook and

Boynton (1952). Three phase uptake of sucrose by bean leaves was demonstrated by Vickery and Mercer (1964).

Surfactants

The degree to which a droplet can wet the surface of a leaf is determined by the chemical and physical configuration of the surface waxes (Eglington and Hamilton, 1967; Martin and Juniper, 1970) and the presence of leaf hairs (Holly, 1964). Wettability is measured by the advancing contact angle of the applied solution - "the angle between the surface of a leaf and the tangent plane of a water droplet at the circle of contact between leaf, liquid, and air" (Martin and Juniper, 1970). Larger contact angles mean a smaller degree of contact between the droplet and the leaf surface or a lesser degree of wettability.

A surfactant is a chemical compound with two opposing characteristics. The combination of both a hydrophilic and lipophilic portion in the same molecule allows the surfactant to be water soluble and yet surface active (Behrens, 1964). Surfactants serve several valuable purposes. Oil solutions of certain chemicals can be held in solution with water by preventing oil droplets of the emulsion from condensing (Behrens, 1964). Surfactants drastically decrease the surface tension of a water solution, allowing the solution to wet and spread on waxy leaves, insuring greater contact of solution and leaf surface, and hence, greater penetration.

Parr and Norman (1965) state that the maximum reduction in surface tension occurs at the critical micelle concentration (generally in the concentration range of 0.01 to 0.1%). This would theoretically allow maximum wettability and thus maximum cuticular penetration. Since many surfactants enhance penetration at concentrations well above the critical micelle concentration, they must have other effects (Holly, 1964) such as dissolving oils and waxes and thereby enhancing penetration (Parr and Norman, 1965).

Dybing and Currier (1961) showed that surfactants which were most effective in reducing surface tension allow the greatest stomatal penetration. Schonherr and Bukovac (1972) have suggested that a surfactant must reduce the contact angle to an angle lower than that of the cuticular ledges of the stomatal aperture to effect instantaneous stomatal infiltration.

Active uptake

Cuticular penetration is considered a diffusion process (Franke, 1967) while subsequent cellular absorption is an energy requiring, active process (Gustafson and Schlessinger, 1956; Sargent and Blackman, 1962, 1965). Various workers have shown foliar penetration to be an active process. The Q_{10} values for absorption of streptomycin by apple leaves suggested metabolic mediation (Goodman and Goldberg, 1960). Kamimura and Goodman (1964c), Greene (1969), and Sargent and

Blackman (1962) showed a light dependency for uptake. Further, use of respiratory inhibitors by Kamimura and Goodman (1964c) inhibited foliar absorption. Uncouplers of oxidative phosphorylation significantly inhibited the penetration of NAA (Greene, 1969). Metabolic uptake of 2,4-D by bean leaves (Sargent and Blackman, 1965) and sucrose by bean leaves (Vickery and Mercer, 1964) has also been demonstrated. Summing up the importance of metabolic energy: (a) foliar penetration is an active process dependent on temperature, light and oxygen; (b) uptake is mainly irreversible and occurs against the concentration gradient; and (c) uptake is subject to metabolic inhibitors (Wittwer, 1964).

MATERIALS AND METHODS

Apple trees, *Malus domestica* Bork., Cv. McIntosh, were selected as the test plant. Studies of foliar penetration in intact apple leaves by Cook and Boynton (1952) and Kamimura and Goodman (1964a) have established the effects of foliar characteristics on penetration. Penetration and properties of excised cuticular membranes have been studied by Goodman and Addy (1963) and Kamimura and Goodman (1964b). These studies have established a basis for the use of apple as a test plant. Silver nitrate ($AgNO_3$), lead nitrate ($(Pb(NO_3)_2$, SADH-succinic-C^{14} (Uniroyal Chemical, Naugatuck, Conn.), and sucrose-UL-C^{14} (ICN Corp., Irvine, Calif.) were selected as the model compounds for the following reasons: (1) $AgNO_3$ -- silver ions are readily reduced to visible metallic silver by endogenous reducing substances within the leaf; (2) $Pb(NO_3)_2$ -- lead ions are precipitated as lead sulfide following treatment of leaf tissue with hydrogen sulfide gas, thereby eliminating the distribution of endogenous reducing substances as a factor in the uptake patterns observed; (3) ^{14}C-sucrose -- use of labeled sucrose by Franke (1964b) has established its usefulness as a test compound; (4) ^{14}C-SADH -- selected as a compound in common commercial use.

Growing plants

Greenhouse grown, 3-year-old McIntosh on seedling rootstock apple trees were the source of leaves. Trees were grown in metal containers in a mixture of equal portions of silty loam, sand, and peat. Trees were pruned to about 30 inches and two lateral shoots were allowed to grow. Fully expanded, healthy appearing leaves were selected for penetration studies.

General methods

The treating solution was applied according to the method of Sargent and Blackman (1962) as modified by Greene and Bukovac (1971). Glass cylinders, 1 cm internal diameter and 1 cm in height were cemented to the leaf discs with Dow Corning Silastic 68-110-RTV silicon rubber (Dow Corning Corp., Midland, Mich.) hardened by T-11 catalyst (Wacker Company, Munich, Germany). Dow Corning RTV catalyst F was also found to be an acceptable catalyst. Approximately 0.1 ml of T-11 catalyst (1 gm RTV catalyst F) was thoroughly mixed with about 7 gm of silicon rubber. The bottom edges of the glass cylinders were touched to the silicon rubber and then placed on 1.5 cm discs cut with a #11 cork borer from freshly harvested leaves. The silicon rubber hardened in about 20 to 30 minutes. Leaf discs were then placed in 15 x 2.0 cm Petri dishes lined with filter paper moistened with distilled water. In all cases, 0.25 ml of treating solution was

pipetted into each glass cylinder. Covered Petri dishes were placed in a shallow water bath maintained at 28°C and illuminated with 1500 ft-c of light from a fluorescent light bank.

Silver nitrate

A 0.1 M $AgNO_3$ solution with or without 0.1% surfactant was used for silver accumulation studies. Surfactants used were X-77, Triton B-1956, and Vatsol-OT which reduce surface tension to approximately 32.5, 30.0, and 28.5 dynes/cm^{-1} respectively (Greene, 1969). Immediately after harvesting and washing the leaf discs with distilled water, the discs were infiltrated with either CRAF III or acetic:alcohol (1:3) killing and fixing solutions and left in these overnight. Discs were passed through a graded ethanol-t-butyl alcohol series to t-butyl alcohol. Dehydrated leaf discs were then infiltrated with tissuemat and embedded. Cross-sections were cut from embedded leaf discs using a Bausch and Lomb #5706 rotary microtome and affixed to slides using chrom-gelatin adhesive. Sections were cleared of paraffin in xylene and mounted in Permount.

Lead nitrate

Accumulation studies with 0.1 M $Pb(NO_3)_2$ were performed in a similar manner to the $AgNO_3$ studies. After an appropriate treatment time, leaf discs were harvested, washed, and gassed with hydrogen sulfide (H_2S) for 5 min to insure

complete reduction of absorbed lead. H_2S gas was generated via the pressure method of saturation. The leaf discs were then immediately infiltrated with acetic acid:alcohol fixing solution and left overnight. Subsequent dehydration, embedding, and preparation of slides was as with $AgNO_3$.

<u>Labeled sucrose and SADH</u>

^{14}C-UL-sucrose (specific act.=10 mC/mM) and ^{14}C-succinic-SADH (specific act.=0.2 mC/mM) were both applied in a solution of 0.1% X-77. A 10 uC/ml concentration of ^{14}C-labeled sucrose and a 2.5 uC/ml concentration of ^{14}C-SADH were used. After the required treatment time, leaf discs were gently washed, cut into 2 mm square blocks using a razor blade, and then submersed in isopentane cooled to -165°C with liquid nitrogen. After 2 min the frozen blocks were transferred to alyophilizer and dried for 48 hrs. Dried tissue was infiltrated with tissuemat under vacuum and, after 24 hrs, embedded. Sections were sliced on a rotary microtome.

<u>Autoradiography</u>

Two methods of autoradiography, liquid emulsion and stripping film, were employed. (1) Kodak NTB nuclear track liquid emulsion (Eastman-Kodak, Rochester, N.Y.) was used. The container of emulsion was heated in a water bath at 43°C for 30 min or until liquid. Slides were dipped into this emulsion, and withdrawn at a steady and rapid rate. The back was wiped clean, and the slides placed on precooled

metal plates ($5^{\circ}C$) to dry. Dried slides were placed in black plastic, lightproof slide boxes with a small container of desiccant until developed. These and all operations with the NTB emulsion were carried out in a darkroom illuminated with a safelight equipped with a Kodak Wratten Red Series 2 safelight filter. Paraffin embedded sections, 8 microns thick, were cut and ribbons attached to the slides coated with emulsion using a smooth clean spatula. After the desired exposure time, slides were dipped into a solution of 0.5 gm cellulose acetate in 10 ml acetone and 100 ml methylethylketone and dried to affix sections to the slide. Slides were cleared of paraffin in xylene, then passed through a graded alcohol (ethanol) series to water. Slides were then placed in Kodak Dektol developer (1:3 stock solution) for 5 min, rinsed in water for 2 min, and cleared in a 30% sodium thiosulfate (hypo) solution for 18 min. (All solutions in this series were cooled to $8^{\circ}C$.) Slides were next placed in running tap water (cold) for 15 min, then run through a graded ethanol series to absolute ethanol, cleared in xylene, and mounted in Permount.

(2) Kodak fine grain AR 10 stripping film was applied to slides in a darkroom illuminated by a safelight equipped with a Kodak Wratten Red Series 1 safelight filter. Stripping film was cut into 3 cm x 4 cm rectangles, gently stripped from the glass plates, and floated emulsion side down for

3 min on filtered, distilled water at 5°C. The slides used had treated leaf sections affixed with chrom-gelatin adhesive, had been cleared of paraffin, and passed through a graded alcohol series to water. A slide thus prepared was brought up under the floating film at a 30° angle and lifted out of the water with the film now attached. These were dried with a gentle stream of filtered air and exposed in light-proof slide boxes containing desiccant for the desired length of time. After exposure, slides were hydrated, developed in Kodak D-19 developer for 5 min, and cleared in Kodak acid fixer for 15 min. Slides were rinsed in running tap water for 10 min, then placed in a 2% w/v aqueous solution of polyvinyl alcohol for 5 min to insure penetration of liquid through the emulsion into the leaf tissue. Slides were passed through a graded alcohol series to absolute ethanol, cleared in xylene, and mounted in Permount.

Stained tissue

Cross-sections were cut on an International-Harris model CTD cryostat. The cuticle was stained with Sudans III and IV according to Norris and Bukovac (1968). Pectins were stained with ruthenium red (Norris and Bukovac, 1968) and hydroxylamineferric chloride (Reeve, 1959a, b). Photomicrographs were taken with a Bausch and Lomb Dynoptic microscope using a Kodak 35 mm "Colorsnap" camera.

Ectodesmata

Ectodesmata were demonstrated by fixation in a modified Gilson solution (Schonherr and Bukovac, 1970a). Gilson solution was made up from 40 ml 30% ethanol, 10 ml formic acid, 5 ml formalin, 2 gm oxalic acid, and saturating amounts of $HgCl_2$. Discs were then mounted whole for viewing, or dehydrated, embedded, and sectioned as described earlier.

Stomatal Penetration

Stomatal aperture width measurements

Dow Corning 3110 silicon rubber encapsulant and Catalyst F were thoroughly mixed in a ratio of 6:1 (w/w). This mixture was applied to suitable leaf discs with a glass rod and hardened within 3 to 5 min. Cellulose nitrate impressions of the silicon rubber impressions were prepared and mounted on glass slides under cover slips. Measurements were made of 50 stomatal aperture widths randomly selected from each of 2 cellulose nitrate impressions at each time checked. Measurements were made with an occular micrometer with a total specimen magnification of 400x. Accuracy to 0.4 microns was possible.

Time-crouse for stomatal opening

The time-course of stomatal opening was prepared by taking stomatal aperture measurements at 15 min intervals from time 0 (lights turned on) through 3 hrs. Opening was studied under the influence of normal air, low CO_2 air, and

high CO_2 air. Leaf discs were placed in covered Petri dishes and incubated overnight at 25°C in complete darkness. The discs in all cases were incubated on a medium of m/15 KCL in M/15 KH_2PO_4 buffer at pH 6.8 (Willmer and Mansfield, 1970). When a reduced CO_2 concentration within the Petri dishes was desired, a small vessel of KOH was included during the dark pretreatment and subsequent light treatment. When a CO_2-enriched atmosphere within the Petri dishes was desired, 200 mg of dry ice was placed in a styrofaom boat within the dish 1 hr prior to light initiation. This dry ice was replenished as needed at 30 min intervals. At time 0, Petri dishes containing the leaf discs were placed in a 28°C waterbath and illuminated with 1500 ft-c fluorescent light. Stomatal width measurements were made as previously described.

Stomatal penetration of silver and lead

Leaf discs were harvested, glass cylinders attached, and incubated overnight in darkness. Treating solution contained 0.1 M $AgNO_3$ or $Pb(NO_3)_2$ in either distilled water or distilled water containing 0.1% X-77 or Vatsol-OT. In all cases, leaf discs were incubated on the potassium enriched medium described earlier. Penetration was followed under CO_2-deficient, normal, and CO_2-enriched air. Following the dark pretreatment, leaf discs were transferred to 1500 ft-c light for 2 hours to allow stomates to open completely (Fig 1,2,3) and the treating solution was then applied. Penetration was allowed to proceed for

2, 5, and 10 min. Discs were harvested, gently rinsed, infiltrated with acedtic acid:alcohol, dehydrated to TBA, and embedded in tissuemat for sectioning. Surface views were obtained by mounting, in Permount, whole discs which had been fixed, dehydrated, and cleared in xylene. Photomicrographs were taken as previously described. Due to the limited supply of ^{14}C-SADH and -sucrose, stomatal penetration studies using these compounds were not attempted.

Preferential Sites of Absorption

Epidermal hairs

Penetration of silver and lead into leaf hairs was followed at 2, 5, and 30 min. Treating solutions contained 0.1 M $AgNO_3$ or $Pb(NO_3)_2$ in distilled water or distilled water containing 0.1% X-77, Triton B-1956, or Vatsol-OT. Discs were fixed in acetic acid:alcohol, dehydrated, cleared in xylene, and mounted whole in Permount for viewing.

Guard cell accumulation

Penetration of $AgNO_3$ and $Pb(NO_3)_2$ into guard cells was followed at 5, 10, and 30 min. Treating solutions were the same as for leaf hair absorption studies. With labeled sucrose and SADH, the standard concentrations were used. Treatment times were 30, 60, and 120 min. With sucrose and SADH, the solution contained 0.1% X-77. Emulsion exposure times were 10 and 17 days.

Penetration over veins

Preferential absorption of $AgNO_3$ and $Pb(NO_3)_2$ over veinal areas was studied. Treatment times with the standard treating solutions were 30, 60, and 240 min. Leaf discs were handled in the usual way. With labeled sucrose and SADH, treatment times were 1, 4, and 8 hrs for cross-sections. For surface views, treatment times were 30, 60 and 120 min. Exposure time for cross-sections was 6 weeks, while surface view exposures were 10 days.

Cuticular penetration

Preferential penetration of $AgNO_3$ or $Pb(NO_3)_2$ over anticlinal or periclinal walls was studied. For the lower surface, treatment times were 30, 60, and 240 min. For the upper surface, treatment times were 1, 4, 8, 12, and 24 hrs. Tissue was handled as previously described following treatment. With labeled sucrose and SADH, treatment times of 1 and 4 hrs lower and 1, 4, and 8 hrs upper were used. The standard treating solutions were used. Exposure times were 4 and 8 weeks in both instances.

Pores, cracks, and fissures

Artificial induction of cracks was accomplished by very lightly cutting the surface to greenhouse grown leaves with a razor blade. The patterns of uptake with these leaves were compared to that observed with field grown leaves. Treating solutions contained 0.1 M $AgNO_3$ or $Pb(NO_3)_2$. Preparation of tissue for viewing and photography was as previously described.

ABSORPTION OF WATER SOLUBLE COMPOUNDS INTO LEAVES OF MCINTOSH APPLE (MALUS DOMESTICA, BORK.)

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ABSTRACT

The sites of preferential absorption of four water-soluble compounds into McIntosh apple leaves have been shown. Absorption into epidermal hairs occurred readily. Preferential absorption over veinal areas and guard cells was noted; however, uptake could be greatly increased by adding a surfactant to the treating solution. Preferential absorption into guard cells occurred over outer anticlinal walls and through the walls delineating the outer vestibule of the stomatal pore. Penetration into this outer vestibule occurred readily when the stomata were open and a surfactant was added to the treating solution. Equal absorption occurred over anticlinal and periclinal walls of epidermal cells. Penetration through the lower surface was much greater than through the upper. The distribution of ectodesmata (areas of the cuticle permeable to polar compounds) was consistent with the absorption patterns observed with the model compounds. The presence of pectin pathways from the pectic layer to the surface of the cuticle could not be demonstrated.

Stomatal penetration of silver nitrate was demonstrated. The level of carbon dioxide in the atmosphere and the surfactant used had a decided effect on penetration. It is concluded that stomatal penetration was dependent on the degree of stomatal opening (as it affects wall angles of the aperture) and the surface tension of the treating solution.

INTRODUCTION

The cuticle, regarded as the most limiting factor in the penetration of substances, is highly lipoidal in nature (Hull, 1964). Lipid soluble compounds are readily absorbed by the cuticle. Water soluble compounds are repelled and much less readily absorbed by this lipoidal cuticle. However, there is ample evidence to show that applied compounds do penetrate into the leaf.

Various preferential pathways of penetration into the leaf have been suggested: over anticlinal walls of epidermal cells, areas over veins (Dybing and Currier, 1961), leaf hairs (Hull, 1964), guard cells (Franke, 1964), and stomates (Currier, Pickering, and Foy, 1964). Several anatomical features have been cited as favoring preferential absorption of water-soluble compounds at these sites. Distribution patterns of ectodesmata have been closely correlated to preferential patterns of uptake over guard cells, veins, and anticlinal walls (Franke, 1964, 1967); however, the presence of ectodesmata, as cell wall structures, is now doubtful (Schonherr and Bukovac, 1970a,b). The existence of continuous pectin pathways from the surface of the cuticle over veinal areas to vein extensions or bundle sheaths has been reported by Roberts *et al*. (1948) in McIntosh apple leaves. Pear leaves do not exhibit these pectin pathways (Norris and

Bukovac, 1968).

The purpose of this investigation was to determine the areas of preferential absorption of water soluble compounds into apple leaves and to demonstrate and correlate the coincidence of ectodesmata and pectin pathways with these sites.

MATERIALS AND METHODS

Fully expanded, mature leaves were harvested from 3-year-old, greenhouse grown apple trees (*Malus domestica* Bork., Cv. McIntosh). The leaf disc method of Greene and Bukovac (1971) was used in treating the leaf tissue.

Four water-soluble compounds were used in this study. Silver nitrate ($AgNO_3$) at 0.1 M was selected since endogenous reducing substances within the leaf readily reduce the silver ions to black silver grains that are easily seen with a microscope. Lead nitrate ($Pb(NO_3)_2$) at 0.1 M was chosen since it could be reduced by hydrogen sulfide (H_2S) treatment (Crowdy and Tanton, 1970). This eliminated the requirement for the presence of endogenous reducing substances in the areas where penetration was actually occurring. Two radioactive compounds were used in this study with autoradiography revealing preferential sites of entry. Sucrose-UL-C^{14} at 10 uC/ml (sp. act. 10 mC/mM) and succinic acid N,N-dimethyl hydrazide (SAFH-succinic-C^{14}) at 2.5 uC/ml (sp. act. 0.2 mC/mM) were used. Stripping film (Kodak AR 10) and liquid emulsion (Kodak NTB) methods of autoradiography (Jensen, 1962) were used to follow their penetration.

For most experiments, 0.1% X-77 (a mixture of alkylarylpolyoxyethylene glycol, free fatty acids, and isopropanol) and aqueous solutions of the test compounds were used. In

stomatal penetration studies, the 0.1 $AgNO_3$ and $Pb(NO_3)_2$ treating solutions contained 0.1% of either X-77 or Vatsol-OT (diocytl ester of sodium sulfosuccinic acid).

Stomatal opening was regulated in several ways. Filter paper was moistened with M/15 KCL in M/15 KH_2PO_4 at pH 6.8 to insure sufficient potassium for stomatal opening (Willmer and Mansfield, 1970). Leaf discs were given a 12 hr dark pretreatment and subsequent 2 hr light treatment prior to application of solution. Small amounts of dry ice were placed in styrofoam containers within the Petri dishes to increase CO_2 and retard opening. Small vessels of KOH were added to decrease CO_2 and enhance opening (Fig 1, 2, 3).

The cuticle staining solution consisted of saturating amounts of Sudans III and IV in 95% ethyl alcohol. A 1:500 aqueous solution of ruthenium red was used for pectin. A second pectin staining procedure used was hydroxylamine-ferric chloride as described by Reeve (1959). Demonstration of ectodesmata was accomplished by use of Gilson fixative as described by Schonherr and Bukovac (1970a).

All photomicrographs were made using a Bausch and Lomb dynoptic microscope equipped with a Kodak "Colorsnap" 35 mm film holder.

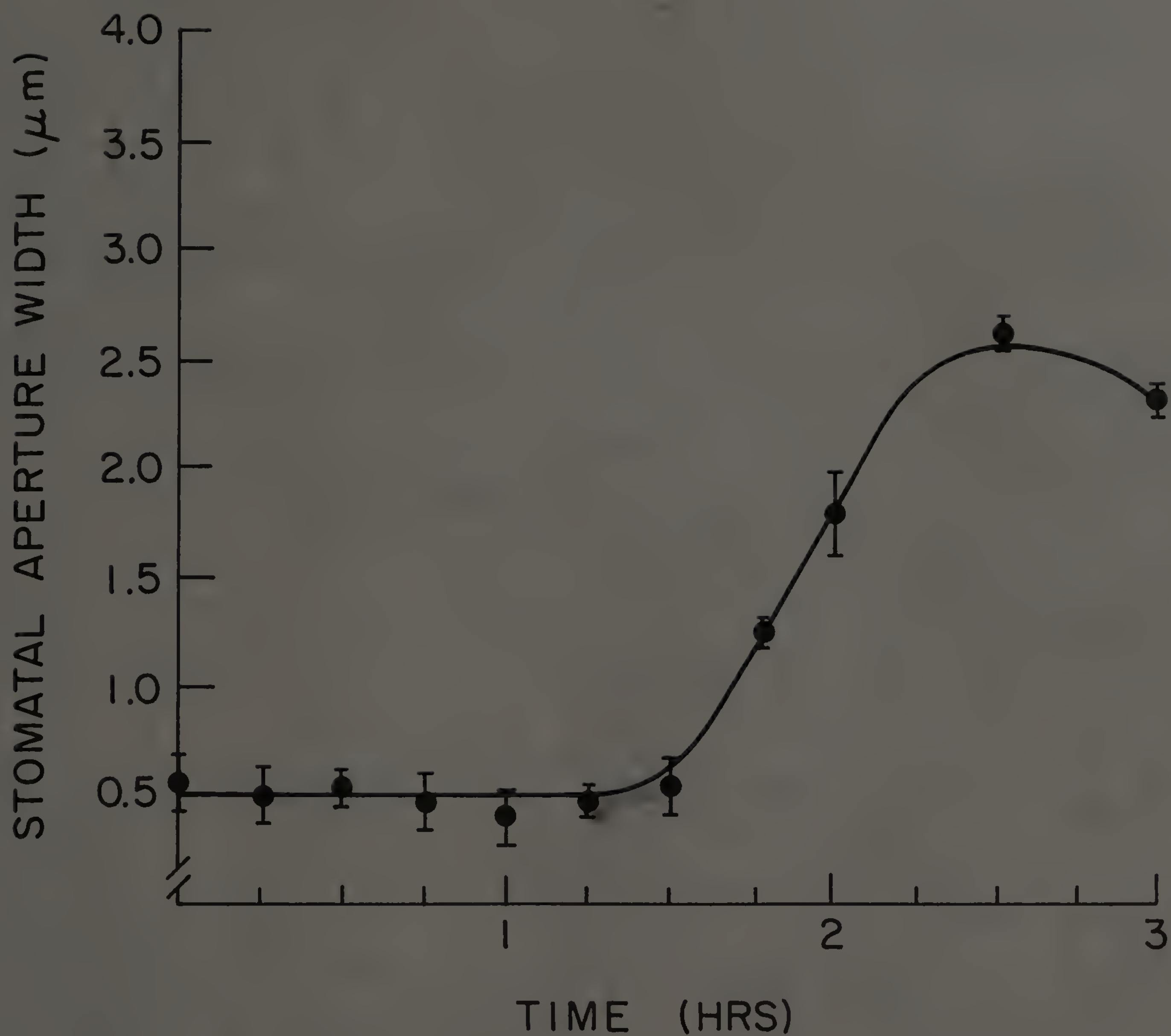

Fig 1. Time-course of stomatal opening (+CO_2); CO_2 added 1 hr prior to light initiation.

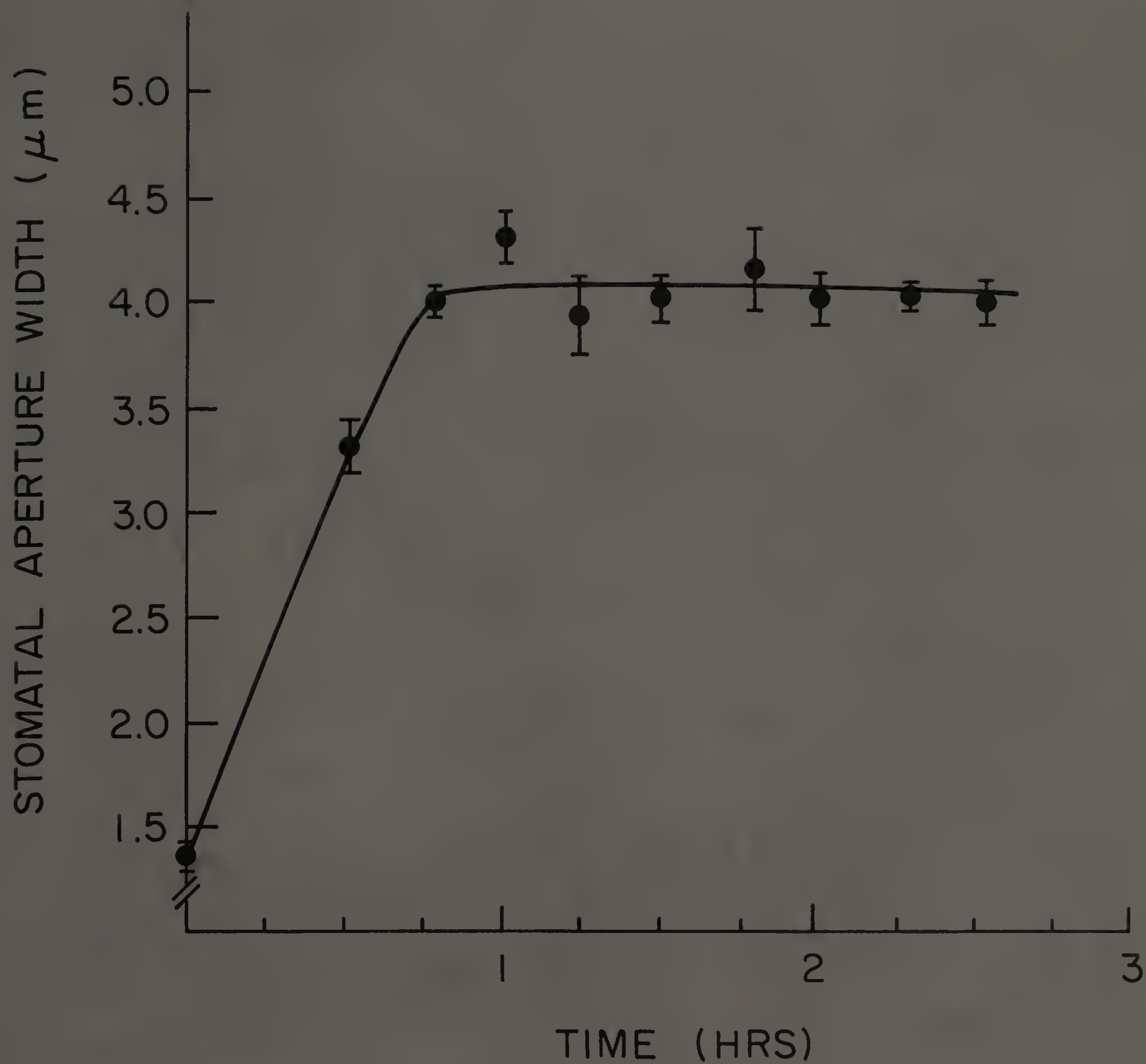

Fig 2. Time-course of stomatal opening in light (air).

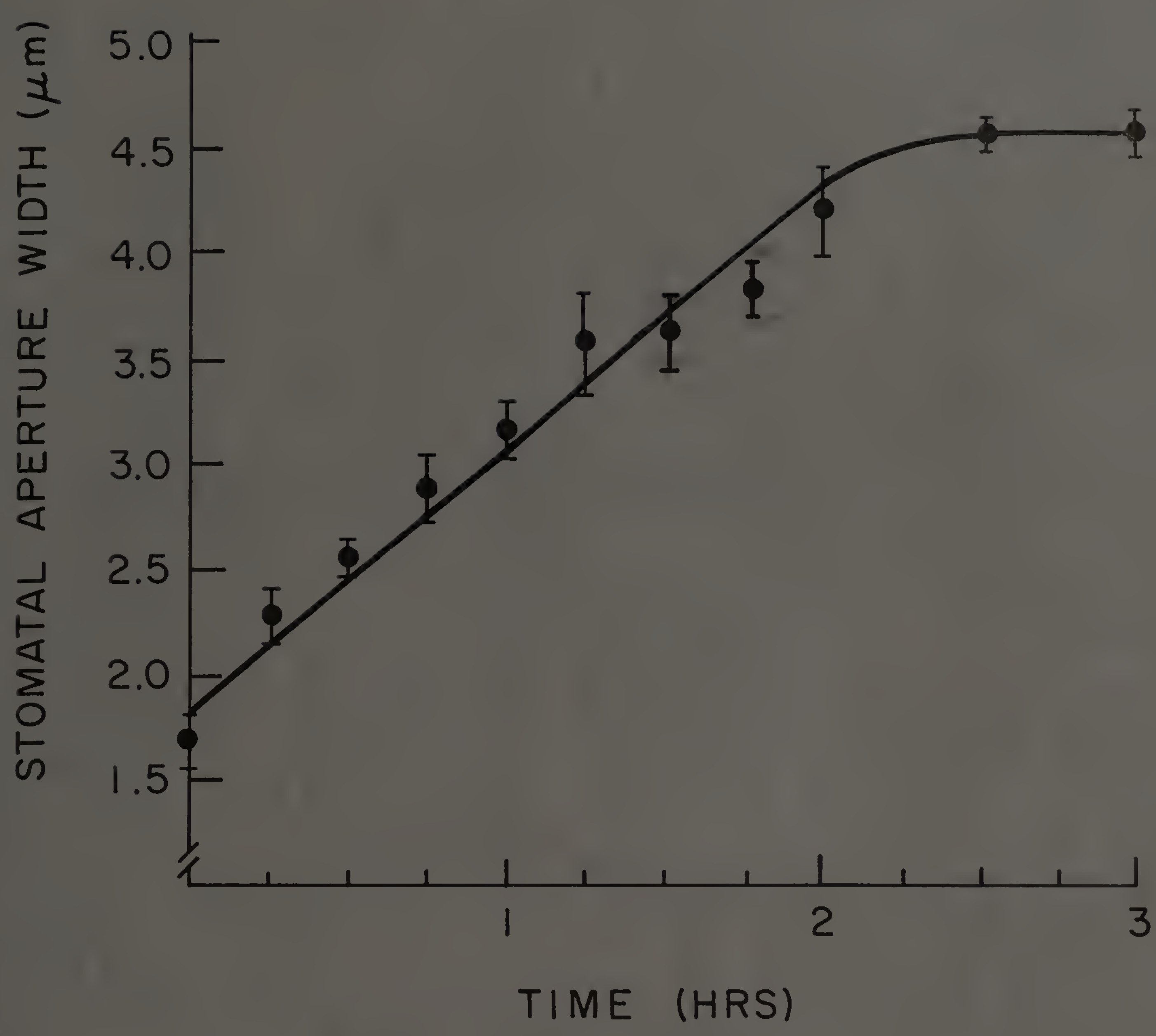

Fig 3. Time-course of stomatal opening in light ($-CO_2$). KOH added 8 hrs prior to light initiation.

OBSERVATIONS

Epidermal hairs

Accumulation of reduced silver and lead sulfide in epidermal hairs was evident within 2 min of treating discs (Fig 4, 5). Accumulation was mainly in the cell walls of the hairs, but some cytoplasmic accumulation did occur. Uptake into the cell walls was uniform and similar for both silver and lead. Uptake into leaf hairs occurred only in certain areas of the leaf when the treating solution lacked a surfactant. However, when surfactants were used, there were few areas with hairs devoid of accumulation. There were no differences in the patterns of uptake observed when X-77 or Vatsol-OT was used.

Guard cells

Preferential uptake of silver and lead into guard cells of leaf discs was observed after 2 min of treating leaf discs (Fig 6, 7, 8, 9). Accumulation occurred in the outer anticlinal walls and in the region near cuticular ledges (Fig 7, 8, 9). Accumulation in the cuticular ledges occurred in amounts only slightly higher than surrounding surface cuticle (Fig 10). Close examination of absorption near cuticular ledges suggests that penetration into the outer vestibule (delineated by the cuticular ledges and the narrow true aperture) is necessary for a pronounced absorption

Fig 4. Epidermal hairs of lower leaf surface over vein treated with $AgNO_3$ in X-77 for 2 min. (100x).

Fig 5. Epidermal hair from leaf treated with $Pb(NO_3)_2$ in X-77 for 2 min. (250x).

pattern near ledges to develop (Fig 10, 11). The guard cell walls forming this chamber display a random distribution of silver or lead sulfide grains, except for deposits at the neck of the aperture (Fig 10). Penetration and subsequent accumulation of silver and lead ions in this chamber occurred when surfactants were present in the treating solution. The increase in ledge area penetration in normal or CO_2-deficient air over that in CO_2-enriched air suggests that stomatal opening is important for accumulation in the outer vestibule (Fig 22).

Substantial quantities of silver accumulated within the protoplasts of many guard cells within 30 min of treating. This accumulation appeared largely as a precipitate along the inner surface of the guard cell wall. No accumulation of lead sulfide within the guard cell protoplasts occurred.

The use of surfactants had a marked effect on the pattern of uptake observed. When aqueous solutions of $AgNO_3$ and $Pb(NO_3)_2$ were used, only small areas of a treated disc showed preferential absorption into the guard cells. The amounts of precipitate near the cuticular ledges in these few areas were greatly reduced. When a surfactant was included in the treating solution, all guard cells in all portions of the leaf discs showed accumulation. Increased absorption near cuticular ledges was noted with Vatsol-OT (Fig 6, 7, 8, 9).

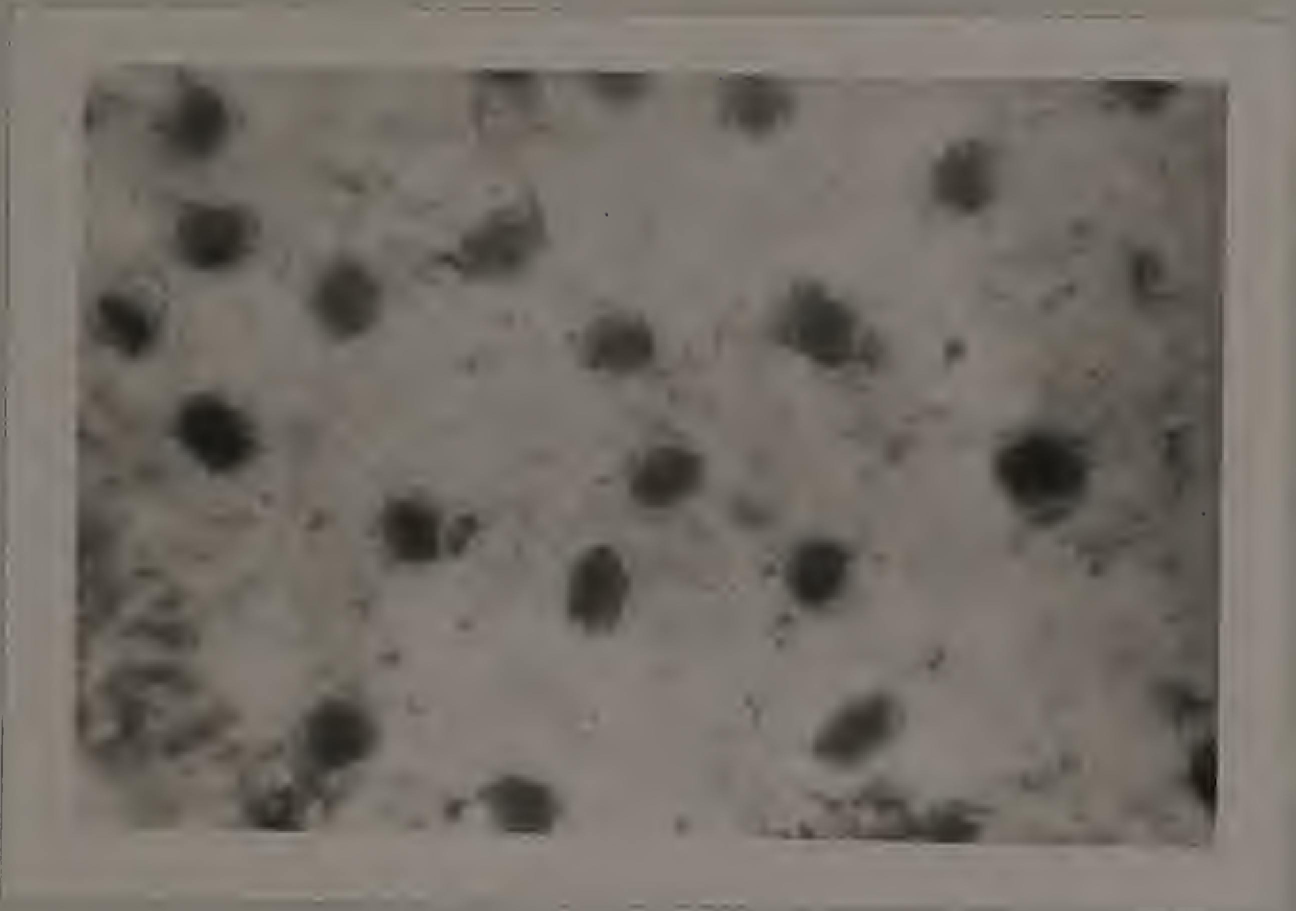

Fig 6. Surface view of lower epidermis of leaf treated for 2 min with $AgNO_3$ in Vatsol-OT. (100x).

Fig 7. Surface view of leaf treated with $AgNO_3$ in X-77 for 2 min. (250x).

Fig 8. Lower surface of leaf discs treated for 2 min with $Pb(NO_3)_2$ in Vatsol-OT. (250x).

Fig 9. Lower surface view of leaf treated with $Pb(NO_3)_2$ in X-77 for 2 min. (250x).

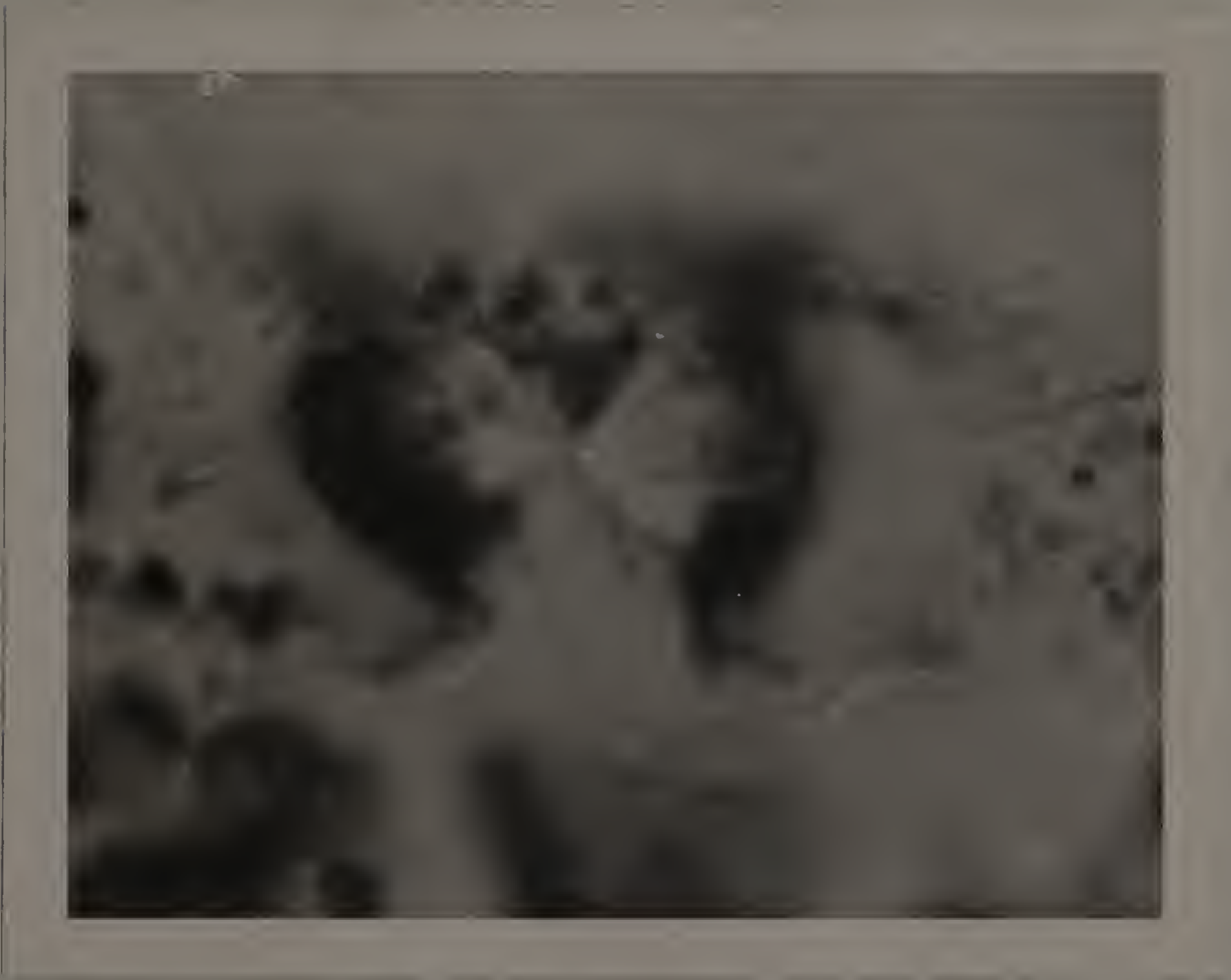

Fig 10. Cross-section showing lower surface of leaf treated with $AgNO_3$ in X-77 for 10 min. (250x).

Fig 11. Cross-section of lower surface of leaf treated with $AgNO_3$ in X-77 for 10 min. (250x).

Veinal areas

A preferential absorption of silver (after 30 min) or lead (after 60 min) was observed over veins and vein extensions when treating the lower surface (Fig 12). Absorption could be detected after just 5 min treatment with $AgNO_3$ (Fig 13). Accumulation was similar in anticlinal and periclinal walls of the lower epidermis below veins when viewed in cross-section (Fig 14). However, when viewed from the surface, uptake appeared in rows running the length of the veins (Fig 13). Close examination revealed that these rows were over anticlinal wall regions of the epidermal cells over veins. This same pattern of uptake occurred with lead.

Leaf discs were also treated with labeled sucrose and autoradiograms of the lower surface were developed. Again a linear or row absorption pattern over veinal areas developed (Fig 15). It was not possible to determine if this occurred over anticlinal or periclinal walls. When treating solutions of silver and lead without surfactants were used, veinal accumulation was rarely observed. However, when a surfactant was added to the treating solution, uptake into veins was widespread.

When treating solutions were applied to the upper surface of leaf discs, veinal area preferential absorption of silver and lead rarely occurred. The addition of surfactants did not enhance veinal accumulation, even up to 24 hr treatment.

Fig 12. Cross-section of leaf treated for 30 min with $AgNO_3$ in X-77. (25x).

Fig 13. Surface view of lower leaf surface over vein (arrow) treated with $AgNO_3$ in X-77 for 5 min. (250x).

Fig 14. Cross-section through lower epidermis over vein; treated with $AgNO_3$ in X-77 for 30 min. (250x).

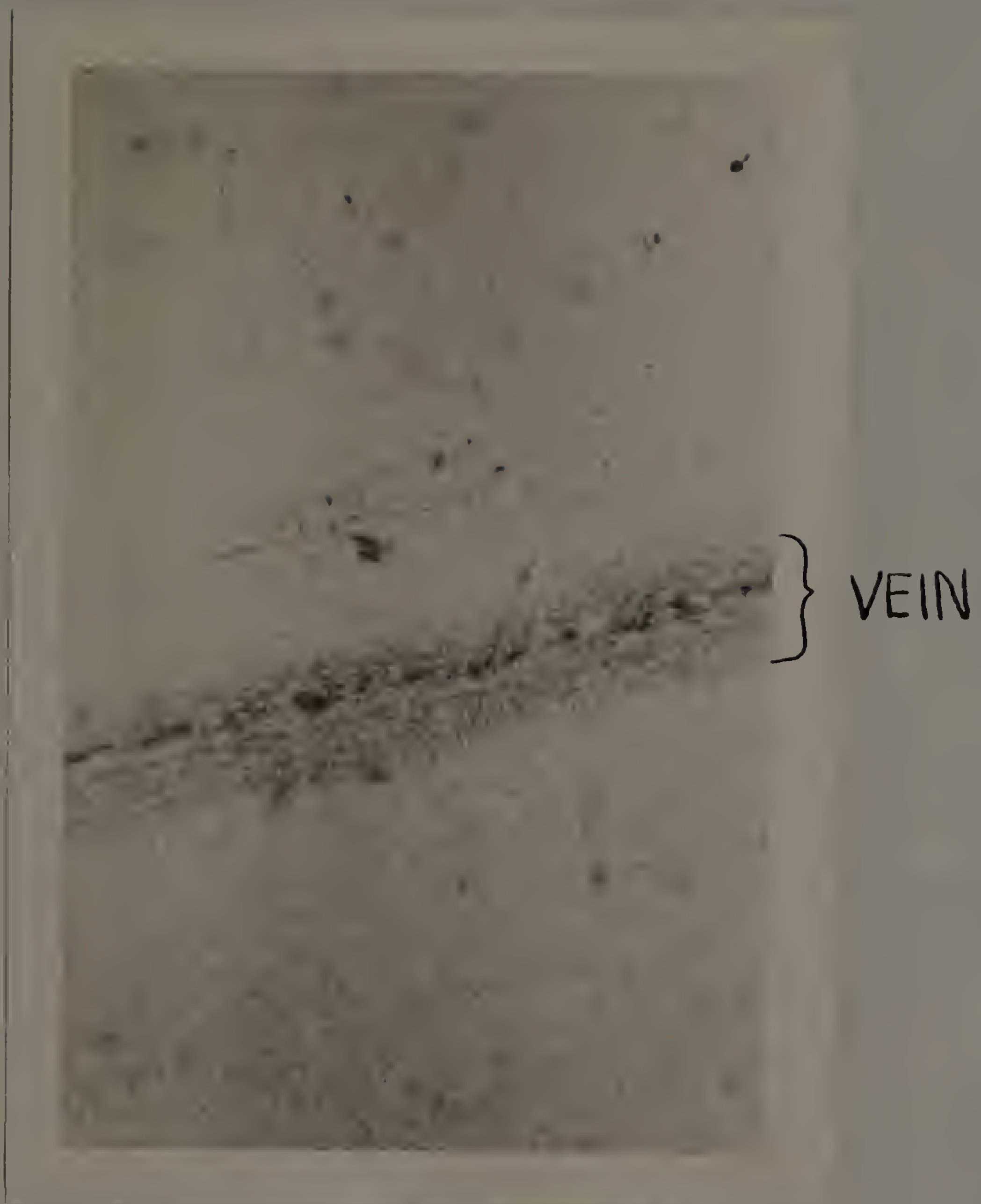

Fig 15. Autoradiogram of lower leaf surface treated with ^{14}C-sucrose in X-77 for 30 min. (100x).

Autoradiography of the labeled sucrose treated upper surface of leaves showed no accumulation of sucrose over veinal areas.

Periclinal vs. anticlinal walls

Penetration of silver and lead through the cuticle over "normal" epidermal cells occurred in both the upper and lower surfaces. The pattern of penetration through the lower surface appears unclear when viewed in cross-section. Accumulation of silver and lead sulfide was evenly distributed throughout the outer periclinal wall and the anticlinal wall of the epidermal cells (Fig 16). Greater accumulation often occurred along the inner periclinal wall (Fig 16). Surface views of silver accumulation showed an even distribution pattern of silver, suggesting no preference of uptake over anticlinal or periclinal walls (Fig 7, 9).

Cross-sections of the upper epidermis of leaves treated on the upper surface showed accumulation of silver and lead sulfide evenly throughout the outer periclinal wall (Fig 17). Anticlinal walls rarely showed accumulation.

Labeled sucrose and SADH followed very similar patterns of uptake. Surface autoradiograms of the lower surface treated with either ^{14}C-sucrose or ^{14}C-SADH showed a random distribution of silver grains over normal epidermal cells (Fig 15). Upper surface treatments yielded similar results. In cross-section, however, some differences did exist. SADH, like silver and lead, accumulated uniformly in the outer

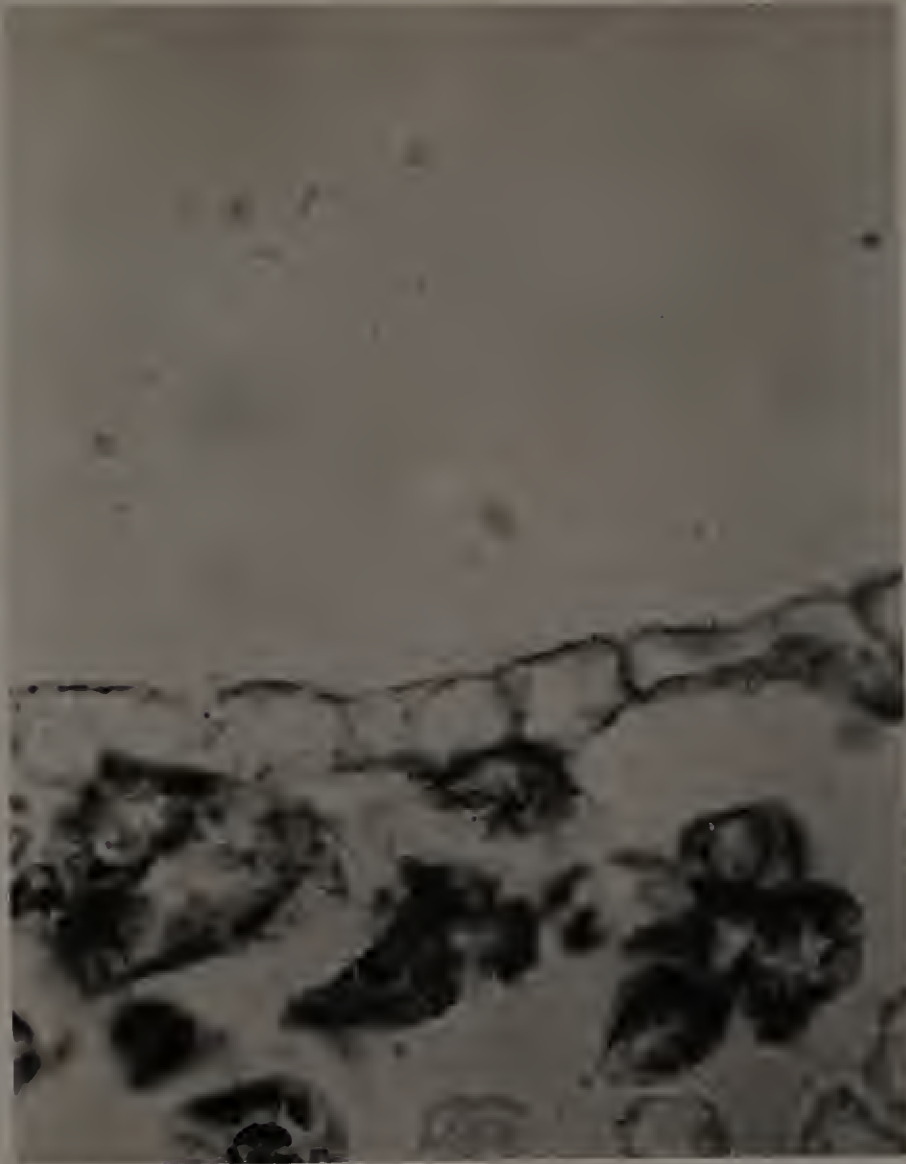

Fig 16. Cross-section of lower epidermis of leaf treated with $AgNO_3$ in X-77 for 30 min. (100x).

Fig 17. Cross-section through upper epidermis of leaf treated for 1 hr with $Pb(NO_3)_2$ in X-77. (100x).

Fig 18. Autoradiogram of cross-section of upper surface of leaf treated with ^{14}C-sucrose in X-77 for 1 hr. (100x).

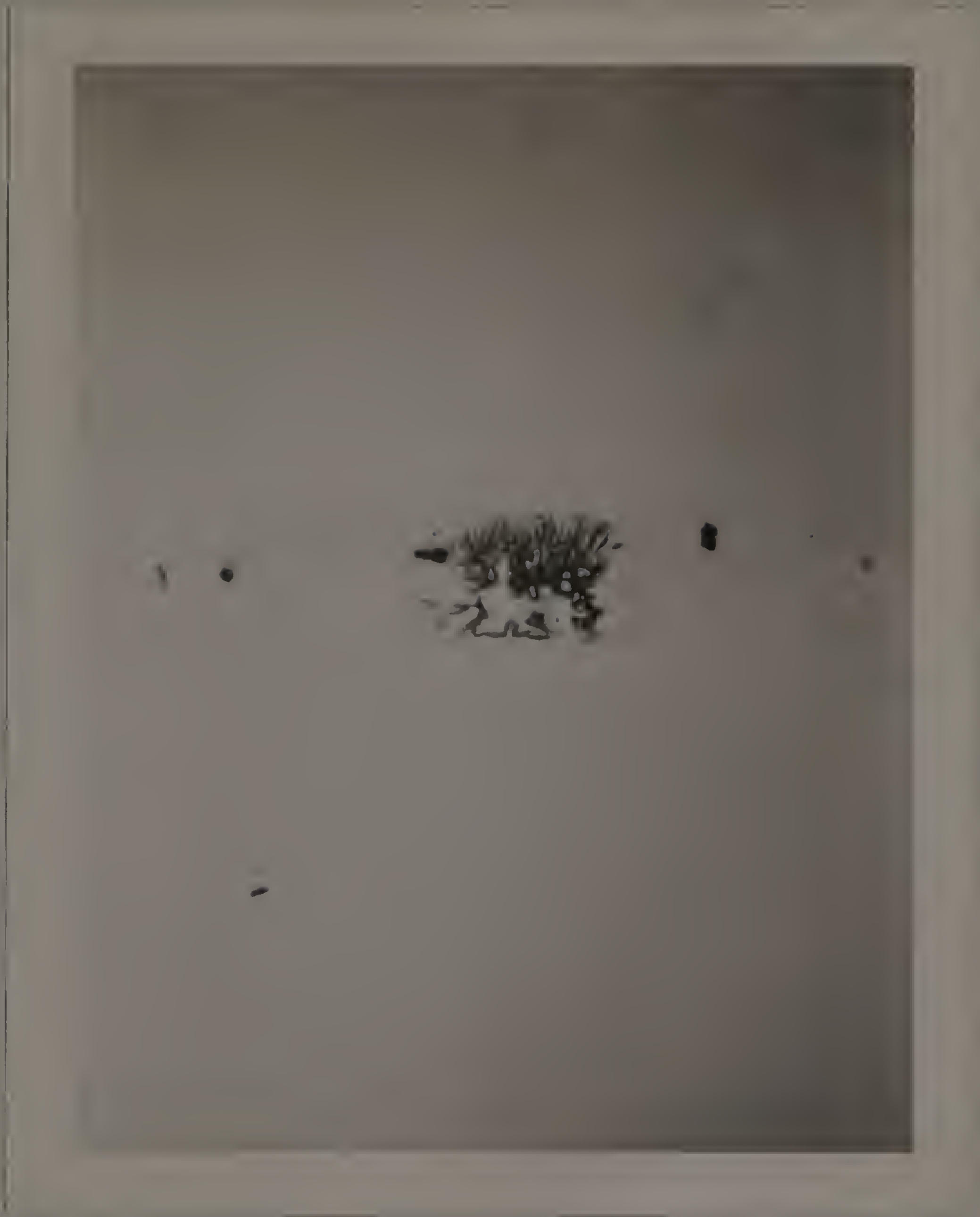

Fig 19. Cross-section of leaf artificially injured with razor blade, then treated with $Pb(NO_3)_2$ in X-77 for 1 hr via the lower surface. (25x).

periclinal wall with very little accumulation in anticlinal walls. Sucrose, on the other hand, was occasionally present in higher amounts in anticlinal than in periclinal walls (Fig 18).

Cracks and other artificial pores

Field grown leaves used in preliminary experiments showed occasional pockets or areas where mass flow of silver or lead into mesophyll tissue had occurred. These were unexplained since none of the above anatomical features were found associated with these areas of mass uptake. However, by artificially inducing cracks in the leaf surface, we could observe uptake very similar to that observed with field grown leaves (Fig 19), suggesting that cracks in the cuticle of field grown leaves could be responsible for the observed areas of mass uptake.

Stomatal penetration

No stomatal penetration could be observed from treating solutions lacking a surfactant. When X-77 was added to the treating solution, instances of stomatal penetration of silver could be found, but these were rare. Penetration was greatly enhanced and silver filled the apongy mesophyll tissue when Vatsol-OT was added (Fig 20). This enhanced stomatal penetration occurred more extensively in CO_2-deficient air than in normal air and rarely in CO_2-enriched air (Fig 21, 22). Stomatal penetration of lead was never

Fig 20. Cross-section through lower surface of leaf treated 10 min with $AgNO_3$ in Vatsol-OT. (250x).

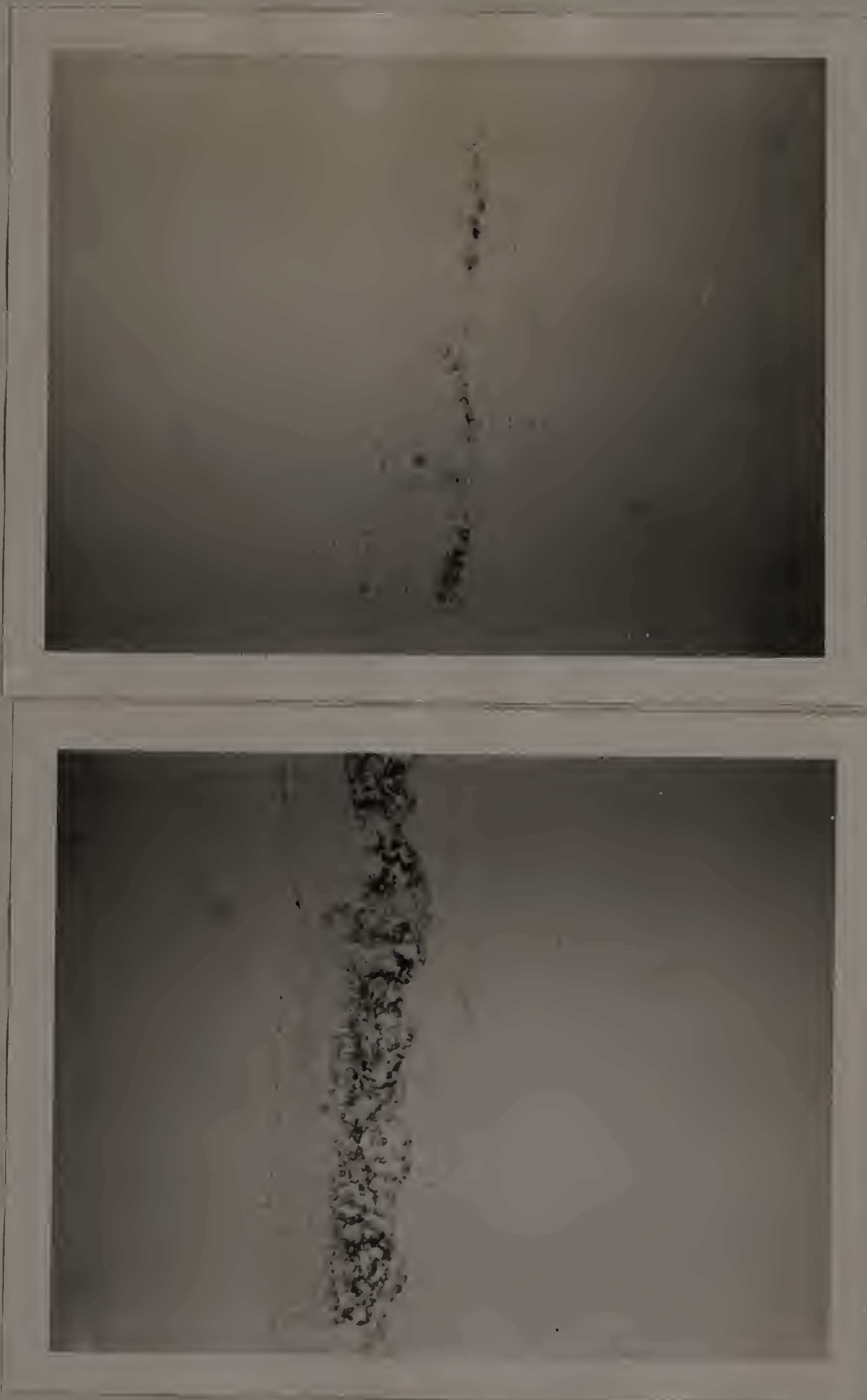

Fig 21. Cross-sections of leaves treated with $AgNO_3$ + Vatsol-OT for 10 min: Top ($+CO_2$); Bottom ($-CO_2$). (25x).

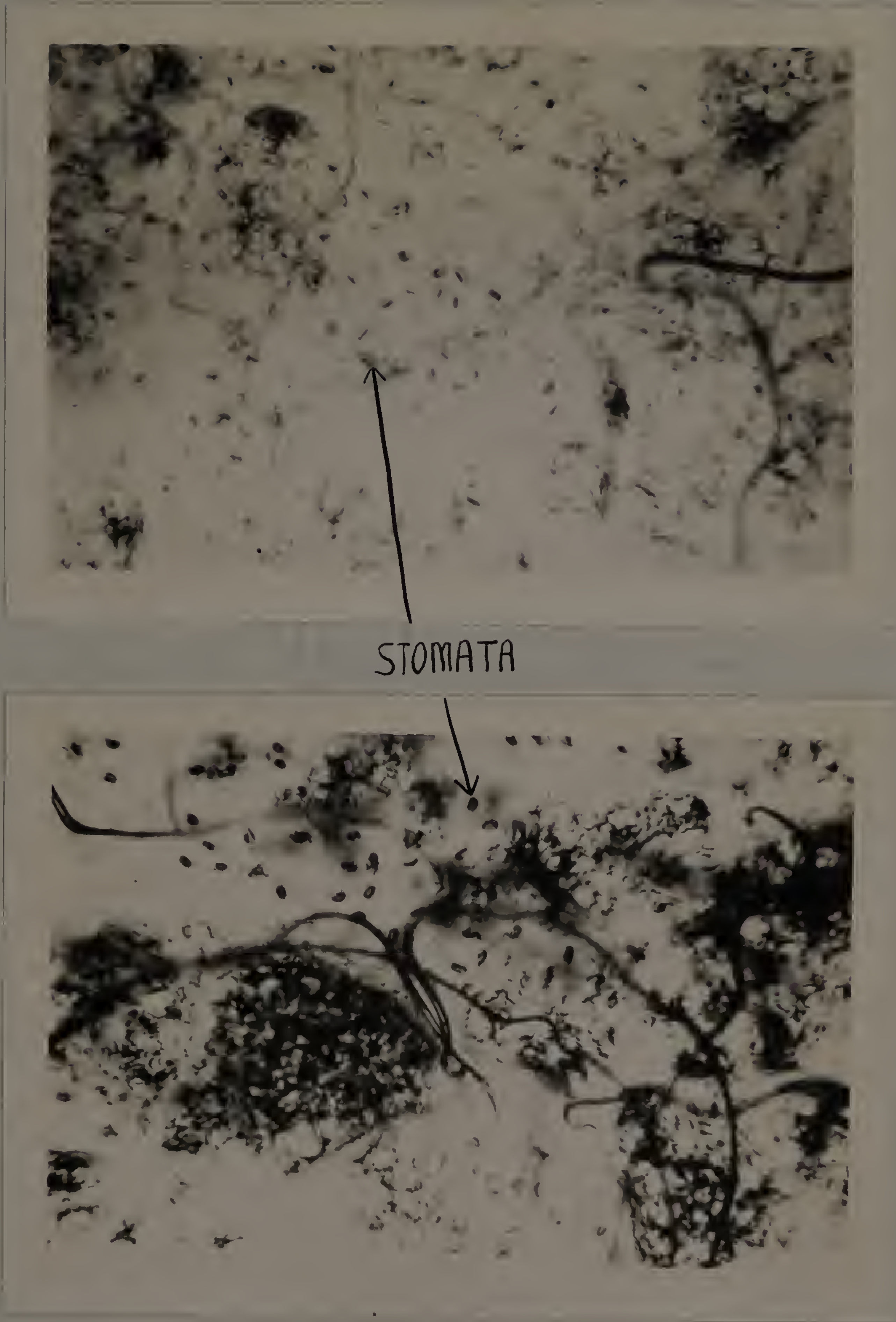

Fig 22. Surface views of leaves treated with $AgNO_3$ + Vatsol-OT for 2 min: Top ($+CO_2$); Bottom ($-CO_2$). (25x).

observed.

A time-course study of stomatal penetration under all conditions showed visual detection of stomatal penetration within 75 sec after applying $AgNO_3$. Dark spots appeared in the disc as silver filled the intercellular spaces of the spongy mesophyll.

Cross-sections of leaves stained with Sudans III and IV reveal a prominant pair of cuticular lips at the outer end of the aperture canal (at the surface) and a second less prominant pair at the base of the canal (projecting into the substomatal chamber). Also evident was an internal cuticle surrounding at least portions of the substomatal chamber.

Pectin pathways

A well defined layer of pectins continuous with the middle lamella of the epidermal cells was observed, (Fig 23, 24). This layer was somewhat thickened over anticlinal walls, but showed no extensions through the cuticle to the leaf surface (Fig 23, 24). No pectin canals could be found extending from anticlinal or periclinal walls over veins to the leaf surface (Fig 23, 24).

Ectodesmata

Ectodesmata (not definable cell wall structures, but areas of the cuticle permeable to polar compounds), demonstrated using Gilson fixative, were prominent surface features (Fig 25, 26, 27). Large accumulations of these

existed over guard cells, especially in the cell wall lining the stomatal pore, and to a lesser degree, over anticlinal walls (Fig 25). Leaf hairs were nearly devoid of ectodesmata. The lower surface over veins showed a marked accumulation of ectodesmata, and these were arranged in rows along the length of the veins along the anticlinal walls (Fig 26). In the upper surface over veins, this same pattern existed but apparent concentrations were not much higher than over surrounding tissue (Fig 27). Views of the upper and lower surface in both cross-section and in surface view showed a randomly scattered distribution of these ectodesmata with a much higher concentration in the lower surface than in the upper (Fig 27).

Fig 23. Cross-section of upper epidermis over vein extension stained with ruthenium red to show pectins. (250x).

Fig 24. Cross-section of upper epidermis of leaf stained with hydroxylamine-ferric chlorine to show pectins (arrow). (250x).

Fig 25. View of lower surface of leaf fixed in Gilson solution. (250x).

Fig 26. View of lower surface of leaf treated with Gilson solution. (100x).

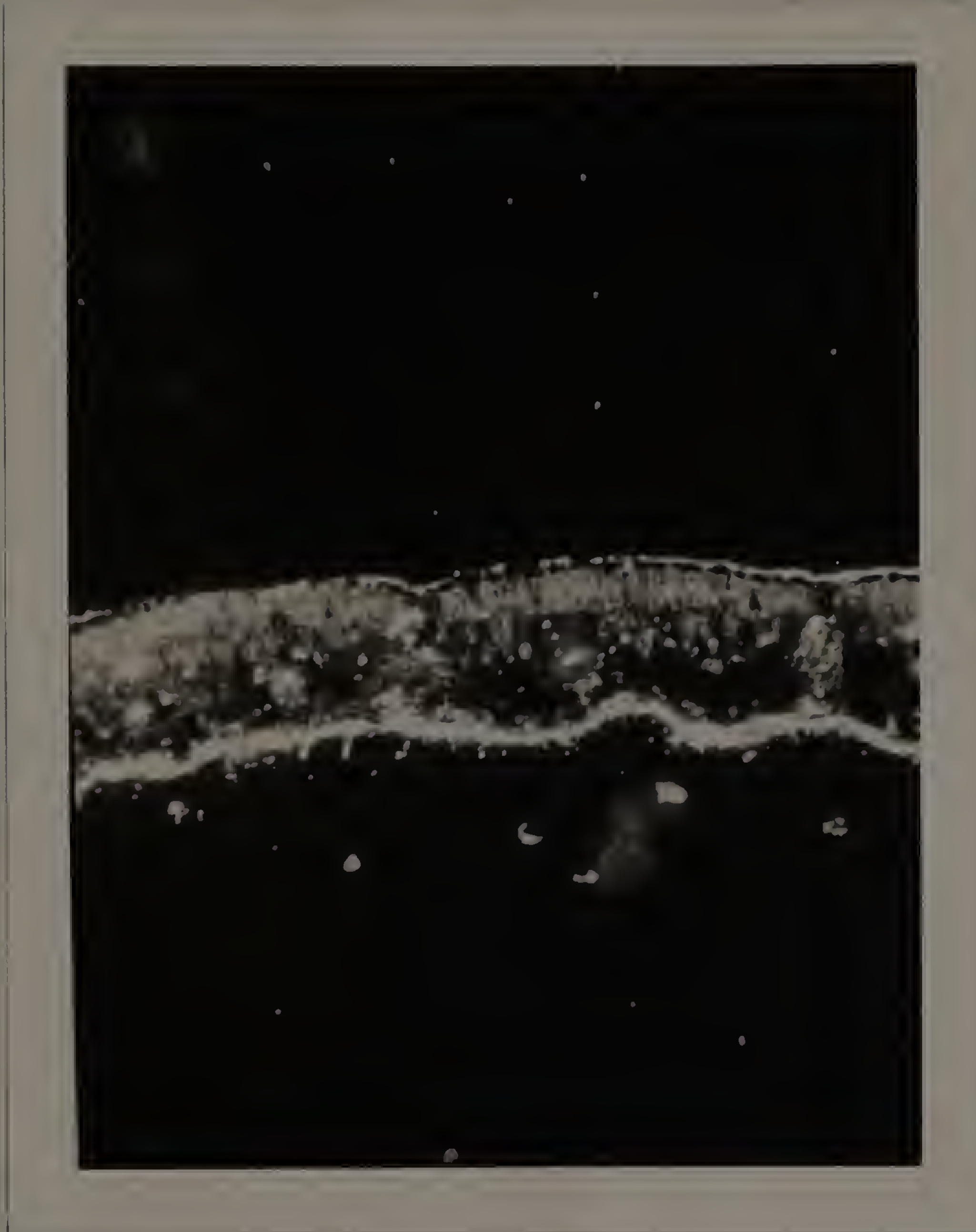

Fig 27. Dark-field photomicrograph of cross-section of leaf fixed in Gilson solution. (25x).

DISCUSSION

Cuticular penetration of water-soluble compounds is the primary mode of entry into the leaf. Various morphological structures and features of a leaf have been suggested as preferential sites of absorption. This report demonstrates preferential sites of absorption of water-soluble compounds in apple leaves and relates these to particular anatomical features.

Special features were investigated that were associated with areas of preferential absorption. The one feature that was consistently associated with these areas was the concentration of ectodesmata (Franke, 1967, 1969). Ectodesmata in this discussion are defined, not as specific cell wall structures, but areas of mercury precipitation in the cell wall (Schonherr and Bukovac, 1970a,b). Ectodesmata were concentrated in: (1) outer anticlinal walls of guard cells, (2) cell walls of the guard cells lining the outer vestibule of the stomatal cavity, and (3) anticlinal walls of epidermal cells over veins. The lower surface also had a much higher concentration than the upper. These areas have been shown to preferentially accumulate both silver and lead.

Schonherr and Bukovac (1970a,b) have suggested that ectodesmata are reflections of areas in the cuticle permeable to polar compounds i.e., mercuric chloride. One must also

consider the possibility that ectodesmata merely indicate localized areas of endogenous reducing substances within the leaf. Silver and mercury ions are polar compounds that are reduced within the leaf to metallic silver or mercuric chloride. Lead, the second ion used in this investigation, must be reduced by an exogenously supplied reducing substance. Since all three compounds exhibit similar penetration patterns, it is concluded that the distribution of reducing substances within the cell wall does not dictate the occurrence of ectodesmata, but rather their occurrence is dependent on areas of the cuticle permeable to polar compounds. It is suggested that both silver and lead patterns of uptake are a demonstration of the presence of ectodesmata in much the same way as the Gilson fixative is.

Regardless of the degree of stomatal opening, guard cells were important sites of absorption and accumulation. This is in agreement with Franke (1964) and Jyung _et al_. (1965). Only limited accumulation in the cuticular ledge area occurred when stomata were closed. Accumulation to a much greater extent was noted when stomata were open and a surfactant was included in the treating solution. Apparently, solutions with reduced surface tension accumulated in the outer vestibule (Fig 10), thus allowing uptake of large amounts of the applied compound through cell walls lining this outer vestibule. Increased absorption through cell walls lining this

chamber may be due to a more polar nature of the cuticle in this region (Turrell, 1947). Preferential accumulation of lead and silver in both anticlinal walls of the guard cells and the walls inside the outer vestibule were closely associated with high concentrations of ectodesmata.

Preferential uptake in the veinal region shown herein is similar to reports by Hull (1964) and Dybing and Currier (1961). Because of the relatively short distance a compound must move to enter the translocation system, uptake over the veins could provide an important mode of entry of a compound into the plant. The importance of ectodesmata being concentrated in the veinal region has been stressed. Further, a penetrating molecule may meet less resistance while diffusing through the cuticle since embedded waxes are discontinuous and less highly oriented over veins (Norris and Bukovac, 1968).

Roberts *et al.* (1948) reported the existence of pectin pathways in McIntosh apple leaves extending from the surface of the cuticle to veins or vein extensions. Such pathways could provide an easy route of entry of water soluble compounds into the leaf. The inability in this investigation to demonstrate pectin pathways in McIntosh apple leaves and the negative results of Norris and Bukovac (1968) shed considerable doubt on their importance. Although the existence of pectin pathways has not been disproved, their occurrence must be considered rare. Therefore, if pectin

pathways exist, they must be considered unimportant in foliar penetration.

Epidermal hairs have several features that make them suitable as sites of preferential absorption. They have a thin cuticle (Martin, 1966) and rise above the hydrophobic leaf surface. Large numbers are present over veins, so penetration into leaf hair protoplasts could mean rapid accumulation in veins with subsequent translocation. Rapid and substantial accumulation of silver and lead occurred in epidermal hairs. Contrary to that found for other preferential sites of absorption, concentrations of ectodesmata were no higher in epidermal hairs than in other epidermal cells.

Preferential absorption into the cuticle over anticlinal walls of epidermal cells has been reported (Dybing and Currier, 1961). However, in this investigation penetration into both periclinal and anticlinal walls was similar. Accumulation occurred often along the inner periclinal wall of epidermal cells. It is suggested that penetration through the cuticle occurred uniformly over anticlinal and periclinal walls. Molecules that entered over the periclinal walls moved to the pectin layer and then migrated laterally to the anticlinal regions between epidermal cells. This may be substantiated by the randomly scattered distribution of ectodesmata over these cells.

Penetration of foliar applied compounds through the lower surface has been shown to be greater than through the upper surface (Fogg, 1948; Greene and Bukovac, 1971; Sargent and Blackman, 1962, 1965). Results in this report support these findings. The upper surface of the apple leaf lacks both stomata and epidermal hairs, and the absence of these could have a marked effect on total uptake. Penetration over veins was greatly reduced in the upper surface, perhaps due to decreased numbers of ectodesmata. Norris and Bukovac (1968) have shown that there is a continuous layer of highly oriented, embedded waxes in the cuticle of the upper surface which would further restrict the penetration of water-soluble compounds.

Two types of stomatal penetration were observed in this investigation. (1) Approximately one third of the stomata in leaf discs treated with a solution containing X-77 showed accumulation of silver in the outer vestibule of the stomatal pore. Silver ions then moved readily into the guard cell walls. Nearly all of the stomata in leaf discs receiving solutions containing Vatsol-OT showed some accumulation in the outer vestibule. (2) Less extensive was the movement of treating solution through the stomatal pore and accumulation in the substomatal chamber.

Movement of aqueous solution through the stomatal pore into the substomatal chamber was dependent upon (1) the

addition of a surfactant to the treating solution to lower surface tension and (2) the degree of stomatal opening. Solutions containing Vatsol-OT and having a low surface tension (25 to 30 dyne cm^{-1}) showed only occasional instances of stomatal penetration. No penetration was shown for solutions lacking a surfactant. The maximum stomatal penetration occurred in leaves that had been treated under light in a CO_2-deficient atmosphere to encourage large aperture widths.

It has been shown with Zebrina leaf stomata that the size of the stomatal aperture, per se, is of little importance in stomatal penetration of aqueous solutions (Schonherr and Bukovac, 1972). Rather, surface tension of the treating solution and stomatal pore morphology are the important factors. It is further pointed out for Zebrina that the surface tension of a treating solution must be reduced below 30 dyne/cm^2 for infiltration to occur. Below this point the contact angle of the liquid formed with the stomatal pore surface would be less than the wall angle and spontaneous infiltration would occur. In this investigation stomatal penetration from a solution containing X-77 (approx 32.5 dynes/cm^2) was observed. It was also observed that stomatal penetration occurred more extensively in leaf discs with stomata having larger aperture widths. The results reported herein and those of Greene and Bukovac (1973) would support the idea that increasing the size of the aperture width would at the

same time increase wall angles in the stomatal pore. This in effect would raise the threshold surface tension required for spontaneous infiltration of the stomata. At any given point, leaf stomatal aperture widths are not uniform. It has been shown that the distribution of aperture widths follows a normal curve (Greene, 1969). It is concluded that pore size may be important in determining stomatal penetration, undoubtedly through regulation of wall angles of the stomatal pore. The increasing percentage of stomata infiltrated with decreasing treating solution surface tension would be dependent on the number of stomata open to a size at which the wall angle was equal to or less than the contact angle of the solution. Thus, increasing surface tension (contact angle) would result in smaller percentages of stomata whose degree of opening (wall angle) allowed spontaneous infiltration.

Stomata could be reliably opened only under conditions of high humidity, constant temperature, and high light intensity. Under field conditions with fluctuating environmental conditions it appears unlikely that stomatal penetration is a major pathway of entry of solutes.

In conclusion, preferential uptake of four water-soluble compounds into guard cells, epidermal hairs, and over veinal areas was found. Absorption through the lower surface was greater than through the upper surface and surfactant dependent.

Stomatal penetration was concluded dependent on stomatal aperture width and the surface tension of the applied solution.

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