ABSTRACT

FOLIAR PENETRATION AND TRANSLOCATION OF SUCCINIC ACID 2,2-DIMETHYLHYDRAZIDE (SADH)

By

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Factors affecting foliar penetration of succinic acid 2,2-dimethylhydrazide (SADH) into bean primary leaves (Phaseolus vulgaris L., cv. "Blue Lake") and translocation of foliar applied SADH using apple (McIntosh) and peach (Suncling) trees were studied.

A direct approach using ^{14}C-SADH and an excised leaf disk system and an indirect approach using a biological response as index of penetration were utilized. In the direct approach, ^{14}C-SADH solution was added to a small glass tube affixed to the surface of leaf disks kept in a controlled specified environment. This allowed penetration studies under constant conditions (light, temperature, humidity, pH and concentration of SADH and surfactant). An indirect measurement of SADH penetration was achieved by applying SADH in various formulations to bean primary leaves and inhibition of internode elongation was utilized as an index of penetration. The rate of penetration was studied by detaching the treated leaves after specified time intervals.

Translocation of SADH in one-year-old, potted apple and peach trees with two shoots each was studied by spraying one of the two shoots with Alar 85. The growth of the treated and non-treated shoots of the treated trees was compared with each other and also with the growth of comparable shoots of control trees (non-treated).

Foliar penetration of ^{14}C-SADH was increased by increasing light intensity (up to 3,600 ft.c.), temperature (5-40° C) and surfactant (Tween 20) concentrations (up to 0.1%). The effects of light, temperature and surfactant were related and partially additive. Penetration was greater with treating solutions of a pH below rather than above the pK_a. This pH effect was observed in the light and in the dark, however, it was reversed in light, when Tween 20 was added to the treating solution. Penetration was linear with SADH concentration from 10^{-4} to 10^{-2}. Osmotic stress and inhibitors (NaN_3, 2,4-dichlorophenoxyacetic acid, phenylmercuric acetate) inhibited penetration of SADH in light but not in the dark. Penetration was related to stomatal density and stomatal opening.

From a theoretical treatment of the problem of entry of liquids into the intercellular air space of leaves through open stomata, and from direct and indirect evidence, it appeared that treating solution

entered the aperture of open stomata but not the intercellular space.

Sites of entry for polar molecules into bean leaves (polar pathways) were established using $AgNO_3$ as tracer. These sties were localized along the cuticular ledges of guard cells, the walls of the guard cells, glandular trichomes and anticlinal walls. The participation of these pathways appeared to be modified by light and Tween 20.

From the data it was concluded that penetration of SADH into bean leaves was not directly linked to metabolism. The above factors affected penetration of SADH by affecting (a) the area of the leaf surface in contact with treating solution (effective surface), that is surface through which penetration can proceed, and (b) affecting the permeability of the effective surface.

The effective surface was increased by all factors which enhanced wetting and led to or increased stomatal opening. The walls of the guard cells inside the stomatal aperature appeared to be much more permeable than the remainder of the leaf surface. Furthermore, the permeability of the cuticular ledges seemed to be greater in open than in closed stomata.

Tween 20 and pH of the treating solution influenced penetration of SADH similarly in the experiments using inhibition of internode elongation as an index of

penetration. The rate of penetration was greater with a treating solution of pH 3.0 or 5.0, than of pH 7.0. Tween 20 added to the treating solution also increased the rate of penetration with little effect on the total penetration, which was almost complete after 12 hours whether the solution contained Tween 20 or not. Besides enhancing the rate of penetration, surfactants also reduced retention of SADH solution by leaves (15 - 25%) and promoted internode elongation of bean plants by up to 60%. This promotion was overcome with SADH.

SADH was not or only slightly translocated in apple and peach trees as indicated by the lack of growth inhibition of the non-treated shoots of the treated trees, whereas shoot elongation was completely inhibited on treated shoots. Growth of the non-treated shoots of the treated peach trees was significantly greater than the growth of comparable shoots of control trees.

The concept of ectodesmata with respect to their suggested role in foliar penetration of polar compounds was critically reviewed. It was concluded that the demonstrability of ectodesmata is closely linked to the occurrence of polar pathways in the cuticular membrane. Such pathways seem to be necessary for penetration of Hg^{++} ions, which are essential in demonstrating ectodesmata.

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INTRODUCTION

The introduction of the polar growth retardant succinic acid 2,2-dimethylhydrazide (SADH) and its promise in commercial horticulture has created a need to attack anew the problem of foliar penetration of polar compounds in general and the problem of polar pathways across the cuticular membrane specifically.

The literature was reviewed with emphasis on (a) leaf structure affecting penetration of polar compounds, (b) permeability of the cuticular membrane to water and other polar molecules and (c) polar pathways across the cuticular membrane. Special attention was given to the participation of stomata and ectodesmata in foliar penetration of polar compounds.

The experiments were aimed at (a) elucidation of the major factors affecting foliar penetration of SADH, (b) relating these factors to morphological and physiological characteristics of the leaves and (c) finding the mechanisms in which these factors affect the polar pathways in the cuticular membrane and the foliar penetration of SADH.

In addition, translocation of foliar applied SADH in selected woody plants was studied to determine the

significance of coverage in application of SADH as a foliar spray.

LITERATURE REVIEW

Succinic Acid 2,2-Dimethylhydrazide

Succinic acid 2,2-dimethylhydrazide (SADH) and maleic acid 2,2-dimethylhydrazide (MADH) were reported by Riddell et al. (90) as broad spectrum plant growth retardants with potential application in the control of plant growth and development. Since MADH was not stable in aqueous solutions, due to intramolecular hydrolysis of the acid (19), SADH has been used almost exclusively in subsequent investigations.

SADH is the active ingredient of the commercial products Alar 85 and B-9. In earlier publications SADH was also referred to as experimental compound B-995 and N,N-dimethylaminosuccinamic acid (DMAS). These names have been used interchangeably in the literature. It should be noted, however, that both Alar 85 and B-9 are formulated with a surfactant (121).

Since its introduction in 1962, many experiments involving SADH have been performed and an extensive literature has been accumulated. Investigations have centered on two topics: (a) effects on growth and development on various plants, and (b) mode of action. Very little is known about other aspects such as foliar

penetration and distribution in the plant, both important with respect to the practical application of a foliar applied growth retardant.

The SADH literature has recently been reviewed, particularly with respect to growth responses (15, 56, 122) and the mode of action (56). Therefore, this review will be confined to publications dealing with foliar penetration of SADH and its translocation and stability in the plant.

Methods of Application

SADH is most commonly applied as a foliar spray. Other methods of application may also be effective, e.g. trunk injection, root absorption from a SADH solution (76) or soil medium (7), uptake through a severed stem or petiole (75) and application to the plumule (131). Thus, SADH can penetrate or be taken up by both roots and above ground organs. However, most of these methods of application are feasible only under laboratory conditions and, since SADH is not stable in soil (76, 121), the most effective commercial mode of application is as a foliar spray.

SADH has been employed as foliar sprays at concentrations ranging from 500 to 50,000 ppm. In tree fruit, 1,000 to 4,000 ppm were most commonly used and within this range the response increased with increasing

concentration (24, 30, 51). Higher concentrations than 4,000 ppm did not induce a greater response, but a second application two weeks after the first prevented resumption of growth of apple trees later in the season (30). Even when used in extremely high concentrations (50,000 ppm) SADH usually was not phytotoxic. Petunias showed no leaf injury when sprayed with 5,000 to 50,000 ppm SADH, though a white residue remained on the surface (14). Similarily, 10,000 ppm applied to the foliage of apple trees did not cause leaf injury (24). There is one report, however, of leaf injury in the peach following application of 5,000 ppm SADH as a foliar spray (25).

Foliar Penetration

High concentrations of SADH are required to produce a biological response. This may suggest that (a) SADH penetrated into the leaves poorly, (b) translocation to the site of action was limiting or (c) both.

There are indications that SADH does not readily penetrate into the leaves. SADH was more effective in inhibiting shoot growth of apple when applied with than without a surfactant (24). Further, complete inhibition of flower formation in Pharbitis nil was obtained with only 100 ppm SADH when administered to the roots in the nutrient solution (131). A comparable effect was obtained by application of 100 µg to the plumule or 300 µg

to the cotelydons (131). The growth response of petunias indicated that washing the foliage within 24 hours after spray application (5,000 ppm) removed most of the chemical (14). Penetration into fruits also appears limited, 60% of the ^{14}C-SADH applied to McIntosh fruits penetrated within four days (26).

Translocation

Martin et al. (75) reported that SADH was rapidly translocated in apple trees and seedlings. Since SADH is highly water soluble, these authors suggested a passive translocation in the transpiration stream and concluded that "coverage should be of less importance." However, following stem injection of ^{14}C-SADH most of the ^{14}C accumulated in the leader directly above the point of injection and very little was found in other leaders (75). That SADH might not be readily translocated in pear trees following foliar application was reported by Wertheim and van Belle (125). When only the top of the tree was sprayed, growth was reduced in the treated shoots but not in those in the non-sprayed lower part of the tree.

Stability in Plants

SADH appears to be quite stable in plants. Comparing the rate of breakdown of SADH, ^{14}C-labeled in two different positions (succinic acid and methyl groups),

Martin and Williams (76) found that degradation was slow and gradual, irrespective of the position of the label. A decrease in radioactivity was found in the ethanol extractable fraction, while the radioactivity in the residue remained constant. The bulk of the label in the extractable portion was SADH. Of the label remaining in the residue after 24 hours extraction with 80% ethanol, 60% was still identified as SADH. Measurements of CO_2 evolution from "Red Delicious" seedlings treated with ^{14}C-SADH revealed a constant loss of $^{14}CO_2$ over 7 weeks, which amounted to approximately 20% of the original dose.

The great persistance of SADH in apple trees was confirmed by Edgerton and Greenhalgh (26). Following foliar application in June or August significant levels of ^{14}C-SADH were found in dormant tissue in January, especially in flower buds. Samples taken 6 months later, in July, revealed traces of ^{14}C-SADH in spur tissue, cluster bases and vegatative buds totaling approximately 10% of the levels found in dormant tissue.

Foliar Penetration of Polar Compounds

Whenever biologically active compounds are applied to plants, the dose is of critical importance. This applies for the use of growth regulators as herbicides as well as to their application in controlling growth and development of arable crops.

Most commonly, these compounds are sprayed on the leaves, but the amount of the growth regulator administered to the leaf surface often does not bear any strict relationship to the amount penetrating into the tissue. The portion of the spray deposit which penetrates into the leaf is dependent upon a number of plant related factors; namely, cuticle structure and chemistry, amount and orientation of waxes, the presence of stomata and trichomes and the physiological condition of the plant in general. Environmental factors also alter penetration: by influencing foliar characteristics (e.g. cuticular waxes), the physiological condition of the plant during the penetration period (turgescence, metabolic activity) and the spray performance on the leaf surface (drying time). Physical and chemical properties of the growth regulator as well as of the formulation may also be of great importance.

The literature on foliar penetration of growth substances has been reviewed on a number of occasions. Van Overbeek (81) discussed foliar characteristics in reference to uptake and translocation of auxins. Foliar penetration of herbicides was reviewed by Currier and Dybing (17) and the role of stomata was stressed by Dybing and Currier (22). The importance of plant surface characteristics in relation to foliar penetration was stressed by Crafts and Foy (16) and Foy (33). More recent reviews

were prepared by Hull (60) and Sargent (95). The role of ectodesmata in foliar penetration was discussed by Franke (43, 44).

Since most of the growth regulators studied were relatively lipophilic, the problem of foliar penetration of polar compounds has received little attention. Alternate polar and apolar pathways across the cuticular membrane have been suggested (16, 91) in an attempt to explain the empirical fact that inorganic ions and other polar compounds penetrate the lipophilic cuticular membrane. Little is known concerning the nature of the suggested polar pathways. Insect punctures, cracks in the cuticle, ectodesmata and entry through the open stomatal pore have been suggested, but direct evidence of their participation and relative importance in foliar penetration of polar molecules is scarce.

Leaf Structure as Related to Foliar Penetration of Polar Molecules

Epidermis

The term epidermis designates the outermost layer of cells on the primary plant body (28). The epidermis of leaves may differ in form, composition and function. In pear, for instance, the epidermis of the upper surface is composed of one type of cells while the epidermis of the lower leaf surface contains guard cells in

addition (79). The amphistomatous leaves of Phaseolus vulgaris have guard cells on both surfaces, which contain chloroplasts while the common epidermal cell is lacking chloroplasts. Bean leaves have in addition glandular and conical trichomes on both leaf surfaces (21).

The cuticle covers the entire epidermis. It extends over epidermal cells, guard cells, accessory cells, trichomes (28) and into the substomatal chambers (4, 79, 85). It seems, now, that a cuticle is generally formed where cells are in contact with the atmosphere (103).

The cuticle is continuous and non-cellular and its thickness and composition varying widely (28). Associated with its main function, the reduction of uncontrolled water loss to the surrounding atmosphere, it is relatively impermeable to water and consequently other polar molecules. Permeability to polar compounds is further restricted by epicuticular waxes which occur in some plant species (96).

Epicuticular Waxes

In some species, the cuticular membrane is covered with epicuticular waxes of varying configurations (3, 63, 64, 78). The continuity and the physical arrangement of the waxes is important with respect to cuticular penetration and spray retention. A continuous wax layer

over the entire cuticle may seriously impair penetration of polar molecules, wettability and spray retention, especially if the waxy covering is rough (2, 23).

Quantitative differences in waxes exist between the upper and lower surfaces. In pear leaves more surface wax was found on the lower than on the upper surface (79). The structure of the epicuticular wax has been shown and described for a number of species and the problem of the formation of the waxy coverings has been discussed repeatedly (60, 63, 64, 68, 96). The subject is still one of great controversy, particularly the question whether or not wax or wax precursers are excreted through pores in the cuticle. With respect to penetration of polar compounds it is, however, immaterial whether or not such pores exist, because they would constitute an apolar rather than a polar pathway. The question of continuity of wax layers remains open, although it appears that most waxy coverings are not continuous films but rather crystallin bodies such as platelets, rodlets, granules or columns (3).

Juniper (63) found less wax on the cuticle of guard cells of pea leaves, while Hull (60) showed dense wax platelets on the guard cells of Prosopis juliflora. In tulip leaves even the cuticular ledges were covered with surface wax (3).

The formation of epicuticular waxes of leaves of Pisum sativum was found to be affected by environmental conditions (64). No surface wax was observed on plants grown in the dark but it formed rapidly on exposure to light. Within 24 hours after transfer to light minute quantities of wax appeared on the adaxial surface and six days later the waxy layer was complete. No changes took place thereafter. Wind velocity also influenced size and density of the wax structures on pea leaves.

Parallel with the formation of epicuticular wax was a dramatic increase in contact angle of water droplets. In dark grown plants the contact angle was 68° and reached a value of 140° after surface waxes were formed.

Leaves of Brassica oleracea and Chrysanthemum segetum regenerated the surface wax if wiped off before leaf expansion was complete (64). This is in agreement with the findings of Schieferstein and Loomis (96). Young expanding maize leaves regenerated their surface wax layer following dewaxing, but fully expanded leaves failed to do so. When the wax was removed from the growing region of a leaf it was replaced along the margins but not in the center (over periclinal wall) of the epidermal cells.

The amount of surface wax of apple leaves was found to decrease with increasing leaf age (74). In "Bramley's Seedling" only half as much wax per unit leaf surface

could be removed from old leaves (52 cm^2) than from young, not fully expanded leaves (21 cm^2). There was more surface wax on the lower than on the upper surface.

Cuticular Membrane (CM)

Using staining techniques, polarized light microscopy and, more recently, electron microscopy, the structure and chemical composition of the cuticular membranes of a variety of plants have been investigated. With the exception of the pear leaf cuticle (79) studies have been carried out on plants of little economic importance such as Clivia (46, 77, 93), Hedera helix and Nicotiana glauca (108, 96), Yucca and Dasyliron (77), Ficus elastica (107) and Philodendron scandens (8). Thus, most of the currently available information on the structure and chemistry of CM's is from more or less xerophytic plants. However, most of the economically important plants are mesophytic and plants of this type have been used predominantly in studying foliar penetration. This has led to the situation that results obtained from mesophytic species tend to be interpreted on the basis of cuticular characteristics of xerophytic plants such as Clivia or Ficus.

With this in mind, cuticular characteristics in reference to permeability will be discussed next. To avoid confusion, the nomenclature suggested by Sitte and

Rennier (107) will be adopted. They proposed the term "cuticle" (proper) for the outermost or oldest layer of the CM. Other cutin containing layers are termed "cuticular layers" whether they contain cellulose or not. Cuticle proper and cuticular layers together represent the cuticular membrane (CM) which can be isolated in some species by digesting the pectin layer which separates the CM from the underlying cellulose wall (80, 127).

The CM appears relatively homogeneous following staining with Sudan stains or under the electron microscope (8, 46, 107), while two or more different layers can be distinguished with polarized light. Of the major components the cutin matrix is isotropic or sometimes weakly negative birefringent (46, 48, 93, 94, 107), oriented cuticular waxes are mostly negative and cellulose (if it occurs) positively birefringent. Embedded oriented waxes and cellulose micelles render the otherwise isotropic CM birefringent. A special case of birefringence is the so-called form double refraction exhibited by a porous solid body (Wiener'scher Mischkörper) (46). Form double refraction can be eliminated by imbibition with a liquid having the same refractive index as the solid body.

The CM of the upper surface of pear leaves exhibited a pronounced zone of negative birefringence

under a narrow isotropic band, indicating the presence of embedded oriented waxes (79). This double refractive wax layer was continuous and had a definite structure, the wax being layered with intervening bands of isotropic material. The degree of birefringence decreased over anticlinal walls and appeared broken over veinal tissue. the embedded wax of the CM of the lower leaf surface showed a lesser degree of orientation.

Oriented embedded waxes have been reported to occur in the CM of many species including Fagus silvatica, Betula pendula, Prunus laurocerasus, Agave americana, Ficus elastica, and Hedera helix (107). In some cases some residual negative biregringence remained even after prolonged extraction with wax solvents (93), which led to the hypothesis that perhaps some of the wax molecules are chemically bound in an oriented position to the cutin matrix.

There is indirect evidence that embedded waxes are located in pores of the cutin matrix (68). Following extraction of the embedded waxes, the remaining cutin skeleton either collapsed (107) or exhibited form double refraction which disappeared on imbibition with a suitable liquid (e.g. benzene).

For Fiscus elastica it has been shown (107) that these pore spaces exist prior to the depostition of wax into the CM. The youngest leaf was covered with a very

thin isotropic membrane. The CM of the next older leaf showed negative form double refraction in its central portion which disappeared on imbibition. During the next stage, the CM gained in thickness by means of interposition of cutin between the already existing CM and the cellulose wall. Up to this stage the CM contained very little wax. The last step in the development was an intensive excretion of wax into the pore space of the now fully developed CM, rendering the oldest portion of it the richest in wax.

Working with Philodendron scandens, Bollinger (8), found that the CM of leaves was already covered with a wax layer when the leaves unfolded and therefore he proposed that cutin and wax were excreted simultaneously.

The CM of Nicotiana glauca increased in weight per unit area until the leaves were fully expanded, but not thereafter (108). The total wax content of the CM increased until the leaves were fully expanded and then decreased. The same was true for Hedera helix and Agave americana (96, 108).

Some data are available concerning the relative amounts of surface and embedded waxes. In pear leaves the amount of embedded waxes was the same for the CM of the upper and the lower leaf surface but the amount of epicuticular wax was twice as great on the lower, than on the upper surface (79). In leaves of Nicotiana glauca

80% of the total wax was epicuticular and only 20% was embedded in the cutin matrix. In Agave americana 95% of the total wax was embedded in the CM. Agave and Nicotiana differed also in the chemistry of the waxes. One third of the total waxes of N. glauca were volatile while practically no volatile wax occurred in A. americana (96).

A distinct polarity gradient exists in the CM. Epicuticular waxes, a high degree of polymerization of the cutin of the cuticle proper and oriented embedded waxes render the outermost layer of the CM quite lipophilic. The CM becomes more hydrophilic towards the pectin layer and the cellulose wall, due to a lesser degree of polymerization of cutin and to cellulose fibrils and pectic materials associated with the cutin matrix.

The thickness of the CM is variable. It is generally thinnest over the periclinal walls of the epidermal cells and thickest over the anticlinal walls. The CM can extend deeply between the anticlinal walls as shown for Clivia nobilis (46) and pear leaves (79). Structure and composition of the CM over the anticlinal walls are not well understood. Negative birefrigence due to embedded oriented waxes was reduced compared to the CM over periclinal walls, indicating a lesser degree of orientation or absence of waxes (46, 79). The

anticlinal ribs of the CM of pear leaves stained only weakly with Sudan stains and showed considerable staining with Ruthenium red (79). This staining pattern suggests that fewer non-polar lipids are present in the anticlinal ribs and more polar groups than in the CM over periclinal walls. It should be noted that Ruthenium red is not a specific pectin stain, since hemicelluloses, alginic acid, proteins, cutin precursors and polysaccharides that contain sulphate may also be stained (94). Thus, it cannot be concluded from the Ruthenium red staining alone that the anticlinal ribs contain pectins.

The upper CM has been considered to be thicker than the lower (33, 80). In pear leaves, however, the CM was thicker on the lower than on the upper leaf surface and the CM overlying veinal tissue was also thicker than that over interveinal areas (79).

Permeability of Isolated Cuticular Membranes

A great advantage in studying the isolated CM is that certain variables can be better controlled. However, if data obtained from such systems are to be extrapolated to "in vivo" systems it must be established that the two systems are strictly comparable.

CM's have been isolated using different methods such as chemical isolation with oxalic acid (92) or

enzymatic isolation (80, 96, 108, 127, 128, 129, 130). Only for CM's isolated according to Orgell (80) have structure, composition and binding capacity of the isolated CM's been tested against fresh, non-isolated CM's (12, 79, 127, 128). From these data it appears that the CM's are not appreciably altered during enzymatic isolation.

The penetration of inorganic ions and other polar organic molecules through astomatous tomato fruit CM's and stomatous onion leaf CM's have been studied (127, 129). In the onion leaf CM the cuticle extended through the stomatal pore thus forming a sac. Penetration of Rb^{+}, Ca^{++}, Cl^{-}, SO_4^{--}, SADH, maleic hydrazide (MH) and urea was greater from the outer to the inner side of the CM (influx) than in the opposite direction (efflux). No difference between influx and efflux were observed using a dializing membrane. With the exception of urea, penetration was a diffusion process following first order kinetics.

There was a distinct relationship between ion binding and penetration. Ion binding was always greater on the inner than on the outer side of the membrane (128, 130), whereas more ions penetrated from the outer to the inner side of the CM's than in the opposite direction. Thus, penetration was greater in the

direction towards the side with the greater ion binding capacity.

Urea penetrated at a much greater rate than any other compound and the penetration rate increased with time. There was little difference between influx and efflux. It appears possible that urea changed the structure and/or composition of the CM by breaking weak bonds and by dissolving components out of it. Yamada (126) reported that an ether soluble fraction could be extracted from CM's with urea.

Compared to the dializing membrane, the CM's used in these studies were relatively impermeable. Eighty per cent of the cations and anions penetrated the dializing membrane in 40 hours, while the comparable figure for the CM's was 0.2 to 2%.

The permeability data reported for apple leaf CM's (50) are difficult to evaluate since the CM's were chemically isolated. Furthermore, data were reported in terms of cpm penetrated, even though different isotopes (^{14}C, ^{22}Na), counting procedures, concentrations and specific activities were used.

Silva Fernandes (105) also isolated CM's from apple leaves chemically. Such CM's were practically impermeable to phenylmercuric acetate and copper acetate. The CM of ivy increased in thickness with increasing age (3 years). This was accompanied by an increase in permeability to

water and a drastic decrease in the permeability to 2,4-dichlorophenoxyacetic acid (2,4 D) (96).

It is very difficult to draw a general conclusion concerning the permeability of isolated cuticular membranes to polar molecules, except perhaps that permeability was low to all compounds with the major exception of urea. Yamada et al. (129) concluded that cations penetrate more rapidly than anions. This was true for Ca^{++} vs. SO_4^{--} and Rb^{+} vs. Cl^{-}, however, it was also true that more SO_4^{--} than Rb^{+} penetrated the tomato fruit CM.

While penetration of lipophilic compounds through enzymatically isolated astomatous apricot leaf CM's was related to the partition coefficient (chloroform: water), no such relationship was found for polar compounds (20). Sucrose, glucose, valerate and α-chloroacetate penetrated at a higher rate than the partition coefficient would suggest. The authors suggested that the mechanism of penetration for polar compounds might differ from that for non-polar compounds. The following illustrates the low permeability of the apricot leaf CM (20) for polar compounds: (a) only 2-3% of the sugars penetrated in 48 hours whereas almost complete penetration of N-n-hexyl- and N-isopropyl α-chloro-acetamide occurred during the same time period, and (b) it was estimated that a water or agar membrane

of comparable thickness is 4×10^6 times more permeable to sucrose than the apricot leaf CM.

Permeability of Cuticular Membranes to Water

If the prime function of the CM is to prevent uncontrolled water loss from leaves, one would expect to find a relationship between its morphological and/or chemical characteristics and permeability to water. The thickness of the CM has been evaluated most often. Kamp (67) has been cited as saying that there is no relationship between thickness of the CM and cuticular transpiration (CT) (61, 107). This is true only if different genera are compared. A comparison of related species, however, reveals a striking relationship between CT and thickness of the CM as shown by the following data taken from Kamp (67):

Species		Thickness of CM	CT
		µm	mg/g fr.wt. x hour
Quercus	*sessiliflora*	2.2	64
Q.	*rubra*	3.0	31
Q.	*coccifera*	8.0	35
Q.	*ilex*	9.5	7.5
Rhamnus	*alnifolius*	1.3	29
R.	*glandulosus*	2.6	25
R.	*alaternus*	3.2	18
Coffea	*robusta*	1.1	34.5
C.	*arabica*	1.9	15
Acer	*platanoides*	1.2	56
A.	*syriacum*	3.5	32
Hedera	*helix* (shade)	3.0	6.8
H.	*helix* (sun)	5.0	2.2

This compilation shows that the CT decreased with increasing thickness of the CM. Possibly CM's of related species are similar in structure and composition and adaptation to environment is accomplished primarily by changes in thickness of the CM.

The CT of plants of different ecological type was measured by Pisek and Berger (84). The greatest CT (higher than 50 mg/g fr. wt. x hour) was observed in Impatiens, Caltha, Stellaria and Veronica followed by a group of plants having an intermediate CT (20-40 mg/g fr. wt. x hour) such as Fagus, Quercus, Convolvulus and Betula and less than 10 mg/g fr. wt. x hour were observed for Rhododendron, Sedum, Hedera, Picea and Pinus. Thus, as the ecotype changed from mesophytic to xerophytic the CT and hence the permeability of the CM to water decreased. From the data of Sitte and Rennier (107) it can be seen that the thickness of the CM tends to increase in the same direction, although no representative of the first group was included.

There is agreement (67, 96) that, with increasing age, the permeability of the CM to water (CT) increased, even though the thickness of the CM also increased. No satisfactory explanation has been given for this observation.

Cuticular waxes severely restricted water permeability of the CM (108). Following extraction of the

waxes the permeability to water of the astomatous ivy leaf CM increased dramatically. Interestingly, the permeability of the CM from shade-grown plants increased more than those from sun-grown plants. This indicated that the cutin matrix must have been different since differences in thickness were small.

There is some evidence that the degree of hydration of the CM affects CT. Incipient drying of the outermost layer of the CM was shown to rapidly reduce CT (67, 84, 112). Incipient drying occurred as soon as water loss from the CM to the atmosphere by evaporation exceeded the water diffusion through the CM. A saturation deficit has been thought to occur which in turn further increased the diffusion resistance to water and therefore reduced CT as compared to the fully hydrated CM.

The relationship between the degree of hydration of the CM and CT was investigated by Härtel (53, 54). CT was markedly affected by pH and the presence of certain ions in the membrane. Leaves pretreated with K-phosphate buffers of various pH values showed maximum CT around pH 6 and 7 and CT decreased above and below these pH values. Leaves which had been killed with chloroform vapor gave the same response to the buffer treatment as intact living leaves.

CM pretreated with solutions containing various cations (0.02 N, pH 7.0) increased in the following order: Li^+, Na^+, K^+, Ca^{++}, Sr^{++}, Ba^{++}, and Li^+, Ca^{++}, Al^{+++}. The strongly hydrated ions Li^+ and Na^+ resulted in the lowest CT, while CT increased with decreasing hydration and increasing valence of the ions. The effect of the cations on CT could be reversed by soaking (leaching) previously treated leaves in water for a few hours. Anions produced a similar effect, CT was increased in the following order by citrate^{---}, tartrate^{--}, SO_4^{--}, Cl^-, Br^-, NO_3^-, I^-, CNS^-. The order of effectiveness of cations and anions was reversed at pH values below 6-7, thus coinciding with the pH-dependent maximum of CT.

Härtel (53, 54) explained these results by assuming that CT is controlled by an ampholyte (cutin) interspersed by small pores. The diffusion resistance in this system would depend on the degree of imbibition and the size of the pores. In membranes having relatively narrow pores (cuticle type) hydration would result in an increase in diffusion resistance, due to the presence of bound hydration water in the pores hampering the diffusion of free water molecules. In presence of strongly hydrated ions in the pores most of the space would be occupied by bound (hydration) water and the movement of free water molecules severely restricted.

In order to explain the effect of pH on water permeability of the CM one must assume that the CM is an ampholyte with an isoelectric point around pH 6 to 7, so that the net charge would increase at higher or lower pH values. Currently, however, the cutin matrix and the pectins are assumed to have a negative charge on dissociation and these groups are completely dissociated at pH 6 or 7. Thus, there is some doubt as to whether the CM behaves as an ampholyte as assumed by Härtel.

Water uptake by needles of Abies cephalonica, Abies alba, Picea omorica and Pinus mugo was also influenced by pH and certain ions (27). Water uptake from solutions of different pH values by needles having a water deficit of 15 - 20% was least at low pH values (1.75, 4.25) and increased when the pH was increased to 7.0. The velocity of water uptake from salt solutions increased in the following order: Li^+, Na^+, K^+, Ca^{++}. The presence of strongly hydrated ions impeded water uptake.

It should be pointed out that the concept developed by Härtel (53, 54) is in contradiction to the concept of incipient drying. While incipient drying is thought to reduce CT by reducing the degree of hydration of the CM, the concept put forward by Härtel argues the opposite, namely, that increase in hydration of the CM

reduces water permeability because the bound hydration water "plugs up" the diffusion paths for water.

Polar Routes Across the Cuticular Membrane

It has been shown that water and other polar molecules do in fact penetrate the lipophilic CM. Härtel (53, 54) assumed the existence of hydrophilic pores in the cutin matrix. The problem of the localization of hydrophilic channels in the CM, through which polar molecules can diffuse, will be considered next. The terms "hydrophilic channel" or "hydrophilic pore" will be used to denote a region through which polar molecules can permeate the otherwise impermeable lipophilic matrix of the CM.

Strugger (118) using berberin sulphate (BS) as a tracer studied CT in relation to the extrafascicular transport pathways in plants. BS is a fluorochrome which shows little fluorescence in solution but strong fluorescence if adsorbed to tissue. Below pH 11 this dye exists as a cation and is undissociated at higher pH. In the cationic form BS is taken up slowly into the cytoplasm and thus it is suitable for studying water movement in the apoplast.

When shoots of Helxine soleirolii were supplied an aqueous solution of BS through the cut stem, the rise of the dye in the vascular bundles could be

observed with a fluorescence microscope. BS moved rapidly in the vascular system and reached the leaves within a few minutes. In the leaves, BS appeared first in the veins and then spread out into the cell wall system (apoplast). Soon, the cuticular ledges of the guard cells, the walls of the stalk cells of the globular trichomes and the anticlinal walls of epidermal cells showed strong fluorescence, while periclinal walls exhibited only weak fluorescence, even after prolonged periods. In the astomatous CM of the upper leaf surface the dye accumulated mainly in the anticlinal walls and in the basal cells of trichomes. Generally, the time required for fluorescence to appear was longer for the upper, than for the lower leaf surface.

Strugger (118) concluded from the pattern of fluorescence that the sites exhibiting strong fluorescence were preferentially involved in CT, the dye being carried passively in the transpiration stream and accumulated where the water evaporated. The distribution of BS in the tissue was much too fast to be accounted for by a diffusion process. If transpiration was reduced by inducing stomatal closure or by wetting the leaves the dye spread more slowly, indicating that it moved passively with the water in the plant.

If an aqueous solution of BS was administered to one leaf of Helxine soleirolii and the other leaves were allowed to transpire freely, BS was taken up by the leaf submerged in BS solution. Its cuticular ledges, basal cells of trichomes and anticlinal walls soon exhibited fluorescence and the dye was observed to move out of the submerged leaf into the transpiring leaves where it accumulated at the preferential sites previously mentioned.

The identity in the pattern of fluorescence indicated that the sites of entry were the same as the preferential sites of CT. The entry of dye into a submerged leaf was greatest in young leaves and when stomata were open. Strugger pointed out that the dye did not enter through the open stomatal pore (no surfactant used). He considered it more likely that the hydration of the cuticular ledges differed in open and closed stomata.

The presence of hydrophilic pores large enough to permit passage not only of water but also of the large, charged BS molecule (MW 822, consisting of four six-membered rings) in the CM of Helxine was conclusively demonstrated (118). After 30 minutes uptake through the cut stem the dye (85) had been taken up and spread into the transpiring leaves. The lower surface was then coated with a mixture of 5% gelatin containing 0.9 M glucose and 0.1 M KSCN immediately before its

solidification. Due to the high osmotic pressure in the gelatin mixture, water diffused out of the leaf into the gelatin carrying with it BS where the pores in the membrane were sufficiently large. BS reacted with SCN^- ions resulting in an insoluble precipitate which fluoresced. Such a precipitate was observed where the gelatin had been in contact with cuticular ledges, with the basis of globular trichomes and occasionally with the back wall of guard cells. Thus, these sites have hydrophilic pores sufficiently large to permit passage of BS. Anticlinal walls and conical trichomes which exhibited strong fluorescence themselves were impermeable for BS molecules as evident from the absence of berberinrhodanide in the gelatin negative of these sites.

Renner and Kallmeyer (89) reported that the pattern of fluorescence differed in leaves having open or closed stomata. Very little BS accumulated in the cuticular ledges of closed stomata, while accumulation was pronounced in open stomata.

This problem was pursued further by Bauer (6). Leaf segments of Rhoeo discolor were floated on 0.1 M NcCl solution which caused their stomata to remain open independent of light. These pretreated segments were then floated on BS solution together with controls having closed stomata. After 2-3 minutes the segments with open stomata showed strong fluorescence in the cuticular

ledges and 2-3 minutes later in the anticlinal walls, whereas the control segments first exhibited fluorescence in the anticlinal walls (4-6 minutes) and only after 10 minutes did weak fluorescence occur in the cuticular ledges.

Since only small quantities of fluorescent dye accumulated in cuticular ledges of closed stomata it can be concluded that transpiration through these ledges (peristomatous transpiration) was reduced as compared to open stomata. Thus, the cuticular ledges of stomata appear capable of changes in permeability as regards stomatal opening.

A differential permeability of cuticular ledges of open and closed stomata was observed also by Maercker (71) using tritiated water and microradioautography. In Sagittaria sagittifolia dense silver grains occurred in the film over the cuticular ledges of open stomata, whereas a uniform blackening of the film over the entire guard cell and adjacent cell was apparent when stomata were closed.

Trichomes of a variety of plants have been shown to be able to take up various fluorochromes (13, 61, 115). In glandular trichomes of Vicia faba and Phaseolus coccineus penetration occurred preferentially through the thin CM over the proximal cells (13).

EXPERIMENTAL

Foliar Penetration of Succinic Acid 2,2-Dimethylhydrazide: Studies Employing Excised Leaf Disks

Materials and Methods

Plant Material

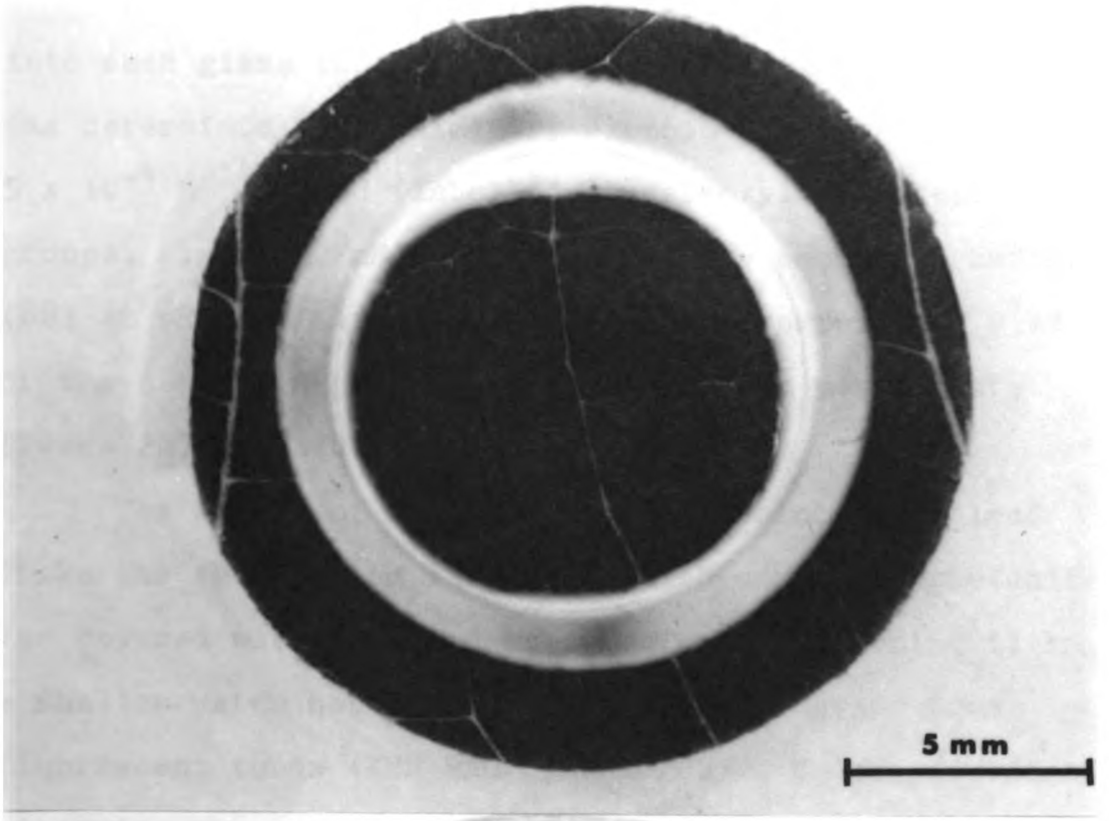
Primary leaves of bean (Phaseolus vulgaris L. cv. "Blue Lake") were used as test material. Plants were grown in flats containing vermiculite, placed in a growth chamber. Temperatures of 30° C day and 25° C night were maintained. Light from fluorescent and tungsten lamps at an intensity of 1 600 ft.c. was supplied for 14 hours a day. Relative humidity was not controlled and averaged 50 - 70% during the light and up to 80% during the dark period. Plants were spaced to prevent mutual shading of the leaves. To minimize possible injury, leaves were not handled by the blade and they were kept dry during watering and free of pests and diseases. Under these conditions the primary leaves reached maximum size at the tenth day after planting. Fully expanded leaves (10 - 12 days old) were used in these studied and unless stated otherwise penetration was followed through the upper surface.

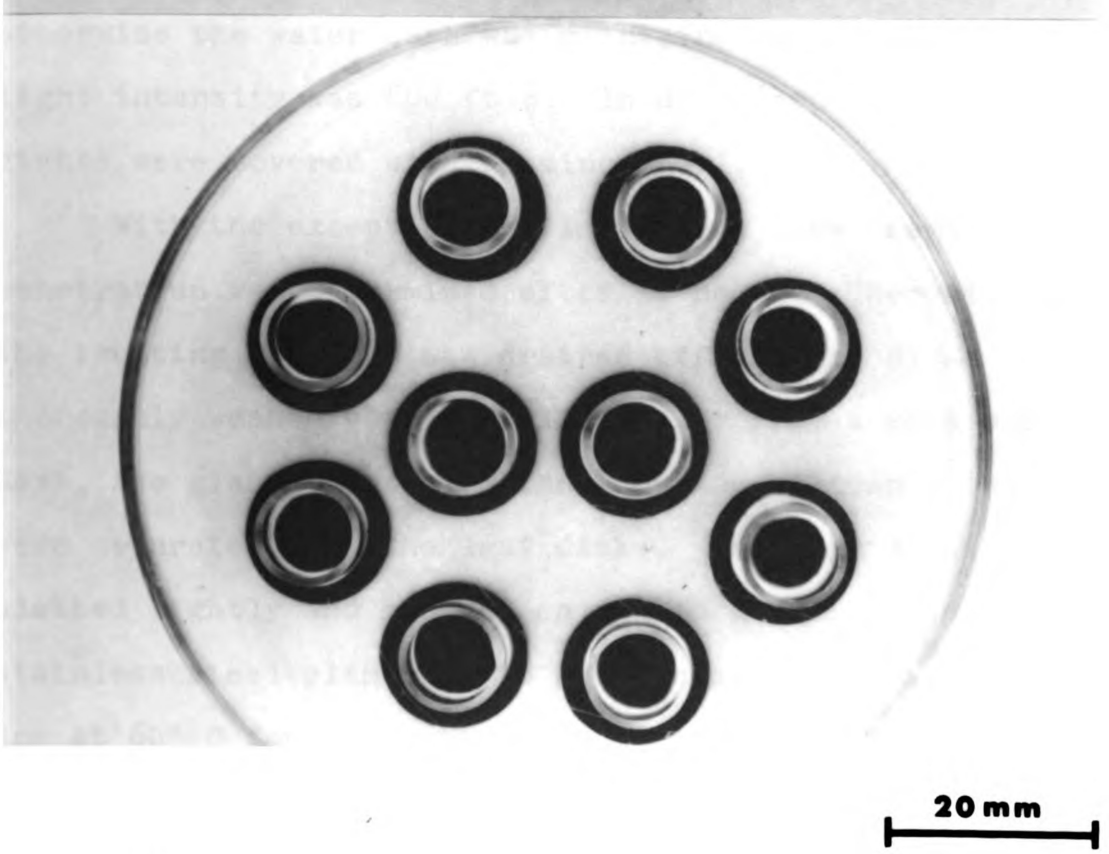
Procedure

Leaf disks, 17 mm in diameter, were punched from the leaves omitting major veins and placed immediately in Petri-dishes (diameter 9 cm) lined with moist filter paper. Eight disks were punched from the two primary leaves of each plant.

A small glass tube (10 mm internal diameter, 7 mm in height) was sealed to the center of each leaf disk using silicon rubber (RTV 11, General Electric). Numerous catalysts had been tested, but only Härter T 1 and T 11 (Wacher Chemie GMBH, Munich, Germany) proved suitable since all others caused varying degrees of leaf injury. The silicon rubber and the catalyst were mixed thoroughly, then one side of the glass tube was dipped into the fluid mixture and lowered immediately onto the leaf disks without excerting any pressure. Curing occurred in 5 - 10 minutes (T 1) to one hour (T 11) depending on the quantity of catalyst added. Silicon rubber, instead of petroleum jelly (95) had some advantages: the surface of the leaf exposed to treating solution is well defined and of uniform size for each leaf disk (0.5 cm^2) (Figure 1), leaf injury is minimized since pressure is not needed for obtaining a tight seal, no leaks occurred with surfactant concentrations up to 1% and temperatures up to 40° C and the silicon rubber is chemically inert.

Figure 1.--Photographs illustrating the penetration units consisting of a glass tube affixed to a leaf disk with silicon rubber.


5 mm


20 mm

A volume of 0.2 ml treating solution was pipetted into each glass tube. The standard treating solution (as determined by preliminary experiments) consisted of 5×10^{-4} M ^{14}C-SADH (labelled in carboxyl or methyl groups, s.a. 2 mC/mM) in 0.05 M citrate phosphate buffer (88) at pH 5.0. If a surfactant was incorporated, 0.1% of the non-ionic polyoxyethylene sorbitan monolaurate (Tween 20) was added.

The bottom of the Petri-dish containing the leaf disks was then placed in a Petri-dish cover and the unit was covered with a second cover before positioning it in a shallow water bath with overhead illumination from fluorescent tubes (KEN RAD F 30 T8/CW). Unless mentioned otherwise the water bath was maintained at 30° C and the light intensity was 600 ft.c. In dark treatments the dishes were covered with aluminum foil.

With the exception of time-course experiments, penetration was determined after 12 hours. Thereafter, the treating solution was drained off and the disks thoroughly washed with distilled water from a wash bottle. Next, the glass tubes with the adhering silicon rubber were separated from the leaf disks. The leaf disks were blotted lightly and mounted on double adhesive tape in stainless steel planchets to keep them flat during drying at 60° C for 24 hours.

The radioactivity was determined using a proportional gas-flow end-window GM counter. Each sample was counted for 2,000 counts and the radioactivity calculated as cpm. The leaf disks were of finite thickness (density was 3.1 mg x cm^{-2}). A self absorption curve was prepared (Appendix II A) using ground leaf tissue labelled with ^{14}C-SADH and the counting geometry was evaluated according to procedures outlined by Schweitzer et al. (102). The geometry was constant up to 20 mg x cm^{-2} density, indicating a constant counting efficiency.

Presentation of Data

Since the ^{14}C-SADH used was of the same specific activity for all experiments, data are reported on a net cpm basis. The data are expressed as rate of penetration: amount (cpm) of SADH penetrated through unit area (0.5 cm^2) in unit time (12 hours) for a 5 x 10^{-4} M SADH solution, except for those experiments in which time or concentrations were experimental variables. Taking self absorption, counting efficiency and specific activity of ^{14}C-SADH into account a curve was prepared from which cpm data can easily be converted into moles (Appendix II B).

Statistical Treatment

A randomized block design was utilized. Each treatment consisted of 10 replicate leaf disks. In

experiments with up to eight treatments leaf disks from each plant were distributed systematically over all treatments such that each replicate disk originated from a different plant. In experiments with more than eight treatments leaf disks were distributed at random over all treatments. The 10 disks representing a treatment were distributed in groups of 2 or 3 over a number of Petri-dishes in such a manner that each Petri-dish contained 4 - 5 replicates of treatments receiving either light or dark. In some cases, however, randomization of replications was not possible, e.g. in the experiment where the leaf disks were subjected to osmotic stress by adding sucrose solutions to the Petri-dishes.

Analysis of variance was performed on the data and comparison among the treatment means was carried out using Tukey's w-procedure (114). The F-test was employed if only two means were compared. For data presented graphically, the confidence interval was calculated for each mean at a 5% level of significance. The total length of the vertical bar drawn through the mean represents twice the confidence interval $(2\ t_{0.05}\ s_{\bar{x}})$.

Preparation of Leaf Cross-Sections

Leaf segments (5 x 3 mm) were killed and fixed using CRAF III fixative (30 ml 1% chromic acid,

20 ml 10% acetic acid, 10 ml 40% formalin, 40 ml HOH), dehydrated in a graded series of tertiary butanol and embedded in Tissuemat. Sections 6 - 7 µm thick were found most satisfactory. They were lightly stained with Fast Green. Permanent slides were prepared.

Leaf segments for observation in surface view were killed, fixed and dehydrated as above and cleared in clove oil. For inspection and photography the sections were mounted in clove oil.

Photography

Photographs were made using a Wild M 20 research microscope equipped with a 35 mm film carrier and an automatic exposure control unit.

Leaf Surface Images and X-ray Analysis

An electron microprobe X-ray analyzer (Applied Research Laboratory Inc., EMX - SM) was employed to obtain images of the bean leaf surface and for studying the distribution of silver in leaves. Sample preparation was similar for both purposes. Leaf disks were freeze-dried, small segments (2 x 3 mm) cut from the disks and mounted on strips of double adhesive tape on a carbon disk of 3.2 cm diameter. The two ends of the leaf segments extending over the adhesive tape were grounded to the carbon disk with Television Tube Koat

No. 49-2 (General Electric). Samples were coated with carbon. The instrument was operated at an accelerating voltage of 20 kV and a sample current of 5 nA.

Radioautography

Radioautograms of leaf disks treated with ^{14}C-SADH or ^{14}C-tryptophan were prepared as follows: At the end of the penetration period the disks were thoroughly washed with water, frozen immediately on dry ice and freeze-dried to prevent movement of the label. The dried leaf disks were mounted on card board and radioautograms were prepared using Kodak "Blue Brand" X-ray film.

Analysis of Procedures

The quantity of SADH which penetrated was deduced from the radioactivity detectable in the leaf disks after washing the treated surface with water. This deduction was legitimate only if (a) surface binding of SADH is small compared to the amount which penetrated and (b) if no appreciable loss of activity occurred during the 12-hour penetration period.

Breakdown of SADH in plant tissue was shown to occur at a very slow rate (26, 75) regardless of the position of the label. The loss of radioactivity (as $^{14}CO_2$) during the penetration period was therefore assumed to be negligible.

Preliminary experiments revealed a marked increase of penetration in light. This could have been due to assimilation of $^{14}CO_2$ originating from breakdown of ^{14}C-SADH in the treating solution. Identification of the absorbed label after penetration seemed desirable.

The separation procedure of Martin et al. (75) was followed, except that methanol was used for extraction. Penetration of ^{14}C-SADH (carboxyl labelled) was allowed for 12 hours in light and the leaf disks were washed, lyophilized, ground and extracted with methanol for 12 hours in a Soxhlet apparatus. The methanol extract was brought to 10 ml volume in a warm air stream and an aliquot was counted. The total activity in the methanol extract was 19,900 cpm and in the residue 407 cpm.

The methanol extract was passed through a Dowex 50 W X8 (50-100 mesh) cationic ion-exchange resin. The column was washed with 60 ml distilled water and eluted with 60 ml 7.5 N NH_4OH. The eluant was brought to a constant volume and an aliquot counted. A figure of 16,650 cpm was obtained for the 50 W fraction, that is 84% of the total activity of the methanol extract. The fraction not bound by the resin gave 1,890 cpm or 9.5% of the total activity of the methanol extract. This resulted in a 93.5% recovery.

Both fractions were chromatographed on thin-layer plates (Silica Gel G) using isopropanol, NH_4OH and water (20:1:4) as the developing solvent. The plates were scanned for radioactivity using an Actigraph II Model 1036. Both fractions separated into two peaks with R_F values of 0.3 and 0.1 respectively (Figure 2). From a comparison with a ^{14}C-SADH standard it was clear that the activity at R_F 0.3 represented SADH. The compound at R_F 0.1 was not identified. This small peak also occurred using the SADH standard and was also observed and considered an impurity by Martin et al. (75). The standard is not published here, instead, the standard solution and 50 W fraction were co-chromatographed and the scan was superimposed over the scan of the 50 W fraction alone (Figure 2 A). There was absolute agreement between the peaks of both scans. Therefore, the bulk of the radioactivity recovered from the bean leaf disks was ^{14}C-SADH.

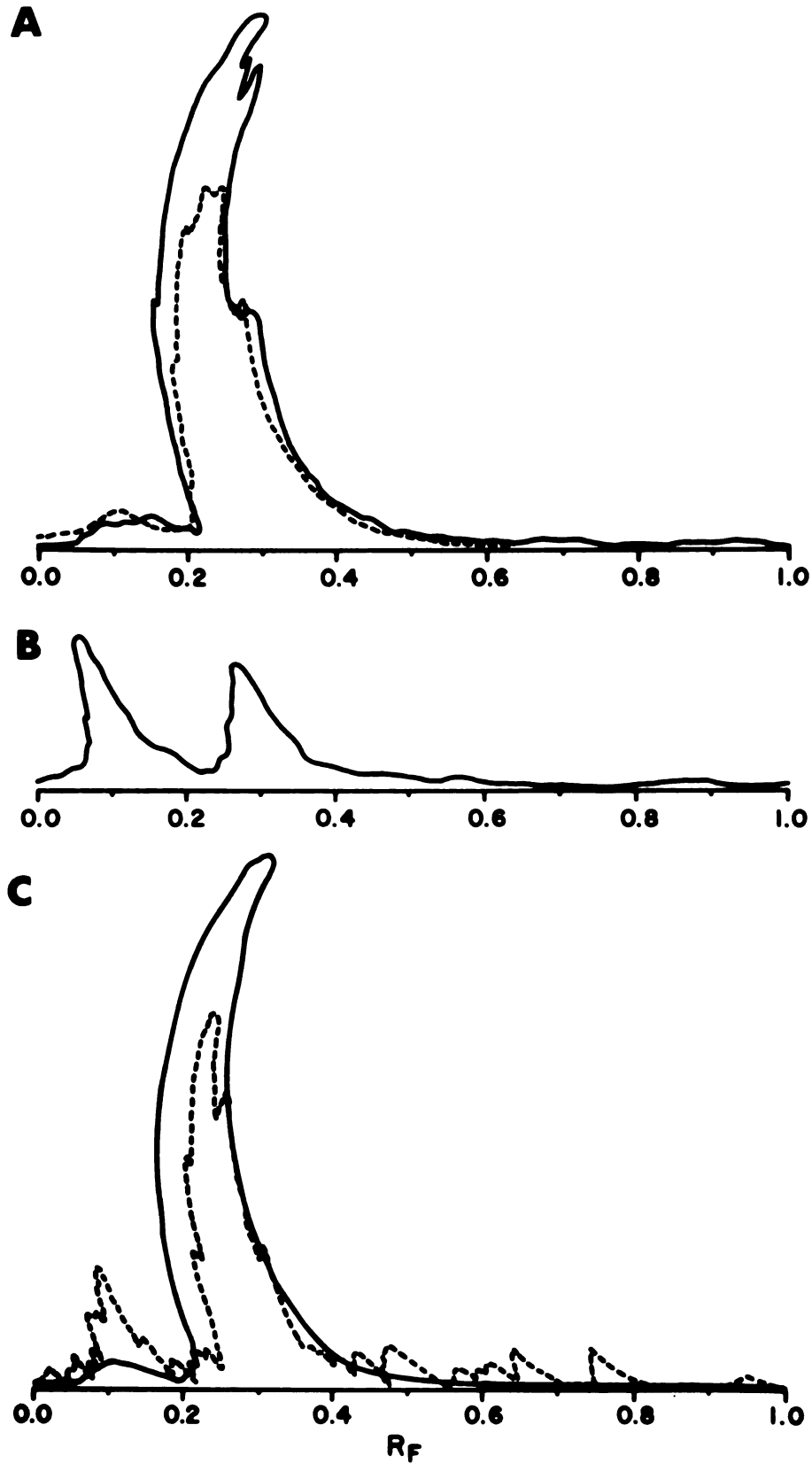
Because of limited quantitites of ^{14}C-SADH available an attempt was made to recover some of the previously used labelled compound. After termination of the penetration experiments the treating solution was collected from the glass tubes, concentrated in a warm air stream, acidified with HCl and then passed through the Dowex 50 W X8 column. The fraction not bound was discarded and the NH_4OH eluent containing ^{14}C-SADH and

Figure 2.--Tracings of radiochromatogram scans of thin-layer plates.

A. Dowex 50 W X8 fraction of the methanol extract from bean leaves treated with ^{14}C-SADH (dotted line) and the same fraction co-chromatographed with SADH standard (solid line).

B. Fraction of methanol extract from bean leaves treated with ^{14}C-SADH not bound to the Dowes 50 W X8 column.

C. Methanol fraction of purified treating solution (dotted line) and the same fraction co-chromatographed with SADH standard (solid line).


A
0.0
0.2
0.4
0.6
0.8
1.0
B
0.0
0.2
0.4
0.6
0.8
1.0
C
0.0
0.2
0.4
0.6
0.8
1.0
R_F

buffer cations was dried in a warm air stream. The residue was extracted with absolute methanol which brought SADH into solution and left NaOH behind. A sample of the recovered ^{14}C-SADH was co-chromatographed with standard ^{14}C-SADH and tracings of the radioscans obtained are presented in Figure 2 C. There was absolute agreement with the standard indicating successful recovery of ^{14}C-SADH. The recovered material was stored under refrigeration until used.

Another experiment was designed to determine the most effective washing medium. Since SADH is very water soluble, distilled water, absolute methanol and 0.02 M SADH, buffered at pH 5.0, were evaluated. Primary leaves of bean were gently agitated in a buffered solution (pH 5.0) containing 24 nC/ml ^{14}C-SADH, removed after 10 seconds and after excess liquid had drained off they were placed each in 50 ml of the prescribed washing medium and gently agitated. After 10 seconds they were removed, the leaf surface determined with a planimeter and discarded. The total radioactivity contained in each 50 ml washing medium was determined. Methanol removed 48 cpm ^{14}C-SADH per cm^2 leaf area and distilled water 42.5 cpm x cm^{-2}, the difference being not significant. With 0.02 M SADH only 14.4 cpm x cm^{-2} were removed. It was therefore decided to adopt washing with distilled water as standard procedure.

Since SADH is very water soluble, it may move freely in the water phase (apoplast) inside the leaf. From the radioautogram it was apparent that the label accumulated in the veins and that some transport to the cut edges occurred (Figure 3). With Tween 20 in the treating solution more label accumulated and appeared more uniformly distributed (Figure 3 B). The silicon rubber sealant did not seem to cause any injury to the cuticle since no preferential penetration took place in the area where silicon rubber was in contact with the leaf surface and the treating solution.

Loss of ^{14}C-SADH from the leaf disk by penetrating through the leaf surface and passing into the moist filter paper, could be a serious source of error. To check this possibility, leaf disks were placed on individual filter disks (2.1 cm in diameter) and penetration through the upper or lower surface was allowed to proceed for 6 and 12 hours in light. Radioactivity of leaf disks and their respective filter disks were determined (Table 1).

There was some loss of radioactivity from the leaf disks. This loss was proportional to the radioactivity in the leaf disk. More radioactivity was lost through the lower surface (5.25 - 6.25%) than through the upper (3.70%). The loss of 4 - 6% of the total radioactivity into the filter paper was accepted since

Figure 3.--Radioautogram of bean leaf disks following penetration of ^{14}C-SADH for 12 hours in light (ca. 1.5 x).

A. SADH

B. SADH plus 0.1% Tween 20


A


B

it appeared to be proportional to the activity in the leaf disks.

TABLE 1.--Loss of ^{14}C-SADH from leaf disks into the underlying filter disks.

Penetration surface	Penetration period hours	Radioactivity		Radioact. of filter in % of total activity
		Leaf cpm	Filter cpm	
Upper	6	118	7.9	6.25
	12	259	14.3	5.25
Lower	6	187	7.2	3.70
	12	414	15.9	3.70

Results

Bean Primary Leaf Morphology

Knowledge of the leaf morphology is essential for a meaningful interpretation of penetration data. Secondary electron micrographs (SEM) of the upper and lower surface of primary leaves of bean are presented in Figure 4. The guard cells appear to be elevated above the epidermal cells (A). The entire stomatal apparatus is approximately 25 - 35 µm long and 15 - 20 µm wide. The aperture between the cuticular ledges measures 7 - 10 by 10 - 15 µm (B). The walls of the guard cells inside the aperture can be seen (B). Glandular trichomes occur on both surfaces of

Figure 4.--Secondary electron micrographs of the surfaces of bean primary leaves.

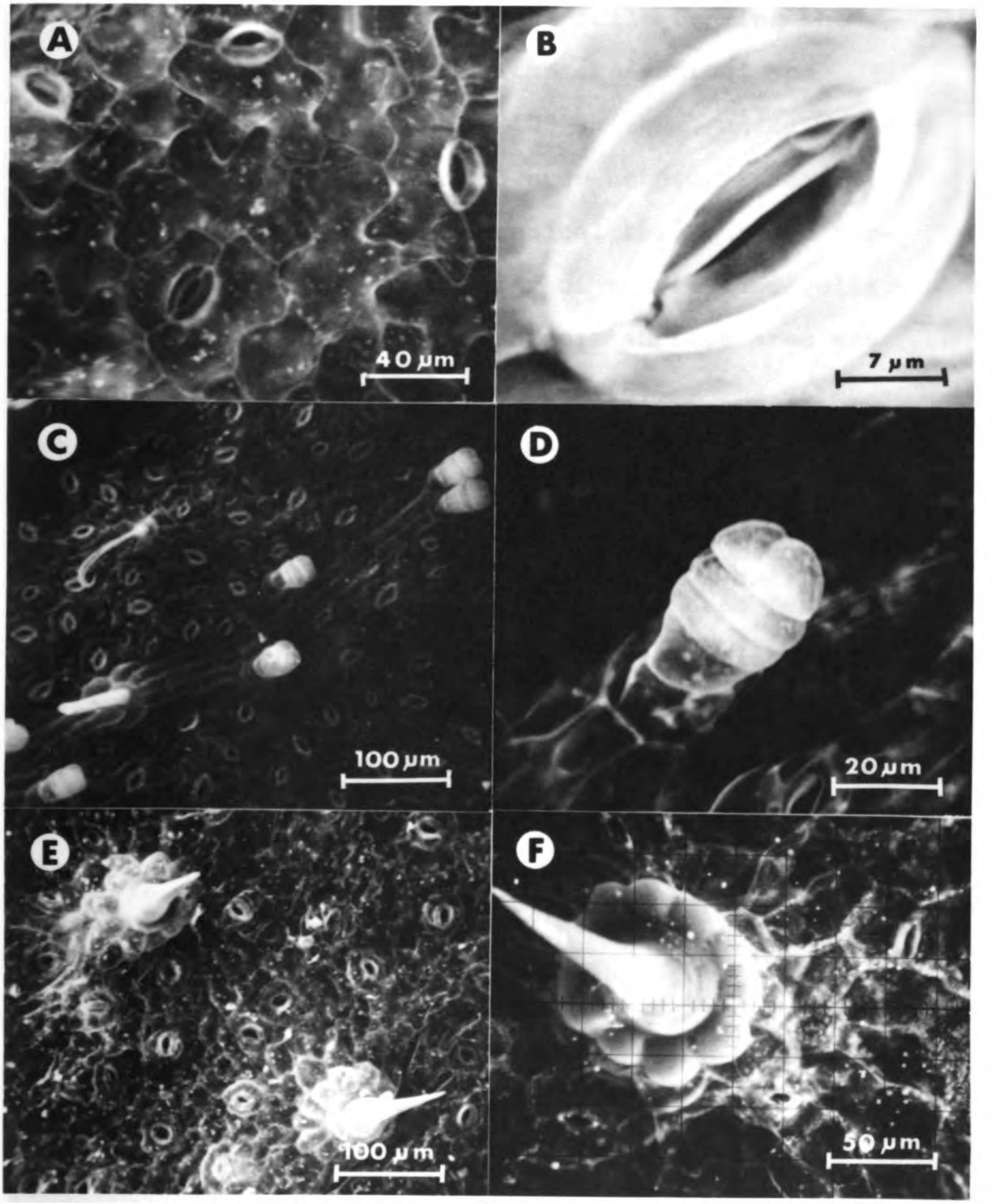
A. Upper surface with stomata.

B. Open stoma showing front cavity and pore.

C. Glandular and hooked trichomes on lower surface.

D. Glandular trichome.

E, F. Conical trichomes on upper surface.


A
40 µm
B
7 µm
C
100 µm
D
20 µm
E
100 µm
F
50 µm

the leaf and tend to be associated with the veins (C). Glandular trichomes are 40 - 50 µm long and consist of a foot cell, the stalk cell and 4 cells forming the "head" (D). The elongated trichomes (conical and "hooked" trichomes) are up to 100 µm long, unicellular and occur randomly distributed over both surfaces (C, E, F). The foot cell of the conical thichomes is surrounded by a characteristic ring of specialized cells.

The number of stomata per unit surface area was estimated using a replica technique (57). The upper and lower surfaces of 10 fully expanded leaves were coated with Rhoplex AC 33. After drying (30 minutes) the thin, transparent replicas were peeled off and mounted on glass slides. Stomata in 10 fields (500 x) of each replica were counted and the stomatal density was calculated (Table 2). The density of glandular trichomes was determined by counting all glandular trichomes on the upper and lower surfaces of 10 leaf disks (0.5 cm^2) directly under the microscope. More stomata (2.78 x) and glandular trichomes (3.70 x) were found on the lower than on the upper leaf surface (Table 2).

The relationship between stomatal density and leaf size and leaf age was also investigated using

Rhoplex replicas (Figure 5). As expected, leaf size and stomatal density were inversly proportional. From the 7th to the 10th day the leaf area increased by a factor of 2.26 while stomatal density decreased by a factor of 2.30. There was no significant change in either leaf size or stomatal density after the 10th day.

TABLE 2.--Density of stomata and glandular trichomes on fully expanded bean primary leaves.

Surface	Stomata per mm^2	Ratio L/U	Gland. trichomes per cm^2	Ratio L/U
Upper (U)	104		154	
Lower (L)	289	2.78	570	3.70

The kinetics of stomatal opening and closing as related to light was determined using continuously operating and recording porometers. The method employed was essentially as described by Raschke (86, 87). Leaf disks from eight different leaves were inserted into the porometer chambers which were submerged in a water bath at 27° C. White light was provided by means of a high pressure xenon arc. The radiation intensity was 15.5 $mW \times cm^{-2}$. The porometer flow of three selected leaf disks (Figure 6) showed that between 60 and 100 minutes were required before maximum opening of stomata occurred. When the light was switched off the initial reduction in

Figure 5.--Relationship between leaf size (plant age) and stomatal density on the upper surface of bean primary leaves.

Growth conditions: 30° C (day), 25° C (night), 1,600 ft.c. of light for 14 hours a day.

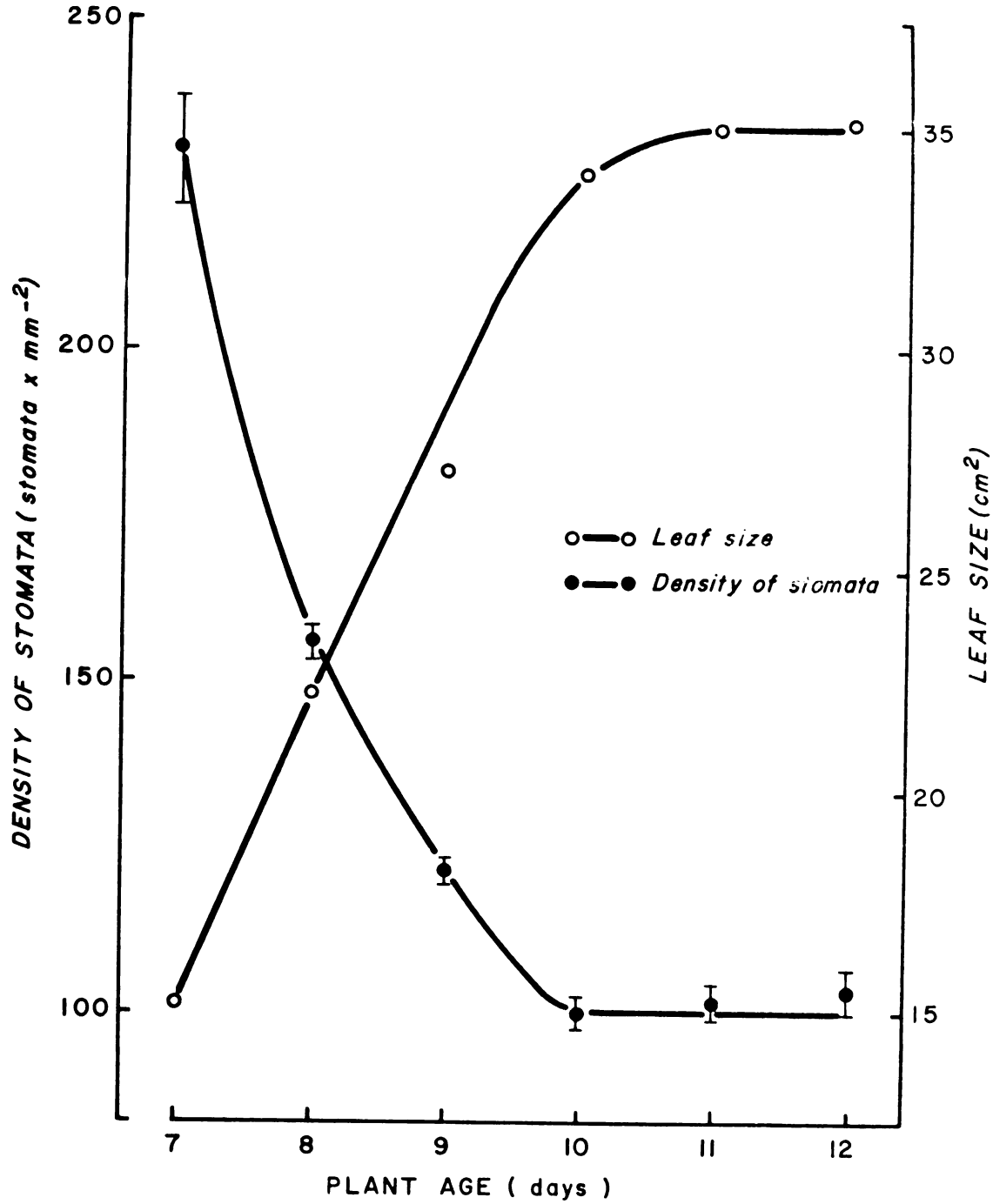
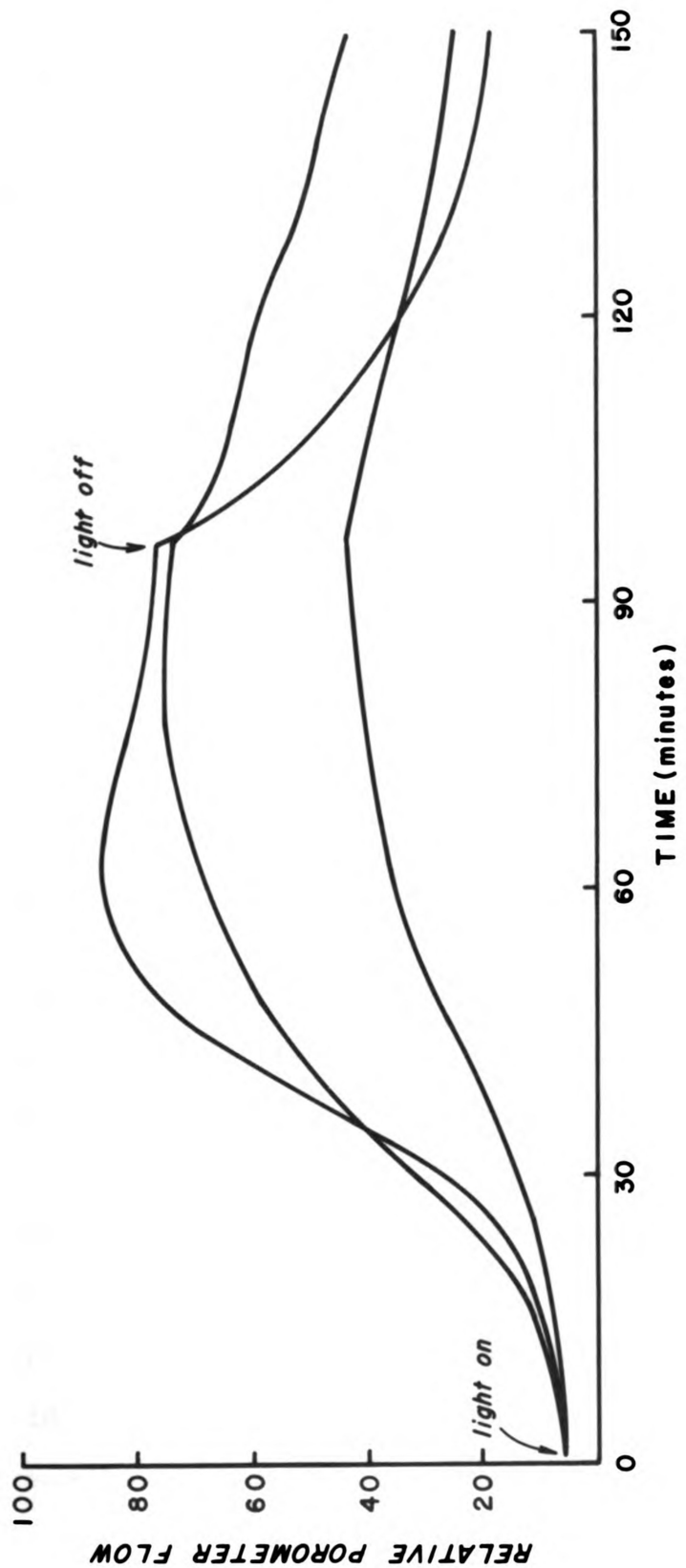

250
200
150
100
35
30
25
20
15
7
8
9
10
11
12
PLANT AGE (days)
DENSITY OF STOMATA (stomata x mm-2)
LEAF SIZE (cm2)
Leaf size
Density of stomata

Figure 6.--Kinetics of stomatal opening and closing of bean primary leaves as indexed by porometer flow. Leaf temperature 27.5° C (light), 27° C (dark), radiation energy 15.5 mW x cm^{-2}.


RELATIVE PORAMETER FLOW
100
80
60
40
20
0
30
60
90
120
150
TIME (minutes)
light on
light off

porometer flow was rapid but even after 50 minutes stomatal opening was greater in all leaf disks than at the beginning of the experiment. The plants were kept in the dark for 10 hours prior to the experiment.

From transverse sections it appears that guard cells and accessory cells from the upper leaf surface are larger than from the lower surface (Figure 7). Guard cells have outer cuticular ledges, while the inner ledges are missing. Also leaves with stomata open were fixed, the significance of the difference in stomatal aperture is questionable, since organic solvents used for fixing and dehydrating could have affected stomatal opening (101).

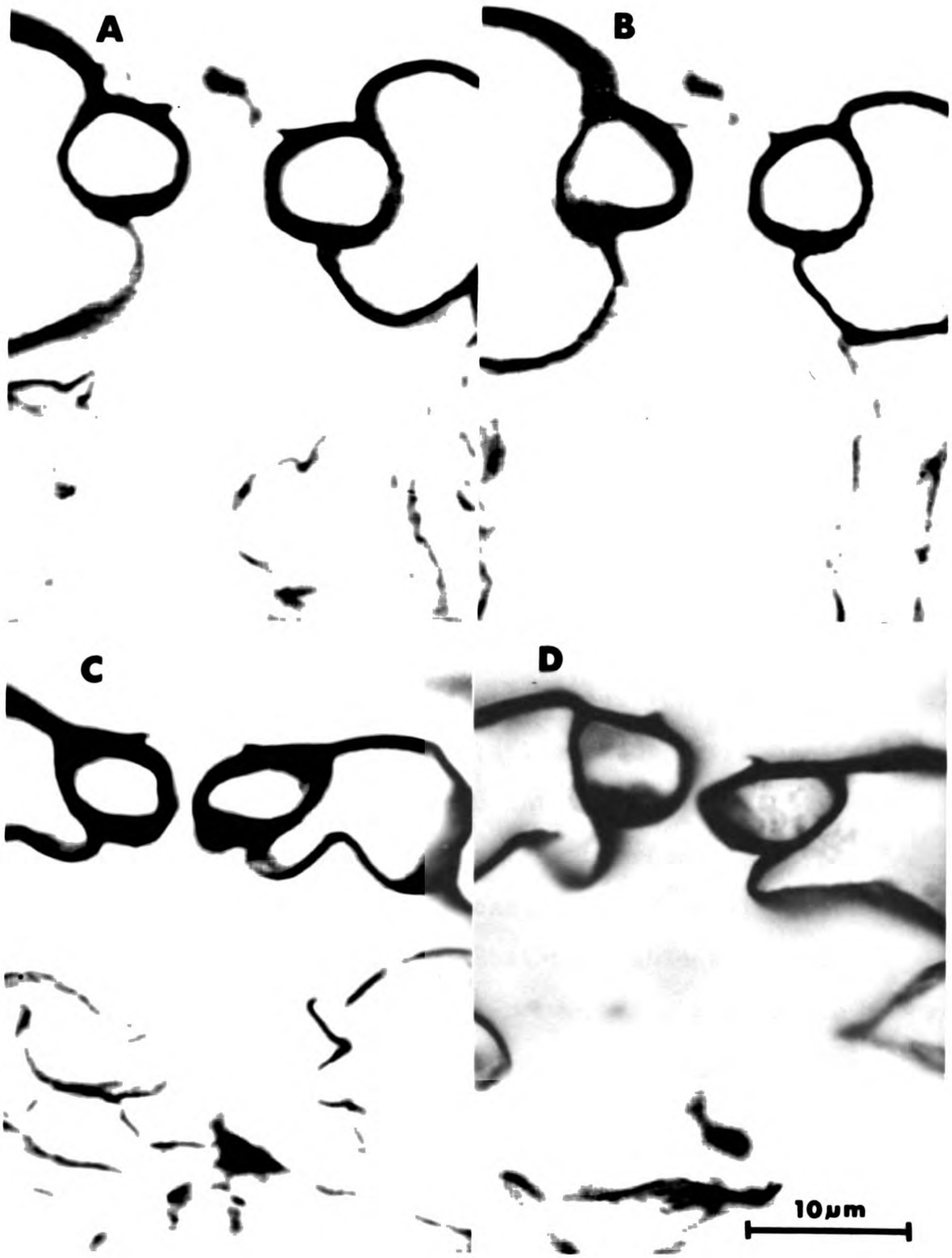
Entry of Liquids into the Intercellular Space Through Stomata, a Theoretical Treatment

The question of aqueous solutions entering the intercellular space through the stomatal pore of leaves is a current topic of discussion. While some workers (108) have claimed that squeous solutions can penetrate stomata readily, others question this (40). Turrell (120) and Dybing et al. (22) are of the opinion that stomatal penetration can occur if the surface tension of the liquid is lowered by a suitable wetting agent. Evidence presented in support of stomatal penetration is largely indirect. Examples are, the establishment of a

Figure 7.--Cross sections of stomata from bean primary leaves.

A and B: From upper surface.

C and D: From lower surface.


A
B
C
D
10µm

relationship between stomatal density or stomatal opening (18) and some parameter of penetration, such as, radioactivity or fluorescence of treated leaves or a biological response of treated plants.

To the author's knowledge no detailed studies determining the major factors controlling stomatal penetration have been reported. The importance of surface tension is often mentioned in this context, although it has been shown that the contact angle was of greater importance in stomatal penetration than surface tension (101). Ursprung (123) concluded from theoretical considerations that the cuticular ledges act as a barrier to stomatal penetration.

Capillary rise in pores of varying diameter has been treated theoretically by Adam (1). On this basis an attempt will be made to establish factors governing the entry of aqueous solutions into the leaf via stomatal pores and to assess their relative importance.

When a liquid enters a capillary of small radius a curved meniscus will be established, unless the contact angle (θ) is exactly 90°, producing a difference in pressure (P_1-P_2) on the two sides of the liquid surface (the concave and convex sides, respectively).

$$P_1 - P_2 = \gamma_L \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \qquad (1)$$

γ_L being the surface tension of the liquid and R_1 and R_2 the principal radii of the curvature (in a capillary of sufficiently small bore the meniscuc will be hemispherical and $(\frac{1}{R_1} + \frac{1}{R_2})$ becomes $(\frac{2}{R})$). For a capillary (circular) with radius r the penetrating pressure P_i (P_1-P_2) is given by

$$P_i = \frac{2\gamma \cos \theta}{r} . \qquad (2)$$

Using the advancing contact angle (θ_A) for a tube with parallel walls (2) becomes

$$P_i = \frac{2\gamma \cos \theta_A}{r} . \qquad (3)$$

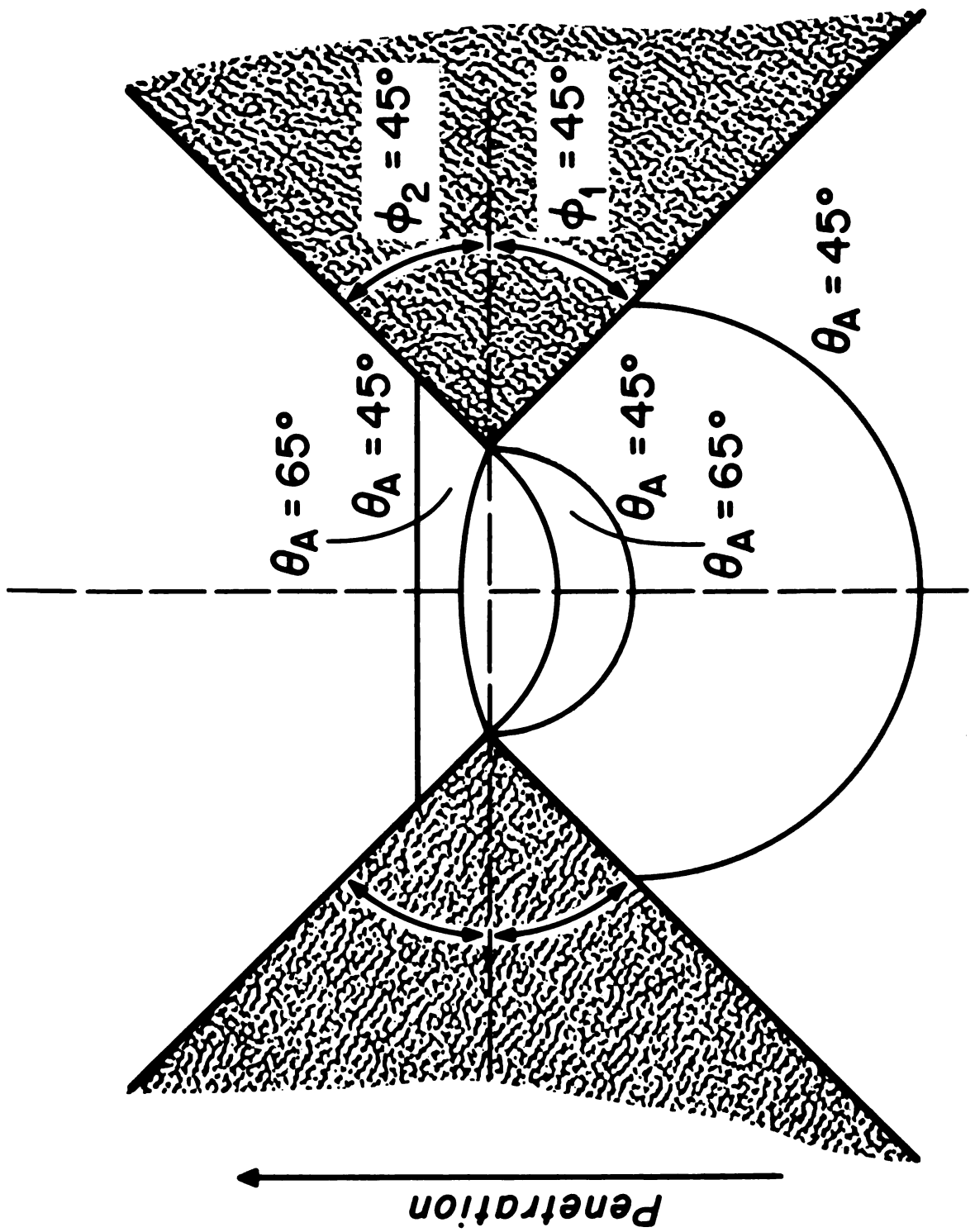
P_i is positive when the meniscus is concave, that is, when θ_A is smaller than 90°. P_i is equal to zero when θ_A is exactly 90° and P_i becomes negative when θ_A is larger than 90°. Therefore, capillary rise will not occur, when the contact angle θ_A is 90° or larger.

A capillary with inclined walls is depicted in Figure 8. The walls have an inclination of 45° to the horizontal axis $(\phi_1=\phi_2)$. If the advancing contact angle of a liquid rising in this tube is 45°, the penetrating pressure P_i will be $\frac{2\gamma}{r}$ before passing the point of inflection and zero after passing. If θ_A is larger than 45°, P_i will become negative as indicated by the convex meniscus for a liquid having θ_A = 65°.

Figure 8.--Schematic diagram illustrating a capillary pore of circular cross-section having inclined walls.

θ_A = advancing contact angle

ϕ = angle of pore walls


$\phi_2 = 45°$
$\phi_1 = 45°$
$\theta_A = 45°$
$\theta_A = 65°$
$\theta_A = 45°$
$\theta_A = 45°$
$\theta_A = 65°$
Penetration

In general terms, before passing the point of inflection the penetrating pressure P_i will be

$$P_i = \frac{2\gamma \sin(\theta_A + \phi_1)}{r} \tag{4}$$

and after passing

$$P_i = - \frac{2\gamma \sin(\theta_A - \phi_1)}{r} \tag{5}$$

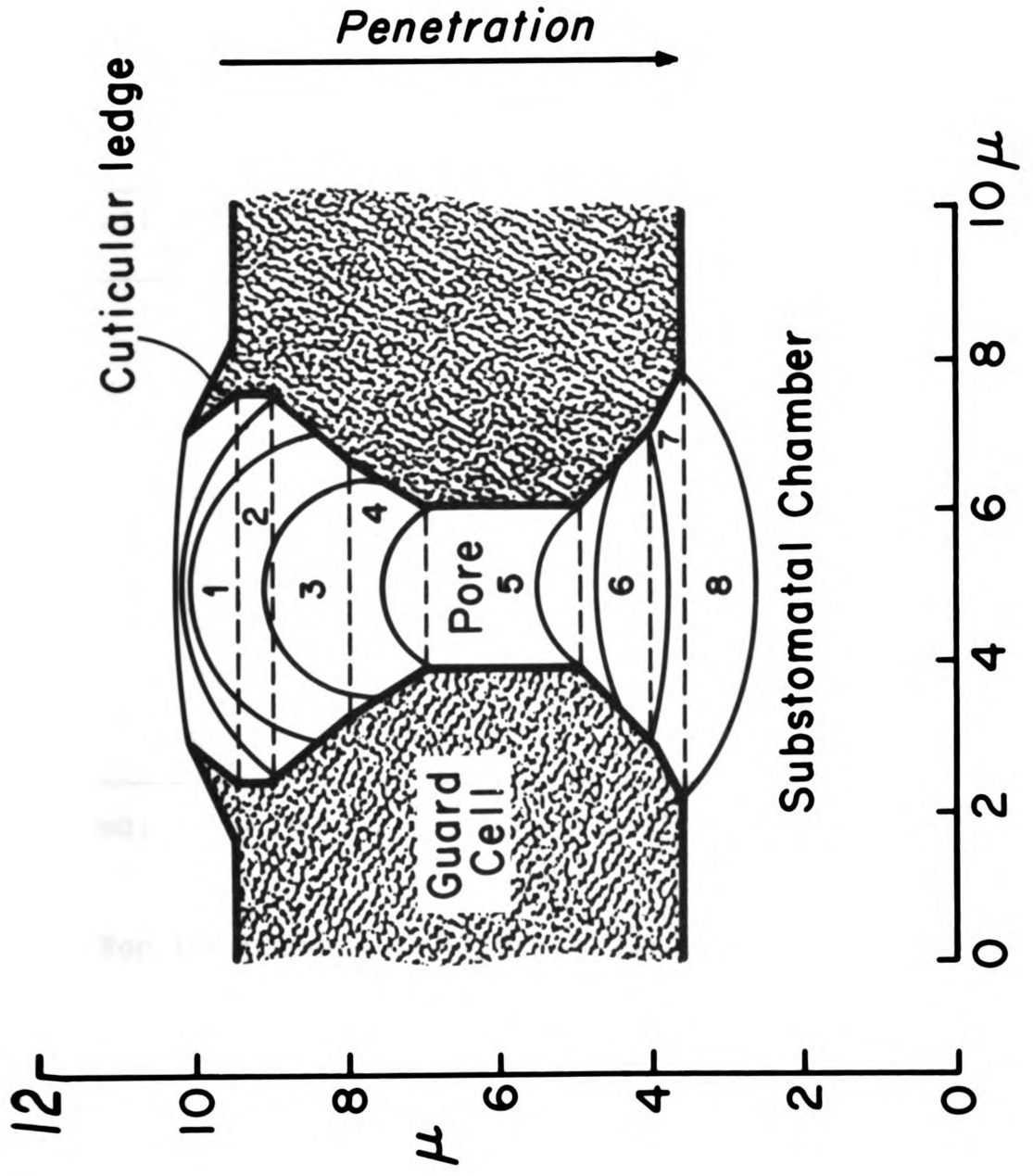
Thus, P_i becomes negative when θ_A is larger than ϕ_2.

A stoma apparatus can be viewed as a capillary of varying radius. For the present purpose terms are defined as follows: A stoma consists of two guard cells and the aperture between them when the stoma is open. The outer boundary (leaf surface side) of the aperture is characterized by the cuticular ledges. In some species ledges are found also on the inner boundary (substomatal chamber side) of the aperture. In stomata without inner ledges the inner boundary of the aperture is a continuation of the inner walls of the guard cells where they progress more or less parallel to the leaf surface (zone 8 in Figure 9). The diameter of the aperture is generally largest in the front cavity and smallest in the pore (Figure 9).

A stoma from the upper surface of a primary bean leaf (Phaseolus vulgaris) prepared from a camera lucida

Figure 9.--Schematic drawing of a cross-section of a bean leaf stoma (drawn to scale).

The penetrating pressure of a liquid ($\theta_A = 35°, \gamma = 30$ dynes x cm^{-1}) penetrating the aperture is illustrated by the radius of the meniscus for each zone.


Penetration
Cuticular ledge
Guard Cell
Pore
Substomatal Chamber
1
2
3
4
5
6
7
8
0
2
4
6
8
10 μ
12
10
8
6
4
2
0
μ

drawing is illustrated in Figure 9. The aperture was subdivided into 8 zones, each being characterized by the angle ϕ which the guard cell wall forms with the horizontal axis (Table 3).

TABLE 3.--Calculated changes in penetrating pressure (P_i) along the stomatal aperture shown in Figure 9.

Zone	Wall angle (ϕ) degrees	Radius (r) µm	Penetrating pressure (P_i)+ dynes x cm^{-2}
1	43	1.9	3,800
2	90	2.5	19,650
3	47	2.5	29,700
4	58	1.6	45,600
5	90	1.0	49,000
6	54	1.0	14,300
7	24	1.9	- 6,030
8	00	2.7	-13,200

+Assumed: $\theta_A = 35°$, $\gamma = 30$ dynes x cm^{-1}.

For the purpose of illustrating the application of formulae 4 and 5 to the problem of stomatal penetration, the following conditions will be assumed: a stomatal aperture as shown in Figure 9; relatively smooth walls of uniform composition throughout the aperture; an aqueous solution containing a surfactant and having a surface tension of 30 dynes x cm^{-1} and forming a contact

angle of 35° with the walls of the aperture, which is constant throughout the aperture and with time.

The first barrier encountered by a penetrating solution is the constriction formed by the cuticular ledge, however, since ϕ = 43° is larger than θ_A (35°) the liquid can enter the front cavity (Figure 9, Table 3). Towards the pore the diameter of the aperture decreases and the slope of the walls increases (larger ϕ) resulting in an increasing P_i. Maximum P_i is attained in the pore (zone 5). After zone 5 the diameter of the aperture increases and the slope of the walls decreases (smaller ϕ), resulting in a decreasing P_i. At the inner boundary of the aperture (zone 8) ϕ becomes zero. From zone 5 to zone 8 P_i decreases but the liquid under consideration cannot advance beyond zone 6, because in zone 7 θ_A becomes larger than ϕ_2 making P_i negative as indicated by the convex meniscus. Only a liquid having θ_A of less than 24° could penetrate further into zone 7 and only a liquid having θ_A = zero could penetrate the substomatal chamber.

Up to this point the gravitational pressure (P_g) has been neglected. Depending on the orientation of the stoma, P_g must either be added or subtracted from P_i. However, P_g is negligible due to the shortness of the stomatal aperture.

$$P_g = hdg \qquad (6)$$

In equation (6), h represents the height of the capillary rise, d the density of the liquid and g the gravitational force (approximately 980 gm x sec^{-2}). For an aqueous solution d is approximately 1 and at h = 6.6 μm P_g is 6.5 dynes x cm^{-2} which is negligible compared to the P_i values calculated (Table 3).

From the model discussed it would appear that the major factors which control the entry of liquids into the stomatal aperture are (a) the geometry of the pore, (b) contact angle and surface tension of the liquid and (c) the diameter of the aperture. These factors shall be considered next.

Geometry of stomatal aperture.--In a capillary with increasing diameter the relationship between θ_A and ϕ determines whether P_i will be negative or positive (equation 5), that is to say, whether or not capillary rise will occur. Most stomatal apertures have two zones in which the diameter increases (52), one is generally direct behind the cuticular ledges (zone 1) and the other beyond the pore (zones 6-8). According to equation 5, a liquid can enter the aperture only, if θ_A is smaller than ϕ_2 of the cuticular ledges. Any liquid capable of entering the front cavity will penetrate at least up to the pore, due to the increasing

P_i gradient. Beyond the pore, P_i decreases and the liquid will advance only up to the point where θ_A becomes equal to ϕ. At this point P_i becomes zero.

Many plants have stomata with apertures of geometry similar to the bean (Figure 9) (28, 52, 70, 120). The aperture tends to widen abruptly at the boundary of the substomatal chamber, thus making ϕ very small or zero (as in zone 8). Only a liquid with θ_A of zero can advance from such an aperture into the substomatal chamber, because any finite contact angle will result in a negative P_i. This appears to be the mechanism which prevents the intercellular space of leaves from becoming infiltrated when wetted with water. The air in the intercellular space probably does not prevent liquids from penetrating into the leaf, because the intercellular space is a continuum and air could escape through some stomata while others are being infiltrated.

Contact angle and surface tension.--The contact angle (θ) formed by a liquid on a solid surface is dependent upon the surface tension of the liquid (γ_L) and the solid (γ_S) and on the interfacial tension (γ_{SL}) according to

$$\gamma_S = \gamma_{SL} + \gamma_L \cos \theta \ . \qquad (7)$$

Thus, the contact angle is affected by the free surface energy of the solid. The largest contact angle a water droplet will form on a smooth surface generally does not exceed 105°. Such contact angles have been observed on lipophilic surfaces, such as stearic acid, paraffin and silicon (2). Roughness generally increases the contact angle if the surface material gives a contact angle larger than 90° on a smooth surface.

Contact angles as large as 140° have been reported for Triticum leaves (32) and pea leaves (64). The problem of wettability of leaves has been dealth with (2, 31, 32, 23) but these studies are irrelevant for the present purpose, because the contact angle inside the stomatal aperture is the point in question. No such information could be found. The surface of the stomatal aperture is generally believed to be more hydrophilic than the leaf surface. Wax coverings probably do not occur in the stomatal aperture. Turrell (120) reported that the guard cell walls of Citrus leaves stained with Ruthenium red in the pore but not with Sudan stains. This indicates the presence of polar groups. Thus, θ_A of a given liquid will probably be smaller inside the aperture than at the leaf surface. However, even with high concentrations of surfactants the contact angle seldom is less than 20 - 25° (23), unless, perhaps, the surfactant interacts with the wall in a way which permits the

solution to penetrate the wall, resulting in a decrease of θ_A with time (1). This may occur with surfactants that are toxic. With solutions which do not penetrate into the walls of the aperture (which exhibit only small changes in contact angle with time) infiltration of the intercellular space is not likely because ϕ will ultimately become smaller than 20 - 25° making P_i negative.

Diameter of stomatal aperture.--Diameters of stomatal apertures for various plants have been published. Most of them were obtained either by direct microscopic examination of leaves (22) or from silicon rubber replicas (66). Such replicas were shown to be not always reliable in measuring the degree of stomatal opening (61).

The diameter of the stomatal aperture is of minor importance for infiltration of the intercellular space, as long as pressure is not involved. According to equations (4) and (5) P_i increases with decreasing diameter of the aperture. It is therefore a misconception to argue that stomatal penetration of bean leaves is difficult to obtain because of their small diameter (22). Since the diameter of the aperture affects only the size of the forces involved (P_i) and not the sign (positive or negative), both entry of a liquid into the aperture and its advancement into the substomatal chamber are not determined by the diameter.

Penetration of SADH

Time-course.--Penetration of SADH into bean leaf disks was linear with time over a 12 hour period (Figure 10). This was true for both surfaces, in light and in the dark and in both the absence and presence of Tween 20. Penetration was greater in light than in the dark and in the presence of Tween 20. From these results it was decided to adopt 12 hours as standard duration for all experiments.

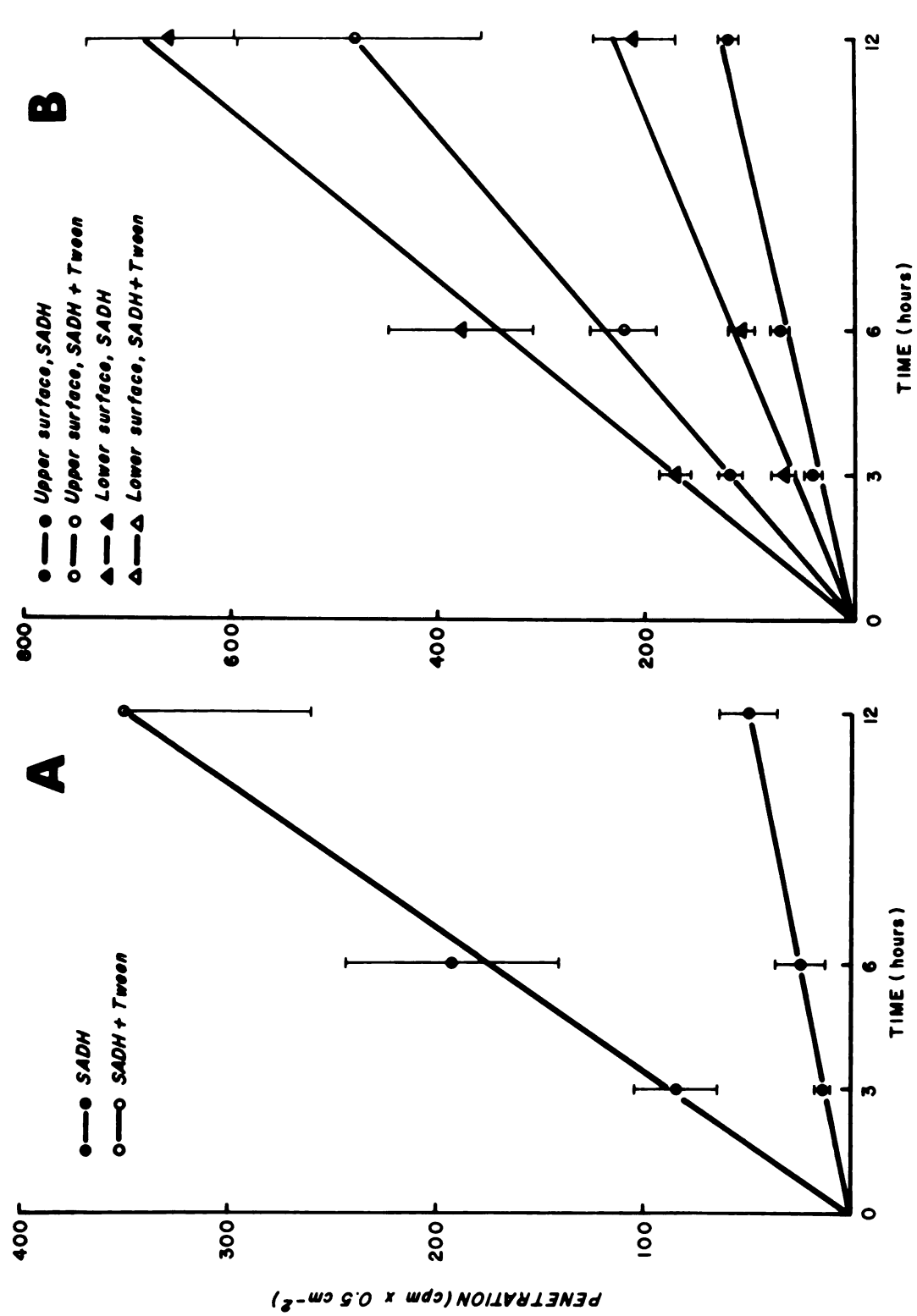
Surfactant concentration.--Tween 20 at concentrations higher than 0.001% increased penetration of SADH in the dark and in light (Table 4). The largest increase occurred when the Tween 20 concentration was raised from zero to 0.01%. A further increase in surfactant concentration to 0.1% resulted in a significant increase in penetration in light but not in the dark. Tween 20 at 1% suppressed penetration in light as compared to 0.1%, and did not significantly affect penetration in the dark.

Light.--For the light intensity studies a light bank providing 3,600 ft.c. was used and lower intensities were obtained by shading the dishes with cheese cloth. The temperature of the leaf disks and the treating solutions in the glass tubes was monitored over a 12-hour period using cooper-constantan thermocouples with a recording potentiometer. There was

Figure 10.--Time course of penetration of SADH into bean leaf disks.

A. Penetration through the upper surface in the dark.

B. Penetration through upper and lower surface in light.


A
B
SADH
SADH + Tween
Upper surface, SADH
Upper surface, SADH + Tween
Lower surface, SADH
Lower surface, SADH+ Tween
PENETRATION (cpm x 0.5 cm⁻²)
TIME (hours)
0
3
6
12
100
200
300
400
600
800

very good agreement between the temperature of the water bath, the temperature of the leaf disks and treating solutions at low light intensities. A ± 0.5° C deviation was found in the dark and at light intensities up to 600 ft.c. The temperature of the treating solution rose 2 - 4° C over the bath temperature under light intensities of 1,200 to 3,600 ft.c., whereas the difference between leaf and water bath temperature was + 1 to + 2° C.

TABLE 4.--The effect of Tween 20 on penetration of SADH into bean leaf disks.

Tween 20 conc.	Penetration			
	Dark		Light	
%	cpm / 0.5 cm^2 x 12 hrs.	%	cpm / 0.5 cm^2 x 12 hrs.	%
0.000	16.3 a*	100	138.9 a	100
0.001	15.3 a	94	160.4 a	118
0.01	49.6 b	304	363.7 b	262
0.1	52.0 b	319	504.7 c	339
1.0	61.7 b	378	345.5 b	249

*Means within a column followed by different letters are significantly different at P = 0.05.

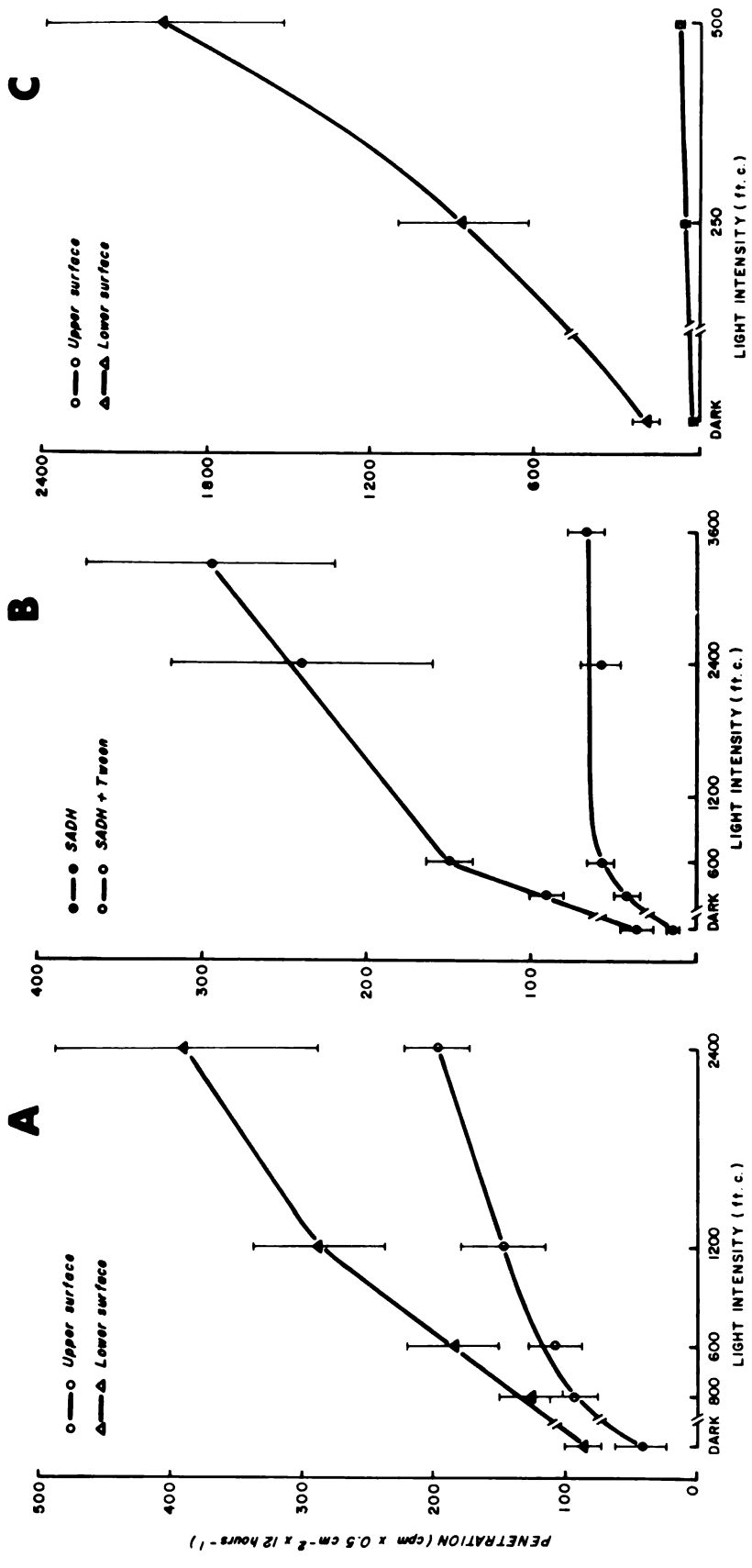
Penetration of SADH into both leaf surfaces increased with increasing light intensity (Figure 11 A).

Figure 11.--The effect of light on penetration of SADH into bean and pear leaf disks.

A. Penetration through the upper and lower surface of bean leaf disks.

B. Penetration through the upper surface of bean leaf disks, with and without Tween 20.

C. Penetration through the upper (astomatous) and lower (stomatous) surface of pear leaf disks (with Tween 20).


A
B
C
PENETRATION (cpm x 0.5 cm⁻² x 12 hours⁻¹)
LIGHT INTENSITY (ft. c.)
DARK
800
600
1200
2400
3600
0
100
200
300
400
500
600
1200
1800
2400
250
o—o Upper surface
Δ—Δ Lower surface
●—● SADH
o—o SADH + Tween

In this experiment, however, only the dark, 300 and 600 ft.c. treatments could be maintained at 30° C leaf temperature, while the temperature of leaf disks receiving 1,200 and 2,400 ft.c. increased slowly and reached 40° C after 12 hours, due to a failure in the cooling system. The further increase in penetration beyond 600 ft.c. is thus, at least partially a temperature effect. When the leaf temperature was maintained at 30° C, little further increase in penetration occurred when the light intensity was raised above 600 ft.c. in the absence of Tween 20 (Figure 11 B). With Tween 20 (0.1%) added, penetration of SADH increased further with an increase in light intensity from 600 to 3,600 ft.c. The light effect on penetration of SADH was more pronounced in the presence than in the absence of Tween 20.

Bean leaves are amphistomatous, therefore it was desirable to establish the effect of light on penetration using hypostomatous leaves. Fully expanded pear leaves ("Bartlet") were selected. Approximately 10 times more SADH penetrated the lower (stomatous) surface in light (500 ft.c,) than in the dark. The upper (astomatous) surface was almost impermeable to SADH, since figures for penetration were 26 cpm/disk in the dark and 72 cpm/disk at 500 ft.c. light. Although this increase was statistically significant the physiological significance might be questioned, since penetration into

the upper surface amounted to only 3.70% of the amount which penetrated the lower surface at 500 ft.c. These data suggest that stomata may be involved in the light increased penetration of SADH.

Temperature.--Variation of leaf temperature was accomplished by changing the temperature of the water bath in which the Petri-dishes were placed. In addition, the water baths were placed in growth chambers in which light intensity was adjusted to 600 ft.c. and the air temperature to ± 2.5° C of the water bath temperature.

The effect of temperature on penetration of SADH was markedly modified by light and Tween 20. Temperature had little effect on penetration in the dark in the absence of Tween 20 up to 35° C (Figure 12 A). A further increase in temperature, however, yielded a strong increase in penetration (Figure 12 B). Increasing temperatures increased penetration of SADH in the dark more effectively in the presence of Tween 20 (Figure 12 A) and a surge was observed between 25 and 35° C. Similarily, temperature affected penetration of SADH more in light than in the dark. With one exception, the increase in penetration per 5° C increment (penetration at higher temperature/penetration at lower temperature) was larger in light than in the dark (from Figure 12 B).

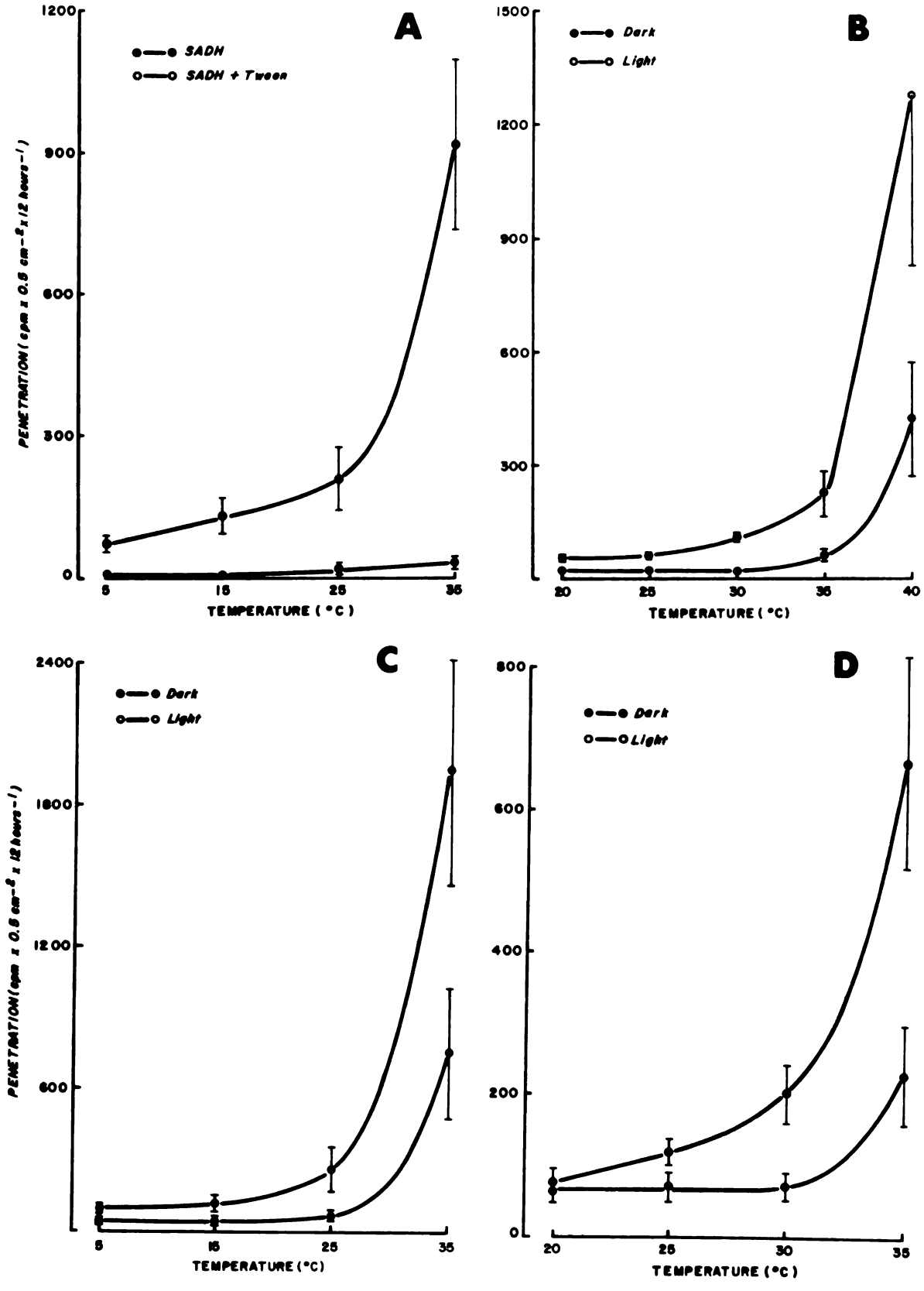
Figure 12.--The effect of temperature on penetration of SADH into bean leaf disks.

A. Through the upper surface in the dark.

B. Through the upper surface with Tween 20 added.

C. Through the upper surface without Tween 20.

D. Through the lower surface without Tween 20.


A
SADH
SADH + Tween
PENETRATION (cpm x 0.8 cm⁻² x 12 hours⁻¹)
TEMPERATURE (°C)
B
Dark
Light
TEMPERATURE (°C)
C
Dark
Light
PENETRATION (cpm x 0.8 cm⁻² x 12 hours⁻¹)
TEMPERATURE (°C)
D
Dark
Light
TEMPERATURE (°C)

Temperature	20/25	25/30	30/35	35/40
dark	1.0	1.0	3.0	6.5
light	1.1	1.6	1.5	8.5

The modifying effects of light and Tween 20 on penetration were partially additive, as the highest penetration rates were obtained in light with Tween 20 added to the treating solution (Figure 12 C).

The lower leaf surface responded similarily to the upper (Figure 12 D). In the dark, temperature had little effect below 30° C but penetration increased four fold between 30 and 35° C. The response to increasing temperatures was more gradual in light and penetration rates increased more rapidly. Thus, light increased penetration of SADH more effectively at higher temperatures.

The effect of temperature as influenced by Tween 20 and light on penetration of SADH can be illustrated by means of the temperature quotient (Q_{10}) for penetration through the upper leaf surface (Table 5). The temperature effect was least in the dark minus Tween, slightly greater in light minus Tween, followed by dark plus Tween and was greatest in light plus Tween for both temperature ranges. The modifying effect of Tween 20 was generally stronger than that of light. The

Q_{10} values for the range of 25/35° C were greater than those for the range 20/30° C. Thus, the temperature effect on penetration of SADH increased with temperature.

TABLE 5.--The effect of temperature, light and Tween 20 on the Q_{10} for penetration of SADH into bean leaf disks.

	Temperature quotients*					
Temp. range	20/30			25/35		
Treatment	- Tween	+ Tween	$\bar{x}$	- Tween	+ Tween	$\bar{x}$
dark	1.60	2.50	2.05	1.20	4.50	2.85
light	1.80	5.00	3.40	2.50	7.40	4.95
$\bar{x}$	1.70	3.75		1.85	5.95	

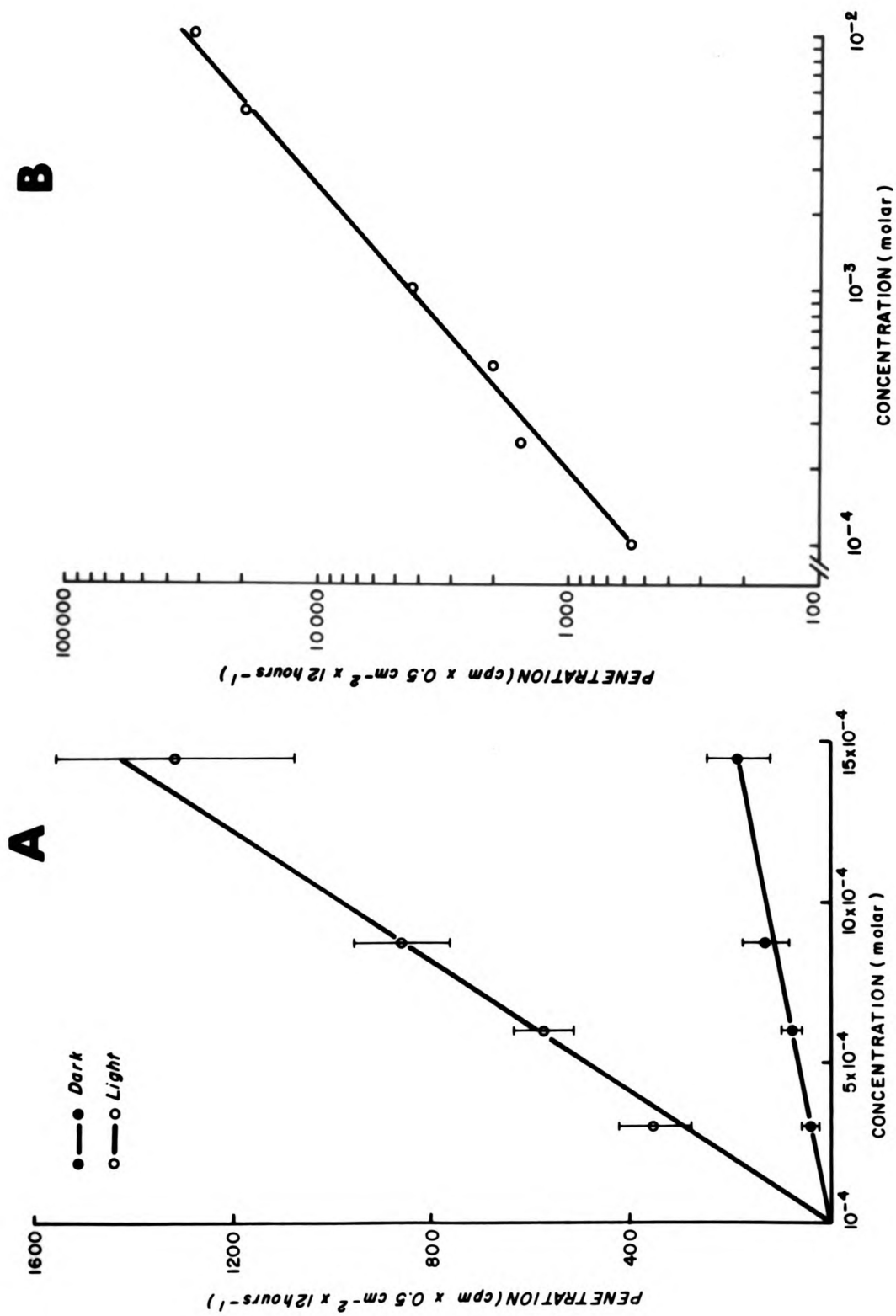
*Calculated from data of Figure 12 A, B, C.

Concentration of SADH.--Penetration of SADH was linear with concentration over a range of 10^{-4} to 15 x 10^{-4} M in both light and dark (Figure 12 A). Only a small portion, however, of the total SADH available penetrated during a 12 hour period; 0.17% of the SADH penetrated in the dark and 1.25% in light. A second concentration study was undertaken, using plants grown at 90% humidity and the concentration range was extended to 10^{-2} M. Penetration was linear with concentration--

Figure 13.--The effect of SADH concentration on penetration into bean leaf disks.

A. Penetration in light and dark with Tween 20 added.

B. Penetration in light with Tween 20 added (plants grown in 90% relative humidity).


A
B
Dark
Light
PENETRATION (cpm x 0.5 cm⁻² x 12 hours⁻¹)
CONCENTRATION (molar)
1600
1200
800
400
100000
10000
1000
100
10⁻⁴
5x10⁻⁴
10x10⁻⁴
15x10⁻⁴
10⁻³
10⁻²

2.72% of the available SADH penetrated within 12 hours (Figure 13 B).

Plant age and leaf size.--Plant age affected penetration of SADH in light but not in the dark (Table 6). Dark penetration was seemingly independent of leaf size or plant age, with one exception--with Tween and 12-day-old leaves. However, there was a close inverse relationship between leaf size and penetration in light. From the seventh to the twelfth day leaf size increased by 66%, whereas penetration decreased to 59% (plus Tween 20) or 52% (minus Tween 20) of the amount which penetrated into leaves of 7-day-old plants. Since leaf size and stomatal density were inversely proportional (Figure 5), a direct relationship therefore existed between penetration of SADH in light and stomatal density.

pH of the treating solution.--Penetration decreased concomitantly with increasing pH in both, light and dark (Figure 14). The dissociation constant of SADH is 1.12×10^{-5} ($pK_a = 4.92$). Thus at pH 4.0 90% of the SADH molecules have undissociated carboxyl groups, only 8.0% at pH 6.0 and only 1% at pH 7.0. Since SADH has been shown to be bound to a cationic resin (75) there must also be a positive charge, probably associated with the hydrazine N. However, no specific information on this point could be found in the literature.

Figure 14.--The effect of pH on penetration of SADH into bean leaf disks.

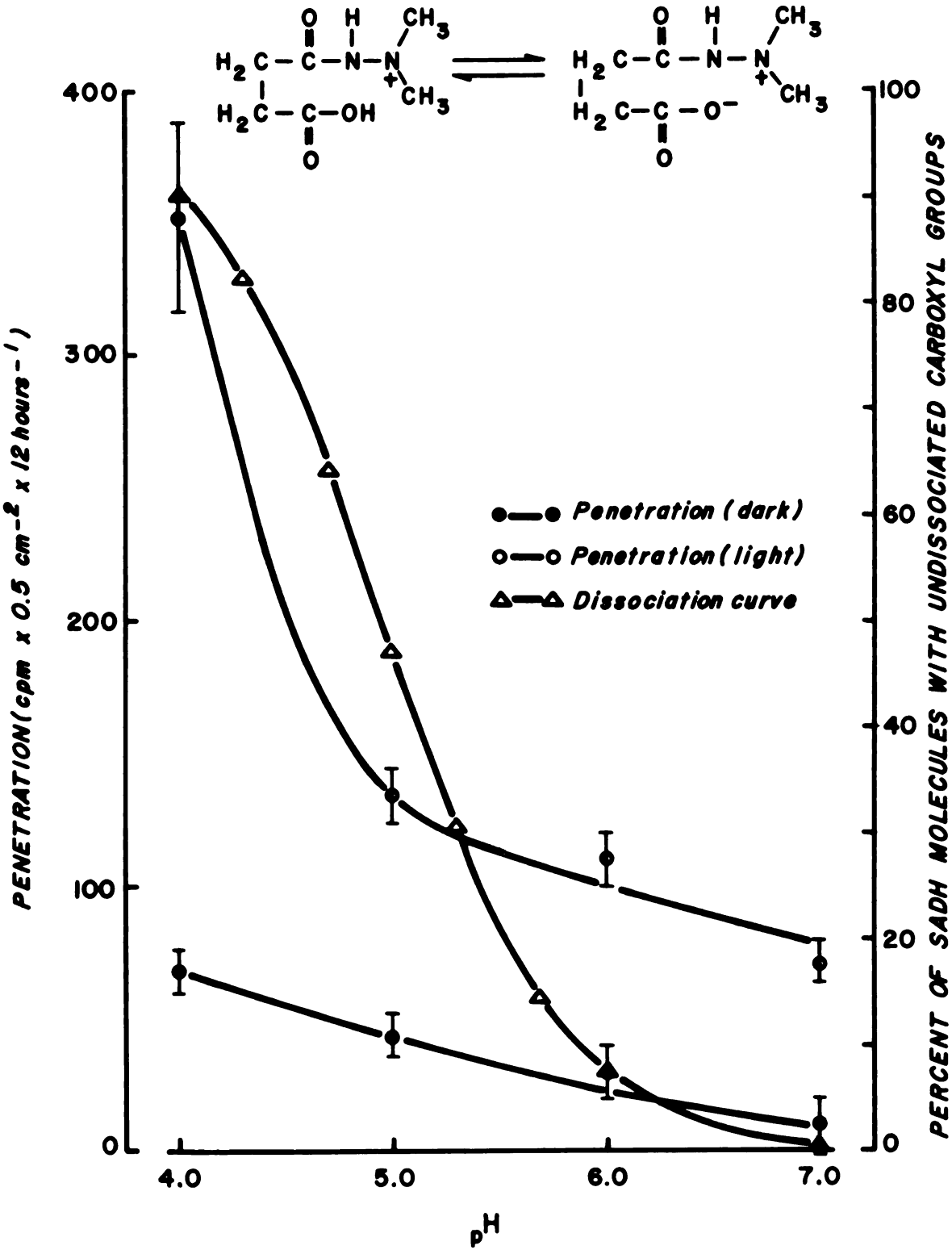

Penetration (dark)
Penetration (light)
Dissociation curve
PENETRATION (cpm x 0.5 cm⁻² x 12 hours⁻¹)
PERCENT OF SADH MOLECULES WITH UNDISSOCIATED CARBOXYL GROUPS
pH
400
300
200
100
0
100
80
60
40
20
0
4.0
5.0
6.0
7.0

TABLE 6.--The effect of plant age and leaf size on penetration of SADH into bean leaf disks.

Plant age	Leaf size	Penetration			
		Dark		Light	
		- Tween	+ Tween	- Tween	+ Tween
days	cm^2	cpm		cpm	
		0.5 cm^2 x 12 hrs.		0.5 cm^2 x 12 hrs.	
7	18	41.3 a*	77.5 a	219.5 a	1947 a
8	18	35.8 a	73.3 a	171.0 ab	2133 a
10	27	22.4 a	70.1 a	146.6 bc	1137 b
12	30	35.5 a	161.3 b	113.8 c	1146 b

*Means within a column followed by different letters are significantly different at P = 0.05.

Penetration of SADH increased threefold in light and twofold in the dark with a change in pH from 6.0 to 4.0 (Figure 14). Thus, the observed pH effect on penetration was influenced by illumination.

The pH effect was also influenced by Tween 20 (Table 7). In both buffer systems, penetration decreased with increasing pH in light as well as in the dark in absence of Tween 20. In the dark Tween 20 increased penetration of SADH markedly but did not alter the pH trend. In light, however, Tween 20 reversed the pH trend. Penetration was greater at pH 7.0 (or 6.0) than at pH 4.0. This was observed in both buffer systems.

The pH effect observed was somewhat surprising since that the molecule remains charged below the pK_a.

TABLE 7.--The effect of pH on the penetration of SADH into bean leaf disks and its modification by Tween 20.

pH	Penetration			
	Dark		Light	
	- Tween	+ Tween	- Tween	+ Tween
	cpm x 0.5 cm^{-2} x 12 $hours^{-1}$			
4.0[1]	19.6 a[3]	61.1 a	56.6 a	286 a
5.0	19.5 a	43.8 b	45.3 ab	354 a
6.0	15.3 ab	31.3 c	35.3 bc	405 a
7.0	7.7 b	23.7 c	22.2 c	428 a
4.0[2]	64.5 a	289.0 a	70.3 a	693 a
5.0	30.0 a	148.0 b	45.9 ab	894 b
6.0	23.5 a	112.0 b	24.4 b	892 b

[1]Citrate-sodium phosphate buffer, 14 day old plants.

[2]Citrate-sodium citrate buffer, 12 day old plants.

[3]Means within a column followed by different letters are significantly different at P = 0.05.

The effect of the ionic character of SADH as determined by pH and the role of Tween 20 (0.1%) on lipid solubility was determined by partitioning ^{14}C-SADH from an aqueous phase (0.05 M citrate-phosphate buffer) into a lipid phase (octanol). Ten ml of each phase were shaken together with ^{14}C-SADH (120,000 dpm) for 6 minutes in a separatory funnel and after separation of the phases

the distribution of the label was determined by counting 1 ml aliquots of each phase.

The distribution of SADH between the two phases was not affected by Tween 20 at any pH and partitioning into octanol was generally low (Table 8). Approximately 3% of the label partitioned into octanol below the pK_a, 1% at pK_a and only 0.1 - 0.2% at pH 7.0.

TABLE 8.--The effect of pH and Tween 20 on the distribution (%) of SADH between aqueous phase and octanol.

Surfactant	Phase	pH of aqueous phase				
		3.0	4.0	5.0	6.0	7.0
- Tween	aqueous	93.0[1]	95.7	98.2	99.2	95.5
	octanol	3.1	2.6	0.9	0.4	0.2
+ Tween	aqueous	93.7	93.6	97.5	100.0	99.2
	octanol	3.5	3.3	1.0	0.3	0.1

[1]The totals for aqueous and octanol phase do not give 100% since recovery was not complete.

Urea.--Urea at 0.1% in the treating solution increased penetration of SADH (Table 9). The increase was generally greater in light than in the dark and urea was more effective in increasing penetration of SADH at higher temperatures.

TABLE 9.--The effect of urea (0.1%) on penetration of SADH into bean leaf disks.

Temperature	Illumination	Penetration		Urea effect
		- Urea	+ Urea	
°C		cpm		%
		0.5 cm^2 x 12 hrs.		
25	dark	6.4	11.2	175[1]
	light	24.4	60.0	246
30	dark	7.7	18.3	238
	light	68.1	246.0	361
35	dark	13.9	23.2	167
	light	72.9	297.0	407

[1]The urea effect is significant at P = 0.05 (except for 35° C, dark treatment).

Osmotic stress.--The leaf disks were subjected to osmotic stress by pipetting sufficient sucrose solution of varying molarity into the Petri-dishes to obtain good contact between the lower leaf surface and the sucrose solution. The upper surface was kept dry. Control dishes contained distilled water. After a 3-hour pre-treatment period in the dark the glass tubes were sealed to the leaf disks and standard treating solution was added.

Penetration of SADH was significantly reduced when the sucrose solution exceeded a 0.2 M concentration (Table 10), which caused plasmolysis and stomatal

closure in light. Penetration into leaf disks subjected to 0.3 or 0.4 M sucrose solution was in light almost identical with the penetration into control disks in the dark.

TABLE 10.--The effect of osmotic stress on the penetration of SADH into bean leaf disks.

Treatment	Plasmolysis	Penetration
		cpm x 0.5 cm^{-2} x 12 $hrs.^{-1}$
control, (HOH)	-	51.1 a[1]
sucrose, 0.1 M, light	-	42.3 a
sucrose, 0.2 M, light	-	46.0 a
sucrose, 0.3 M, light	+	23.2 b
sucrose, 0.4 M, light	+	24.5 b
control, (HOH), dark	-	25.6 b

[1]Means followed by different letters are significantly different at P = 0.05.

Inhibitors.--Plants with fully expanded leaves were placed with their roots into test tubes containing 50 ml 10^{-3} M inhibitor solution. Sodium azide, 2,4-dichlorophenoxyacetic acid (2,4 D) and phenylmercuric acetate (PMA) were used. The solutions were aerated and uptake permitted for 8 - 10 hours in the dark at 25° C. Control plants were kept in distilled water. After termination of the inhibitor treatment the leaves were utilized for penetration studied.

All three inhibitors reduced penetration of SADH in light but were ineffective in the dark (with the exception of 2,4 D) (Table 11). The stomata of the inhibitor treated leaf disks remained closed in light as was evident from direct microscopic examination.

TABLE 11.--The effect of pre-treatment with inhibitors on penetration of SADH into bean leaf disks.

Inhibitor	Penetration			
	Dark		Light	
	cpm 0.5 cm^2 x 12 hrs.	%	cpm 0.5 cm^2 x 12 hrs.	%
control[1]	78.9 a[3]	100	619 a	100
NaN_3	59.1 a	75	183 b	30
2,4-D	21.1 b	27	247 b	40
control[2]	45.8 a	100	170 a	100
NaN_3	41.2 a	89	62 b	36
PMA	45.8 a	100	103 c	60

[1]With Tween 20

[2]Without Tween 20, different experiment.

[3]Means within a column followed by different letters are significantly different at P = 0.05.

Transpiration.--To test the effect of transpiration on penetration the standard procedure was modified. Larger leaf disks (3 cm diameter) were used. The glass

well was sealed to the center as usual. The treating solution (buffered at pH 7.0) contained 2 uC/ml ^{14}C-tryptophan (s.a. 8.95 mC/mmole) and 0.1% Tween 20. A penetration period of 6 hours was allowed in light (600 ft.c.) at 30° C. To minimize transpiration in one set of leaf disks, the leaf area around the glass tube was kept moist and the Petri-dishes were covered with a glass cover as usual. With a second set of disks the Petri-dishes were not covered and the leaf area around the tube was not wetted. The filter paper on which the leaf disks rested was maintained moist. Turgidity of the leaf disks and degree of stomatal opening were checked periodically in parallel leaf disks. The transpiring leaves did not wilt and the stomata remained closed in both sets of leaf disks. (The failure of stomata to open in light was probably due to the fact that the plants had been in light for 12 hours prior to the experiment.)

After termination of the penetration experiment, the leaf disks were carefully washed with distilled water, frozen immediately on dry ice and then freeze dried. The radioactivity of the leaf disks was determined and radioautograms were prepared.

Cuticular transpiration increased penetration of SADH 15-fold. While only 158 cpm/disk were found in leaf disks held at high humidity, 2,481 cpm/disk were

recovered in leaf disks permitted to transpire. This difference is also evident from the radioautogram (Figure 15).

Polar Pathways Across the Cuticle of Bean Primary Leaves as Revealed by Accumulation of Silver

Preliminary experiments have shown that silver is rapidly taken up by bean primary leaves from a silver nitrate solution. On reduction it becomes visible as black metallic silver grains. The same uptake procedures were followed as with SADH, but 0.01 M $AgNO_3$ solution (with or without 0.1% Tween 20) was added into the glass tubes and penetration was generally limited to 1 hour.

Preferential reduction and accumulation of silver occurred in cuticular ledges of guard cells, inside the guard cells themselves, in glandular trichomes and anticlinal walls. The pattern of accumulation was affected by light and Tween 20 (Figures 16, 17, 18). Compared to the dark treatment, more silver accumulated in light (Figure 16). Generally all cuticular ledges were labelled in light while only a fraction of the guard cells showed black ledges after penetration in the dark. If Tween 20 was added, much more silver penetrated and silver grains were present along the anticlinal walls (particularly around the guard cells) in addition to cuticular ledges. However, the Tween effect was more pronounced in light

Figure 15.--The effect of cuticular transpiration on penetration of ^{14}C-tryptophan into bean leaf disks.

A. Cuticular transpiration restricted.

B. Cuticular transpiration promoted.

Figure 16.--Sites of preferential entry, reduction and localization of silver in the upper surface of bean primary leaves (surface view).

A. Penetration in the dark.

B. Penetration in light.

C. Penetration in dark, plus Tween 20.

D. Penetration in light, plus Tween 20.

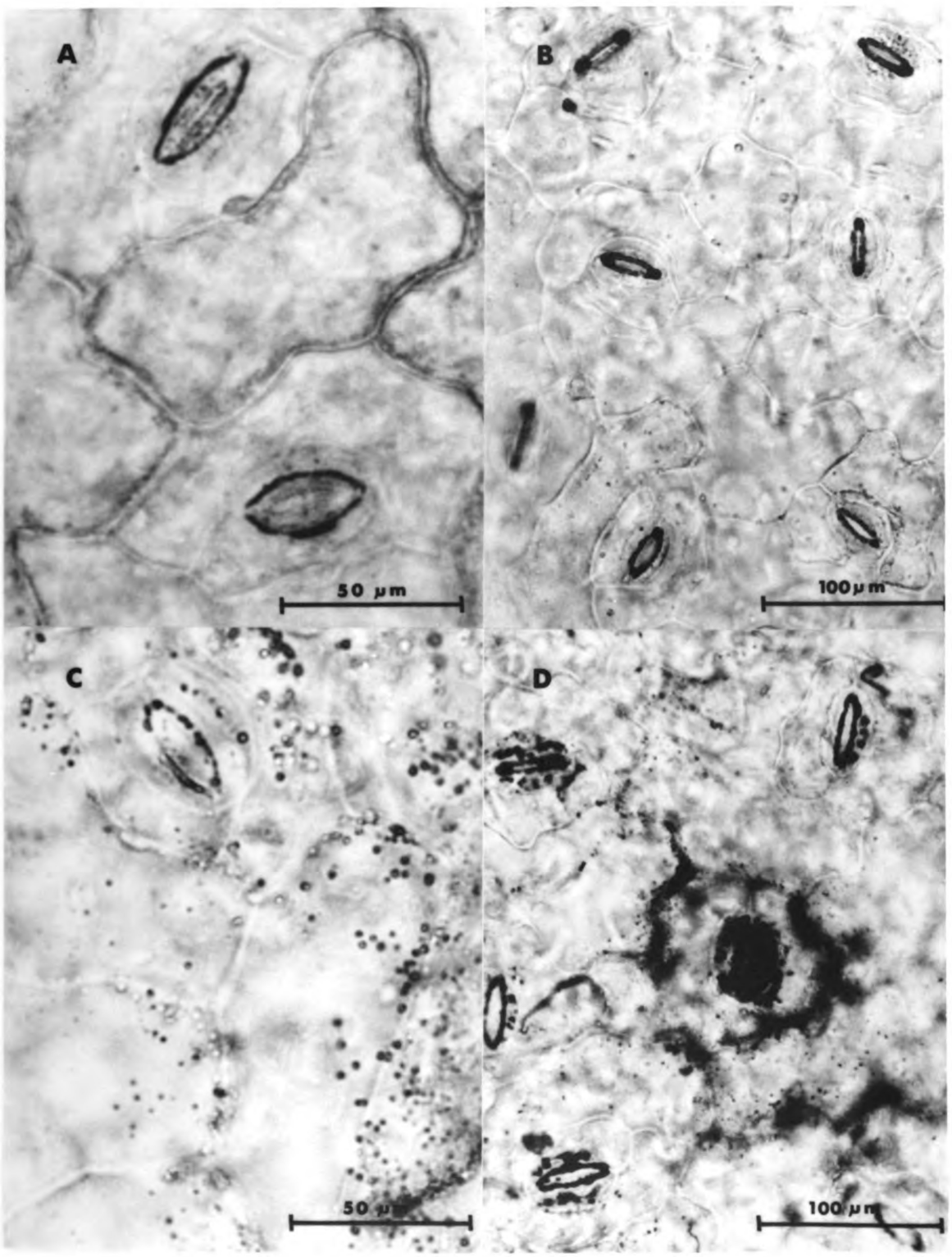

A
50 µm
B
100µm
C
50 µm
D
100 µm

Figure 17.--Sites of preferential entry, reduction and localization of silver in the upper surface of bean primary leaves (surface view).

A. Penetration in light.

B. Penetration in light, plus Tween 20.

C. Penetration in light, focus on cuticular ledges.

D. Same as C, focus inside the aperture.

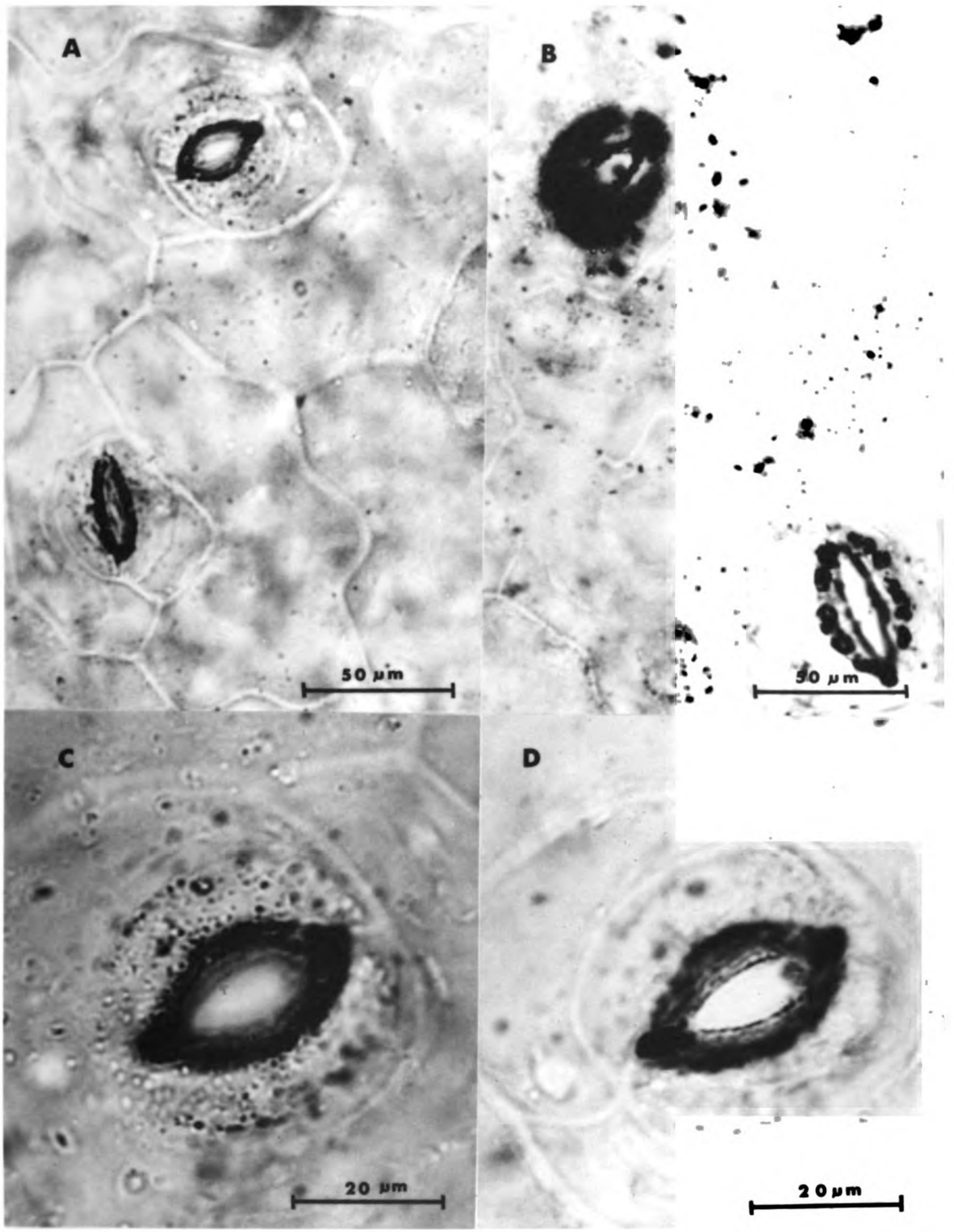
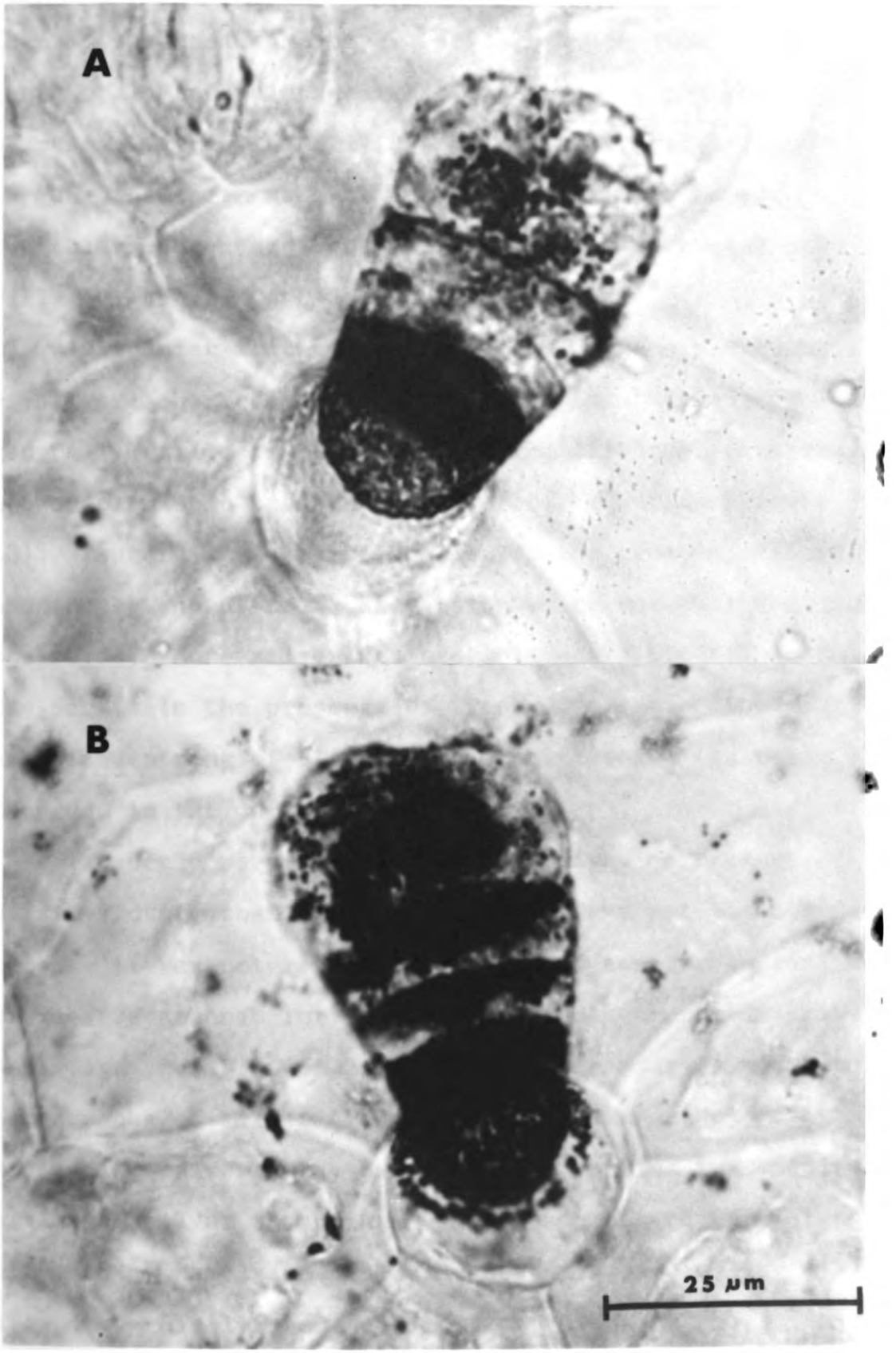

A
50 µm
B
50 µm
C
20 µm
D
20 µm

Figure 18.--Sites of preferential entry, reduction and localization of silver in the upper surface of bean primary leaves (surface view).

A. Penetration in the dark, plus Tween 20.

B. Penetration in light, plus Tween 20.


A
B
25 µm

than in the dark. Moreover, in light with Tween 20 all guard cells accumulated silver (probably in chloroplasts) (Figures 16 D, 17 B). Silver was rarely found in guard cells in absence of a surfactant, although cuticular ledges and occasionally even the stomatal pore walls (Figure 17 C, D) accumulated silver.

Glandular trichomes accumulated large quantities of silver, whereas the conical and "hooked" trichomes generally failed to do so. Glandular trichomes exhibited a characteristic pattern of silver distribution (Figure 18). The stalk cell was labelled to the greatest extent and in the "head"-cells silver tended to accumulate along the walls. More silver was accumulated in light than in the dark. In the presence of Tween 20 even the foot cell exhibited a ring of black silver grains which was not observed in the dark.

It could be argued that silver might be present at sites other than shown, but that it was not visible because it was not reduced. Therefore, comparable leaf disks were scanned for silver with an electron microprobe X-ray analyzer. A comparison of the secondary electron micrographs of silver treated leaf disks and the corresponding silver X-ray oxcillographs confirm the localization of silver in the same areas as observed with light microscopy, namely in cuticular ledges, guard cells, around guard cells and occasionally following the

outlines of anticlinal walls (Figure 19). Silver was also present in glandular trichomes and their foot cells. There was, however, some silver in the cuticular membrane over periclinal walls of the epidermal cells or in epidermal cells themselves which was not observed with the light microscope. A line scan across a stoma (Figure 20) showed two distinct peaks where the scan crossed the cuticular ledges and the concentration of silver in the walls of the pore was well above background.

Cross sections from leaf disks which had been treated with silver nitrate confirm the observations made in surface view. Following treatment in the dark with Tween 20 only the cuticular ledges were labelled (Figure 21 A). Leaves treated in light with Tween 20 had in addition to labelled cuticular ledges, silver inside the guard cells (Figure 21 B, C). Accumulation of silver in anticlinal and periclinal walls is illustrated in Figure 21 D. Silver absorbed by glandular trichomes did not move into adjacent epidermal cells (Figure 22 A).

In no case was silver found in the intercellular space or the substomatal chamber, although thousands of stomata were examined. Palisade and spongy parenchyma cells have been shown to be able to reduce silver which may enter the intercellular space. Localization of silver in the substomatal chamber was accomplished after

Figure 19.--Sites of preferential entry and localization of silver in the upper surface of bean primary leaves, as evident from silver X-ray oscillographs.

A, C and E. Secondary electron micrographs.

B, D and F. Corresponding silver X-ray oscillographs.

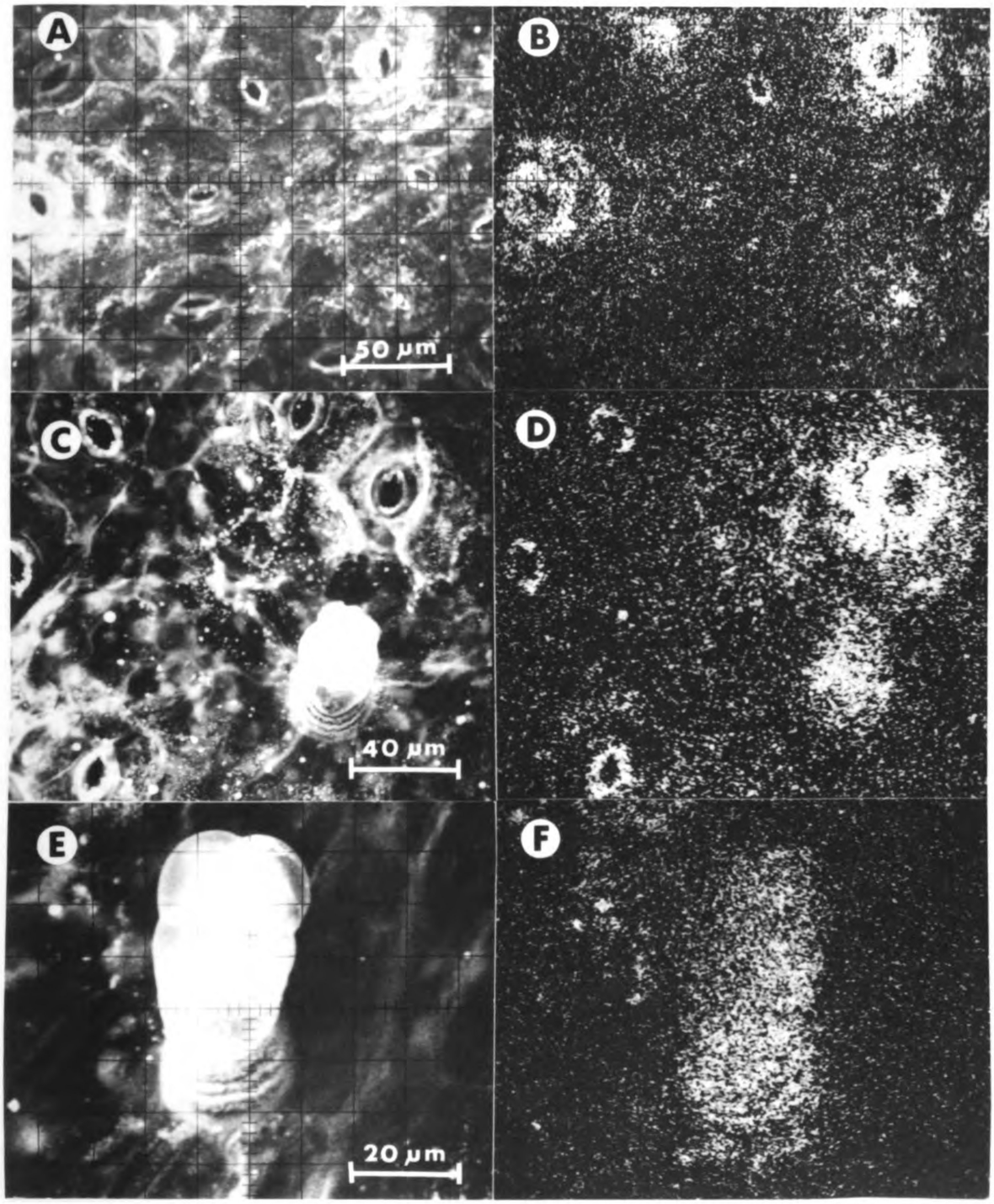
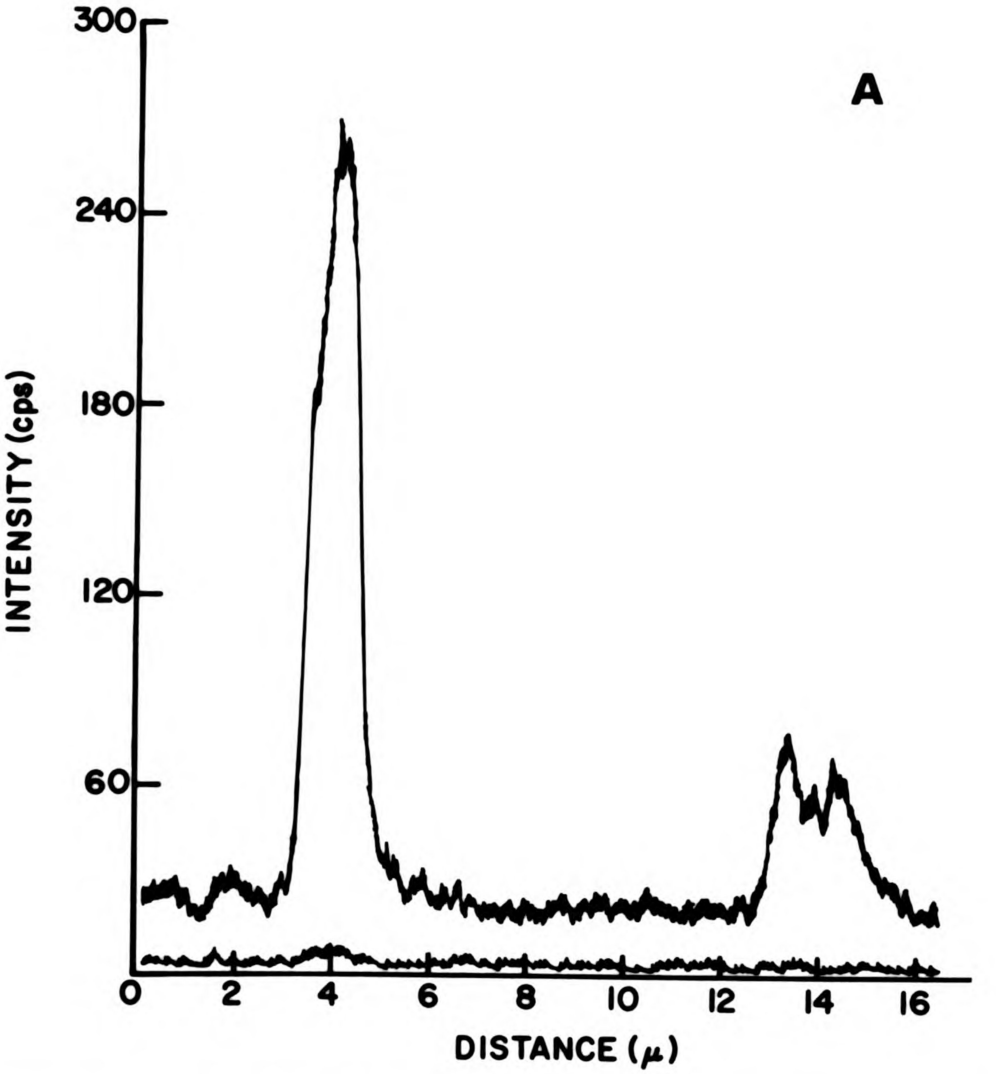

A
50 µm
B
C
40 µm
D
E
20 µm
F

Figure 20.--Sites of preferential entry and localization of silver in the upper surface of bean primary leaves, as evident from silver X-ray analysis.

A. Line scan scross the central horizontal axis (X-Y) of the stoma depicted in B (SEM) (lower curve is background).

B. Secondary electron micrograph of a stoma.

C. Corresponding silver X-ray oscillograph.


A
300
240
180
120
60
INTENSITY (cps)
0
2
4
6
8
10
12
14
16
DISTANCE (μ)

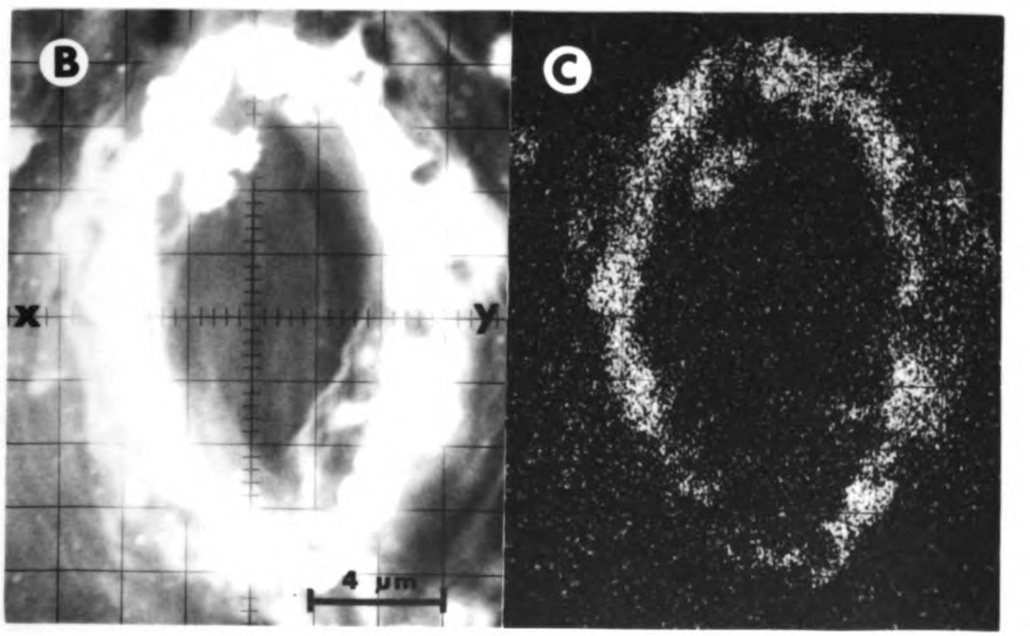

B
X
Y
4 μm
C

Figure 21.--Sites of preferential entry, reduction and localization of silver in bean primary leaves (cross-section).

A. Silver in cuticular ledges after penetration in the dark, plus Tween 20.

B, C. Silver in cuticular ledges and guard cells after penetration in light, plus Tween 20.

D. Silver in anticlinal and periclinal walls after penetration in light, plus Tween 20.

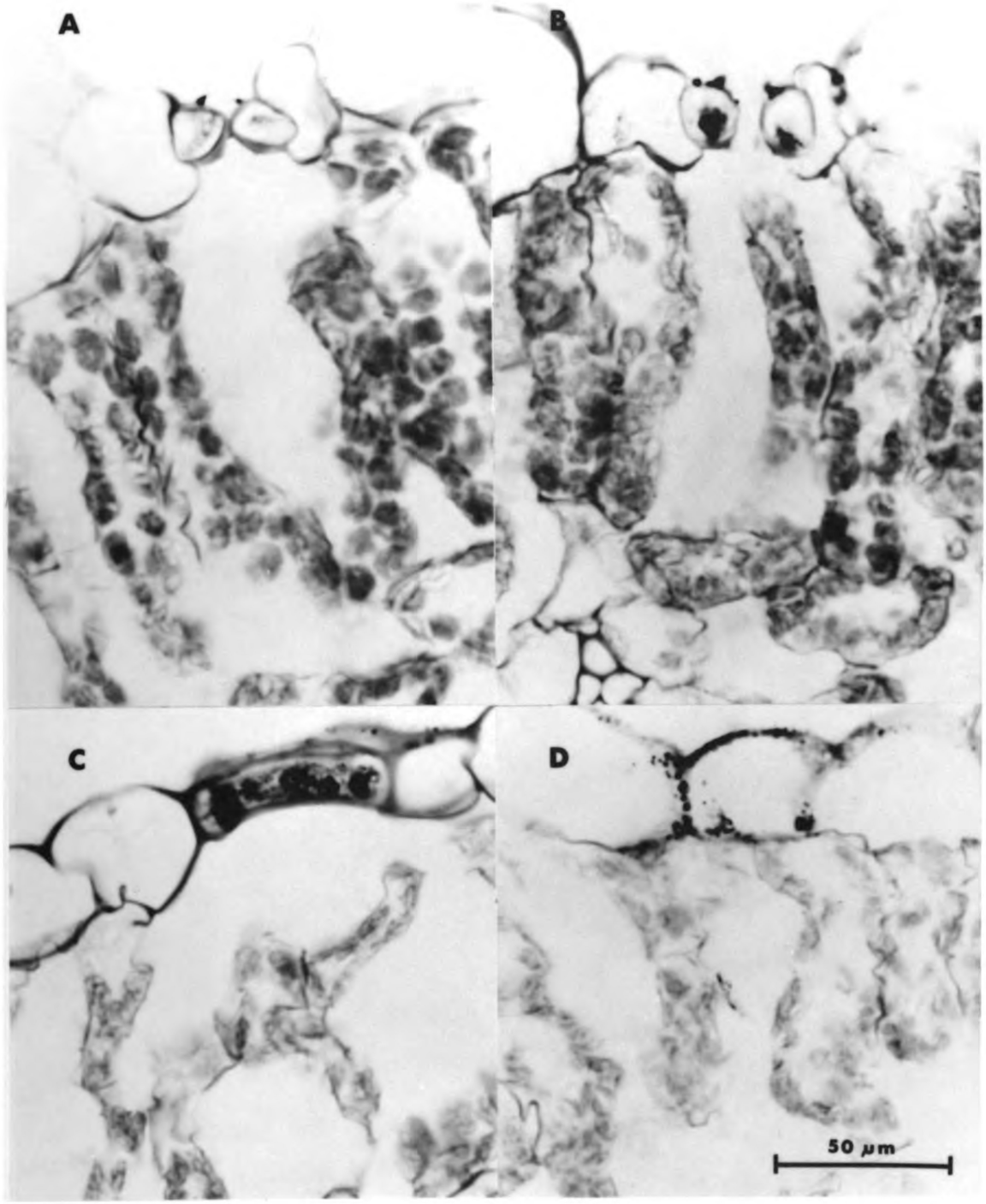
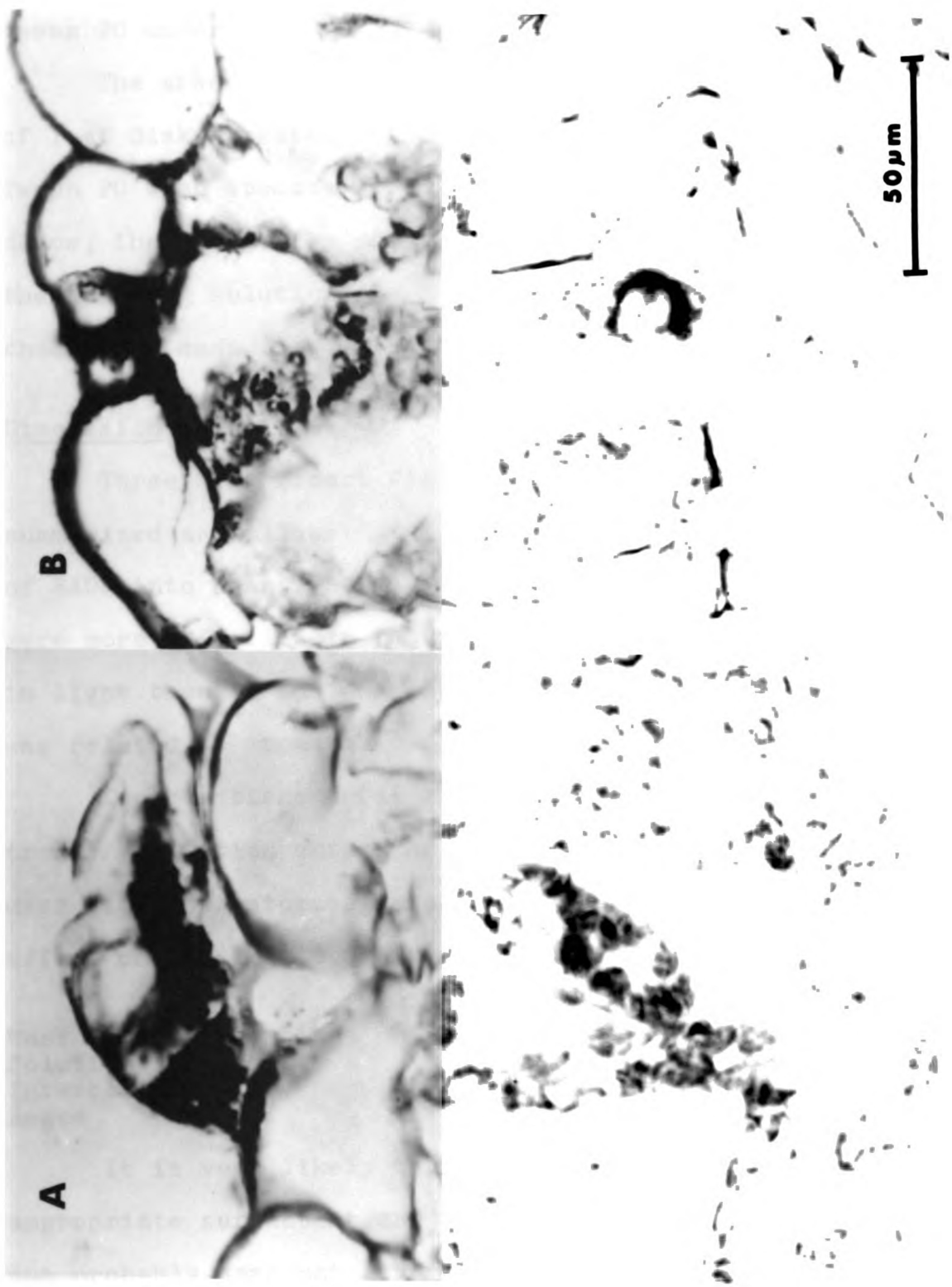

A
B
C
D
50 µm

Figure 22.--Sites of preferential entry, reduction and localization of silver in bean primary leaves (cross-section).

A. Silver in glandular trichome after penetration in light, plus Tween 20.

B. Silver in guard cells and substomatal chamber (palisade cells) after infiltration of leaf disk under vacuum with $AgNO_3$ solution plus Tween 20 in light.


A
B
50µm

infiltrating the leaf tissue with $AgNO_3$ solution plus Tween 20 under vacuum (Figure 22 B).

The absence of silver in the substomatal chamber of leaf disks treated with $AgNO_3$ solution plus 0.1% Tween 20 when stomata were open, is good direct evidence, that under the conditions of the experiments, the treating solution did not enter the substomatal chamber by mass flow.

Discussion

Three significant findings of this study can be summarized as follows: (a) light increased penetration of SADH into bean leaf disks, (b) all factors studied were more effective in increasing penetration of SADH in light than in the dark and (c) penetration in light was related to stomatal density and stomatal opening.

Two questions arise immediately. (a) Did SADH treating solution enter the intercellular air space by mass flow when stomata were open? (b) Is the light effect on penetration indicative of active uptake?

Mass Flow of Treating Solution into the Intercellular Air Space

It is very likely that a solution containing an appropriate surfactant can enter the stomatal aperture, but probably does not advance into the substomatal

chamber. Aqueous solutions form a definite contact angle with the wall of the aperture which ultimately will become larger than the slope of the walls of the aperture of the stoma (Figure 9). At this point the penetrating pressure becomes zero and further movement into the aperture will cease. Assuming a contact angle of 24° and the surface tension of the solution to be 30 dynes x cm^{-1}, at least 10 cm of water would be required for infiltration of the intercellular space. This is a conservative estimate. The treating solution in the glass tubes in this study was 3 mm high. Thus, penetration of treating solution into the intercellular space through open stomata must be ruled out on a theoretical basis.

Infiltration of a leaf can be clearly seen macroscopically. Infiltrated areas appear dark in incident light and bright in transmitted light. These patterns were never observed with treating solution. However, they could be easily induced with methanol.

Direct evidence that an aqueous solution containing 0.1% Tween 20 did not enter the intercellular air space by mass flow was obtained with the use of silver nitrate as a tracer.

Penetration of SADH was linear with time (Figure 10). The time required for bean stomata to open fully was 60 to 100 minutes (Figure 6). Therefore, if infiltration was to occur in light one would not expect

penetration to be linear with time, but rather a pronounced break resulting from infiltration, during the first 3 hours and then a leveling off.

The radioactivity observed was too low to fully account for infiltration of the intercellular air space. The volume of a bean leaf disk was approximately 400 µl and assuming 25% of the leaf as being composed of intercellular air spaces, then the intercellular space volume would approximate 100 µl. If this space would have been filled with treating solution (1 µC/ml) the leaf disks should have had an activity of ca. 22,000 cpm. However, the activities observed rarely exceeded 1,000 cpm.

It is therefore concluded that mass flow of treating solution, containing Tween 20, into the intercellular air space of leaf disks having open stomata did not occur in this study.

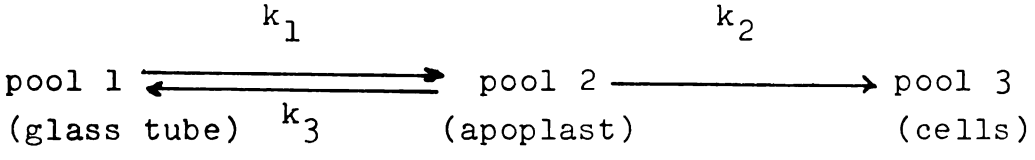
Active Uptake as a Limiting Factor

Within physiological pH ranges, the SADH molecule is ionized and it is unlikely that it will diffuse into the plasmalemma which is a lipid membrane. The uptake of SADH into the cytoplasm is more likely an active process.

The first barrier in foliar penetration, however, is the cuticular membrane. Penetration through the cuticular membrane is generally assumed to be a diffusion

process and has been shown to follow diffusion kinetics (20). SADH is water soluble, hence it may diffuse in the apoplast after traversing the cuticular membrane. Uptake of SADH into the cells would then occur from the SADH in the apoplast. Thus, there are potentially 3 pools in which SADH can be contained; the glass well (pool 1), the apoplast (pool 2) and the leaf cells (pool 3). The cuticular membrane separates pools 1 and 2 and the plasmalemma pools 2 and 3.

At the beginning of the experiment only pool 1 contains SADH, while pools 2 and 3 are empty. SADH will diffuse through the cuticular membrane at a rate k_1 into the apoplast (pool 2) and as soon as it appears there the adjacent cells can take SADH up at a rate k_2.



The concentration of SADH in pool 2 will be determined by the penetration rate k_1 and the rate of uptake k_2. If more SADH penetrates into the apoplast than is taken up into the cells (k_1 greater than k_2), then the SADH concentration in pool 2 will increase until equilibrium between pools 1 and 2 is reached ($k_1 = k_3$). A process of this nature would follow first order kinetics and the time required to reach equilibrium would

depend on the capacity of pool 2 and on the penetration rate k_1 provided a constant concentration in pool 1.

The concentration of SADH in the leaf disks (pool 2 + pool 3) increased at a linear rate over a 12-hour period (Figure 10). This may occur under two conditions. (a) The capacity of pool 2 (apoplast) is large relative to the penetration rate k_1. The total SADH concentration which could accumulate within 12 hours, therefore, remained very low and negligible compared to the SADH concentration in pool 1. Thus, k_1 remained constant. (b) Pool 2 is small but the concentration of SADH in pool 2 remained negligible compared to pool 1, because SADH was taken up by the leaf cells at a rate k_2 which was of a similar magnitude to k_1.

For the first case, SADH uptake into cells would be immaterial, because pool 2 is so large (relative to k_1) that the concentration of SADH in it remains negligible compared to pool 1 even in the absence of uptake into the cells. In the second case uptake of SADH into the cells, or no uptake at all, would result in an increase in SADH concentration in pool 2 and the penetration rate k_1 would then decrease with time.

Only in the second case could active uptake of SADH into the leaf cells affect the penetration rate k_1. It could be argued that the penetration of SADH was greater in light than in the dark, because in the dark

energy-dependent active uptake of SADH into cells was limiting, causing an accumulation of SADH in the apoplast, which in turn depressed the penetration rate k_1. If this was true, penetration in the dark could not have been linear with time. The penetration rate k_1 at the beginning (when the SADH concentration was still low in pool 2) would have been higher than at the end, and the penetration curve would level off with time. Furthermore, the assumption that active uptake is rate limiting in the dark could not explain why addition of Tween 20 and decreasing the pH of the treating solution resulted in an increased penetration rate which was constant with time.

The data from the concentration experiments also indicate that active uptake was not rate-limiting (Figure 13). Penetration of SADH was linear with concentration over a wide range (up to 10^{-2} M). Current concepts assume that carriers are involved in active uptake (65). With increasing concentrations of SADH these carriers must ultimately become saturated and an increase in concentrations beyond this point would not increase penetration. The fact that this saturation was never achieved is a further indication that active uptake was not the rate-limiting factor in penetration of SADH.

Finally, the increasing Q_{10} with increasing temperature was also evidence against active uptake as a

rate-limiting factor as well as the occurrence of temperature quotients as high as 7.4. A Q_{10} of this magnitude is not associated with metabolic processes.

It is therefore suggested that SADH penetrated the cuticular membrane by diffusion and that the penetration rate was constant over 12 hours because k_1 was low compared to the capacity of the apoplast such that the SADH concentration in the apoplast remained negligible relative to the concentration in the glass tubes. Some of the SADH in the apoplast was probably taken up into the cells but it had little or no effect on the penetration rate.

This concept of apoplastic accumulation of SADH is supported by the fact that SADH was lost from the leaf disks into the underlying filter disks (Table 1). This loss was proportional to the concentration of SADH in the leaf disk and specific for each leaf surface. More SADH was lost through the lower surface than through the upper. The ratio of per cent lost through the lower surface/per cent lost through the upper surface (1.69 for 6 hour and 1.42 for 12 hour period) was almost identical to the ratio of per cent penetrated into the lower surface/per cent penetrated into the upper surface (1.60).

If penetration of SADH into bean leaf disks was strictly a diffusion process as assumed, then temperature,

light, Tween 20, pH and urea must have affected the penetration rate directly by: (a) affecting the surface area of the cuticular membrane in contact with the treating solution (effective surface), (b) affecting the permeability of the cuticular membrane or (c) affecting both permeability and effective surface area.

Factors Affecting the Effective Surface Area

The apparent leaf surface area inside the glass tube was measured as 0.5 cm^2. The true surface area, however, was greater due to topography of the cuticular membrane and the presence of trichomes. A distinction between inner and outer surface must also be made. The outer surface may be defined as that area of the cuticular membrane accessible when stomata are tightly closed. The inner surface is defined as the surface inside the stomatal aperture whose size depends on stomatal density, degree of stomatal opening and size of the stomatal apparatus. For the upper surface of bean leaves the inner surface was estimated to be approximately 0.8 to 1.2% of the apparent outer surface. All factors which tend to increase stomatal opening will increase the inner surface. Of the experimental variables involved in this study, light, temperature and pH can affect stomatal opening (55, 113), while water, which is otherwise an important determinant, was not a factor

because the leaf disks were in a constant environment of 100% relative humidity.

In most plants stomata close in the dark and remain closed. Light affects the degree and the speed of opening. Using maize leaves, Raschke (87) has shown that the relative stomatal width ($\sqrt[3]{\text{porometer flow}}$) was directly proportional to CO_2 assimilation. With increasing light intensity, stomatal width increased rapidly at first with a subsequent leveling off as the light intensity approached saturation. The speed of stomatal opening was proportional to light intensity.

The effect of temperature on stomatal opening in Vicia faba leaves was investigated by Stalfelt (113). In leaves, floating on water and exposed to air and light (20,000 lux), stomata were closed at 5° C. With increasing temperature stomatal opening increased almost linearly up to 40° C.

Small et al. (109, 110) found that the guard cells of closed stomata (dark) had generally a lower pH than those of open stomata in light. Stomata of epidermal strips floating on buffer solutions of different pH values remained closed at low pH values (3-4) and opened at higher pH values (5-6). The response differed with the buffer ions used; nevertheless, stomata opened in the same pH range found in guard cells of open stomata in intact leaves.

The inner surface will be an effective surface only if the surface tension of the treating solution is low enough to permit entry into the stomatal aperture. Thus, addition of a surfactant to the treating solution not only enhances wetting of the outer leaf surface but also makes the inner surface available for penetration when stomata are open. This cannot be viewed as an either-or mechanism. Stomata vary in size and degree of opening. Even in the dark some stomata are more or less open, that is to say, the number of closed stomata will decrease and the degree of opening will increase as the light intensity is increased.

Some stomata may be sufficiently large and of a geometry such that even a treating solution without a surfactant can enter (Figure 17 C, D). Even under these circumstances a surfactant may increase the effective inner surface, because the treating solution could advance deeper into the aperture. Therefore, the effect of stomatal opening and addition of a surfactant on the effective inner surface is quantitative rather than qualitative.

The inner surface is probably not involved in penetration in the dark when no surfactants are employed, whereas, addition of a surfactant may allow the solution to enter those few open stomata. Solution can probably enter a small number of stomata in light in

the absence of a surfactant, particularly under high light and temperature conditions. In light and with a surfactant the treating solution will penetrate into the aperture of many or all stomata. The effective inner surface will be largest when stomata have attained their greatest aperture.

The conclusion, therefore, is that all factors which tend to increase stomatal opening and wettability will increase penetration by increasing the effective surface of the cuticular membrane. These factors are light, temperature pH and Tween 20. The following observations in this study can be totally or partially explained by the arguments presented above: the greater penetration in light than in the dark, the gradual increase in penetration with increasing light intensity and increasing temperature and the relationship between light, temperature and Tween 20.

In the dark and without Tween 20 temperature affected penetration of SADH only slightly (Table 5) because stomata remained closed and there was little change in effective surface. With increasing light intensity and/or temperature penetration of SADH increased because the number of open stomata and their aperture increased. Temperature and light were more effective in increasing penetration in the presence of

Tween 20, because of increased effective surface, particularly the effective inner surface.

There is a possibility that the sudden increase in penetration in the dark at high temperatures (Figure 12) was due to stomatal opening in the dark. It has been reported (73) that stomata of Xanthium leaves open widely in the dark at temperatures between 27 - 36° C.

Inhibitors reduced penetration in the light but were ineffective in the dark (Table 11). This would be difficult to understand if the inhibitor affected a metabolically-linked uptake process, but their effect is easily explained on a basis of a reduced effective surface, since all inhibitors prevented stomatal opening in light (9, 132). The lack of activity in the dark would be expected since the stomata would be closed. Stomatal closure by plasmolysis (Table 10) affected penetration of SADH in the same way. (An effect of stomatal closure on the permeability of the cuticular ledges will be considered later).

The close relationship between stomatal density and penetration in light and its absence in the dark (Table 6) is further evidence for the importance of stomatal opening in penetration of SADH. It must be pointed out, however, that the relationship between stomatal density and penetration is not as good when the upper and the lower surfaces are compared. Only 1.6 times (Table 1) or

1.8 times (minus Tween 20) and 1.4 times (plus Tween 20) (Figure 10) more SADH penetrated into the lower than into the upper leaf surface, although stomatal density is 2.78 times and the density of glandular trichomes 3.7 times greater on the lower than on the upper surface. Stomata and glandular trichomes may well differ anatomically and/or physiologically between the two surfaces.

It is uncertain whether or not the pH of the treating solution can affect opening of stomata in intact leaves as it does in isolated epidermal strips. It could be speculated that Tween 20 increased penetration of SADH more effectively at high pH values (5 - 7) than at low pH values (4), because stomatal opening was inhibited at pH 4.0 (Table 7). Since Tween 20 reversed the pH effect in light, but not in the dark, the possible involvement of stomata should be recognized.

Factors Affecting the Permeability of the Cuticular Membrane

In order to discriminate between different mechanisms of penetration of pharmaceuticals across the lipid membranes of the digestive tract, the terms "non-ionic diffusion" and "filtration" were coined. Partitioning of undissociated weak electrolytes into lipid membranes is called non-ionic diffusion and penetration of charged molecules (dissociated) via polar channels in the lipid

membrane, a process linked to water movement, is called filtration (124). This concept will be adopted for the present discussion.

Non-ionic diffusion.--Lipophilic molecules may penetrate a lipid membrane by partitioning. In a homologous series there was good agreement between the partition coefficient (chloroform:water) and cuticular penetration (20). Weak acids and bases, although often quite polar, can also penetrate a lipid membrane by partitioning in the non-dissociated state. Therefore, penetration of weak electrolytes through lipid membranes is markedly affected by pH, while penetration of non-ionizable molecules is not (106).

Below the pK_a, penetration of a weak acid is proportional to the concentration of the weak acid. With increasing pH above the pK_a, the portion of the total concentration ($HA + A^-$) which penetrates becomes smaller and smaller. It was therefore postulated that only the undissociated molecule (HA) can penetrate while the anion (A^-) cannot. If this was true, penetration would have to be proportional to the concentration of the molecule (HA) at all pH values and independent of the concentration of the anion (A^-). However, Simon and Beevers (106) have shown that this was not the case. As the pH was increased above the pK_a, penetration became greater than one would have expected from the concentration of HA.

Thus, while the penetration of the anion (A^-) was low and negligible at pH values below the pK_a, an increasing portion of the anions penetrated as the pH was raised above the pK_a. Thus, the contribution of the anion to the total amount penetrated can become substantial 3 to 4 pH units above the pK_a.

Simon and Beevers (106) did not offer an explanation for how ions, which are not supposed to partition into the lipid membrane, can nevertheless penetrate. This penetration probably proceeds through localized hydrophilic channels which have been shown to occur in lipid membranes (118, 124). Härtel (53) reported a pH-dependent change in the permeability of cuticular membrane to water, with a maximum permeability around pH 7.0. At this pH most weak acids are completely dissociated and the greater penetration of ions observed at higher pH values might thus be the consequence of an pH-dependent permeability change of the polar channels in the lipid membrane.

Theoretically, non-ionic diffusion should not be involved in penetration of SADH through the cuticular membrane, since SADH appeared to be charged at all pH values used. Above the pK_a (4.92) SADH is probably largely a zwitter ion and below the pK_a it is cationic. Consequently, the solubility of SADH in octanol was very low (Table 8). Still, more cationic SADH partitioned

into octanol than the zwitter ion form and if the cuticular membrane of bean leaves is of similar lipophilic nature as octanol, these pH-dependent differences in partitioning could account for the greater penetration of SADH at pH 4.0 than at higher pH values (Figure 14). It is important to note that Tween 20 (0.1%) did not increase the lipid solubility of SADH at any pH which indicated that the increase in penetration with Tween 20 cannot be attributed to increasing the lipid solubility of SADH.

Filtration.--Important characteristics of filtration are: (a) Movement of molecules is confined to localized permeable channels in the otherwise impermeable lipid matrix of the membrane. (b) Molecular size of a penetrating molecule is inversely related to the penetration rate and very large molecules may not penetrate at all (124). (c) Filtration appears to be linked to water movement through these channels (124). (d) Undissociated molecules if sufficiencly polar and not too large in size probably also move by filtration but this fraction is very small compared to movement by non-ionic diffusion. Charged molecules, on the other hand, seem to move entirely by filtration (124).

Sites of polar channels in the cuticular membrane, as revealed by fluorochromes (6, 118) and radioautography (71), are localized in the cuticular ledges of guard

cells, glandular trichomes and anticlinal walls. This was confirmed for the bean by using silver ions, which by their nature must penetrate the cuticular membrane through hydrophilic channels.

Silver ions in pure water are not reduced by light, however, Ag^+ in contact with organic matter is reduced in light to metallic silver. Ag^+ can also be reduced by reducing substances present in the membrane (72) and in the cuticular ledges (71). The latter processes are responsible for reduction in the dark. If many ions are reduced at one site, the reduced silver becomes visible with the light microscope.

These data demonstrated that silver entered the cuticular membrane and was reduced preferentially in the cuticular ledges, glandular trichomes and anticlinal walls, particularly around the stomata. Silver was also found in the walls of the stomatal aperture and, in smaller quantitites, in the periclinal walls.

Silver accumulated in defined areas over the anticlinal walls and in the membrane of the foot cells of glandular trichomes only if Tween 20 was incorporated in the $AgNO_3$ solution. These channels seem to be available for penetration only if the surface tension of the solution was reduced. The Tween effect on SADH penetration in the dark, which could not be explained completely on the basis of surface changes, because stomata remain

closed in the dark, can thus be explained on the basis of change in permeability.

More silver accumulated in all sites in light than in the dark. If Tween 20 was added to the treating solution, silver was also found inside the guard cells. Light reduction may have been involved, but two other factors discussed previously were probably also involved: the greater permeability of cuticular ledges in open stomata as compared to closed stomata and water movement, similar to that which accelerated transfer of fluorochromes (6, 118). The 15-fold increase in penetration of tryptophan brought about by cuticular transpiration is an impressive example as to how important water movement can be with respect to cuticular penetration.

The increase in penetration of SADH with urea is probably the consequence of increased permeability of the cuticular membrane. Urea has been shown to increase the permeability of isolated cuticular membranes from onion leaves and tomato fruits (129).

As long as the nature of the polar channels in the cuticular membrane remains obscure, the problem of filtration can be discussed only in vague terms. With respect to penetration of SADH it would be interesting to know whether a net charge on a molecule will affect filtration, a likely possibility if the matrix of the polar channels contains dissociable groups. It would be

even more important to know the relative permeability of the walls of the stomatal aperture. At the present time it is known that these walls are relatively polar in Citrus (120). Since the permeability of the cuticular ledges appears to be greater in open than in closed stomata, the relative importance of the inner surface and the cuticular ledges for penetration of SADH must remain obscure, because the two factors cannot be studied separately. To fully understand these implications it must be born in mind that the inner surface was estimated to be 0.8 to 1.2% of the apparent outer surface and that penetration of SADH increased up to 10 fold due to light (Figure 11).

This dramatizes the need for specific research in this direction. The role of water movement is also not well understood. It could be that metabolism affected penetration of SADH indirectly via its effect on the water status and movement in the leaf disk. In this case specific research is also required.

Foliar Penetration of Succinic Acid 2,2-Dimethylhydrazide: Studies Employing a Growth Response As An Index for Penetration

Material and Methods

Bean First and Second Internode Assay

An indeterminate type of bean (Phaseolus vulgaris L. cv. "Blue Lake") was used as the test plant. Seedlings were grown in a growth chamber at 28° C during the light (14 hours) and 22° C during the dark (10 hours) period. Light was provided by fluorescent and tungsten lamps, adjusted to 1,300 ft.c. at the level of the primary leaves. Humidity was not controlled and varied between 60 - 70% (light) and 70 - 80% (dark). Plants were cultured in light soil in 3-inch peat pots. One plant was selected and permitted to develop per pot.

To obtain a growth response from the internode between the primary leaves and the first trifoliate leaf (subsequently called first internode) plants were treated before elongation of the terminal bud began. Optimum time of treatment was 7 - 8 days after planting. At this stage the primary leaves were 50 - 80% expanded.

Treatment consisted of dipping one primary leaf momentarily in an aqueous solution of SADH (technical), buffered at pH 5.0 with 0.01 M citrate-phosphate buffer (88). Treated plants were arranged in the assigned

experimental design as soon as the treating solution had dried on the leaf.

Two general types of experiments were performed, (a) the treated primary leaf either remained attached to the plant or (b) it was detached at specified intervals following treatment. Control plants were similarly treated with buffer or buffer plus surfactant and, if the experiment involved removal of the treated leaf, this was detached from the control plants at an intermediate time interval.

Surfactants used in this study were Tween 20 (polyoxyethylene sorbitan monolaurate), Igepal CO 880 (Nonylphenoxypoly (ethyleneoxy) ethanol, here coded P 303), and Buffer X (principal functional agents: alkylarylpolyethoxyethanol, free and combined fatty and phosphatidic acids). Tween 20 and P 303 are both non-ionic and Buffer X is a blend of non-ionic and anionic surfactants.

When the second internode had ceased elongating (ca. 3 weeks after planting), the length of the first and second internode were determined and the experiment terminated.

Randomized block experimental design with 5 to 8 replications was utilized and analysis of variance performed on the data. Comparison of the means was by use of Tukey's w-procedure (114).

Details of specific experiments will be presented with the appropriate section.

Determination of Retention of SADH by Bean Leaves Following Dipping

Fully expanded primary leaves of 10-day old plants, grown under conditions mentioned earlier, were detached and dipped for 10 seconds in treating solution (buffered solution of ^{14}C-SADH at 25 nC/ml concentration, without or with surfactant added). After removal from the treating solution the leaves were held in a vertical position to allow the excess liquid to drain off and then washed in methanol (2 leaves in 50 ml) to remove the SADH adhering to the leaf surface. The leaf area was determined with a planimeter. Residual radioactivity was determined after the methanol wash on disks punched from randomly chosen leaves. No significant radioactivity could be detected.

Five 5-ml aliquots of the methanol wash from two leaves (50 ml) were plated in stainless steel plachets and assayed for radioactivity. Each treatment consisted of 10 leaves with a total surface of ca. 700 cm^2. Analysis of variance was performed (randomized block design) and significance determined by the F-test.

Determination of Penetration of ^{14}C-SADH Into Attached Bean Leaves

Fully expanded primary bean leaves were dipped into a 5 x 10^{-5} M ^{14}C-SADH solution either without or with Tween 20 (0.1%) added. Zero (= retention), 1/3, 1, 2, 4, 8 and 24 hours after dipping 5 leaves per time-treatment were detached and each washed in 50 ml distilled water. The leaf area was determined with a planimeter. Two 5- ml aliquots were plated and counted for radioactivity from each wash solution and the radioactivity removed per unit leaf area was computed. The difference in radioactivity removed per unit leaf area between zero time and the subsequent sampling intervals was considered to have penetrated.

Penetration of Alar 85 Into Apple Leaves

Ten-week-old McIntosh seedlings, 12 - 13 cm high and having 12 - 14 fully expanded leaves each, were selected as a second test specimen. The plants had been raised on light soil in peat pots (3 inch) on a greenhouse bench at 22° - 24° C night and 25 - 28° C day temperature. These conditions were maintained during the duration of the experiment but supplemental light from fluorescent lamps (1,500 ft.c.) was given to insure vigorous growth during the months of October and November.

Plants were sprayed at 8 a.m. with 1,000 ppm (active ingredient) Alar 85 to a point just short of run-off. Control plants were not sprayed. Leaves appeared dry 20 - 30 minutes after spraying. To prevent the Alar spray from contaminating the soil, the pots were placed in plastic bags which were tightly closed around the base of the stem, with an intermediate layer of soft blotting paper wrapped around the stem.

One-third, 1, 6 and 12 hours after spraying the leaves of 10 plants (replications) per time interval were thoroughly washed with tap water to remove the Alar residue which had not penetrated. One group of 10 plants was not washed. The plastic bags were removed from the pots after the wash, plants arranged in the assigned design and cultured for a further 4 weeks. The growth increment obtained during these 4 weeks was determined and used as a biological index of penetration of SADH during the time interval between spraying and washing.

Results

Concentration Response

To establish the relationship between SADH concentration and internode length one primary leaf per plant was dipped into SADH solution. Five concentrations from 5×10^{-4} to 5×10^{-2} M SADH were used. The treated leaves remained attached to the plants.

SADH inhibited elongation of the first and second internode (Figure 23). The concentration response curves are sigmoidal. The first internode was more sensitive to low concentrations (5×10^{-3} M) while the second internode was more sensitive to high concentrations (5×10^{-2} M). The lowest concentration required to attain a significant inhibition of internode elongation was 10^{-3} M for the first and 5×10^{-3} M for the second internode.

Surfactant Effects

All surfactants tested reduced retention of the treating solution by bean leaves as compared to the retention of a solution lacking a surfactant (Figure 24). The reduction in retention was statistically significant at a surfactant concentration of 0.01% and a 10-fold increase in surfactant concentration did not result in a significant further reduction of retention, although this was considerable for Tween 20. The differences between the surfactants were not significant. At a concentration of 0.1% P 303 reduced retention to 74%, Tween 20 to 78% and Buffer X to 85% of that quantity of SADH retained by bean leaves dipped in a solution lacking a surfactant (control).

Penetration of ^{14}C-SADH into bean leaves was enhanced by Tween 20 (0.1%) (Figure 25). One hour after

Figure 23.--The effect of SADH concentration on elongation of the first and second internodes of bean plants.

Elongation of the first internode was significantly ($P = 0.05$) inhibited at SADH concentrations of 10^{-3} M and higher, and the second internode at 5×10^{-3} M and higher.

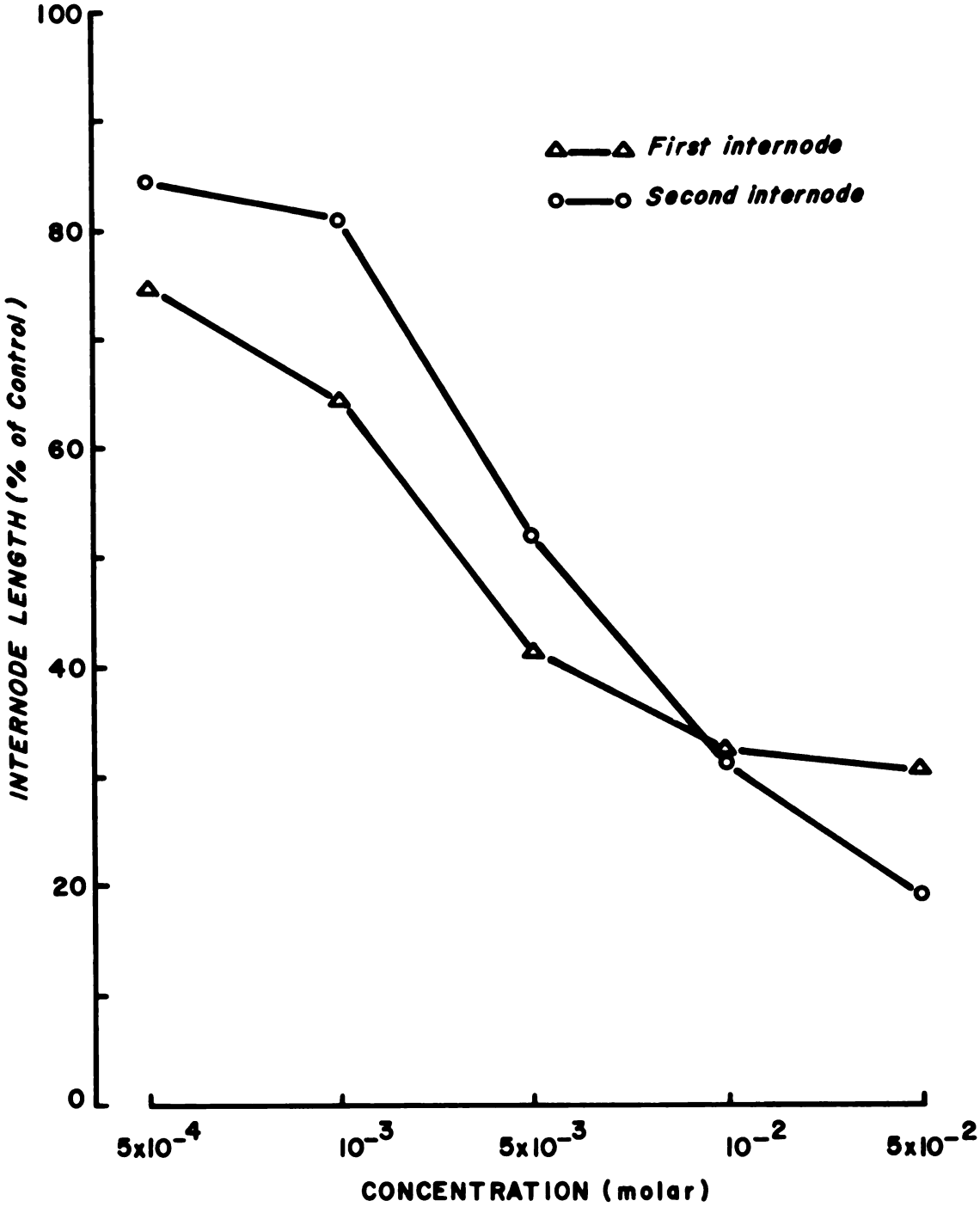

100
80
60
40
20
0
INTERNODE LENGTH (% of Control)
First internode
Second internode
5x10⁻⁴
10⁻³
5x10⁻³
10⁻²
5x10⁻²
CONCENTRATION (molar)

Figure 24.--The effect of surfactants and surfactant concentration on retention of treating solution by bean primary leaves.

Differences between control (lacking surfactant) and 0.01% surfactant concentration are statistically significant (P = 0.05). Differences between surfactants are not significant.

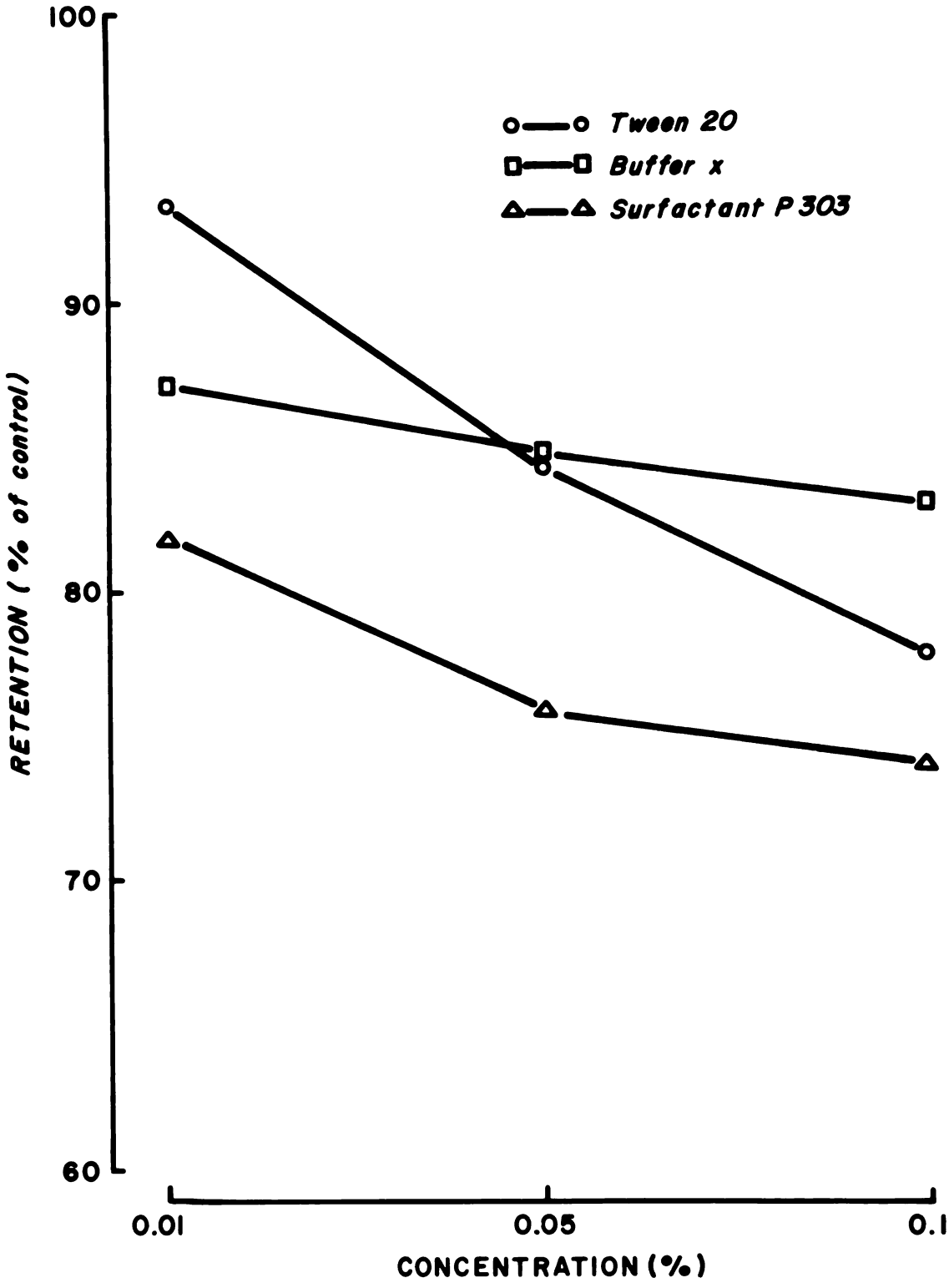
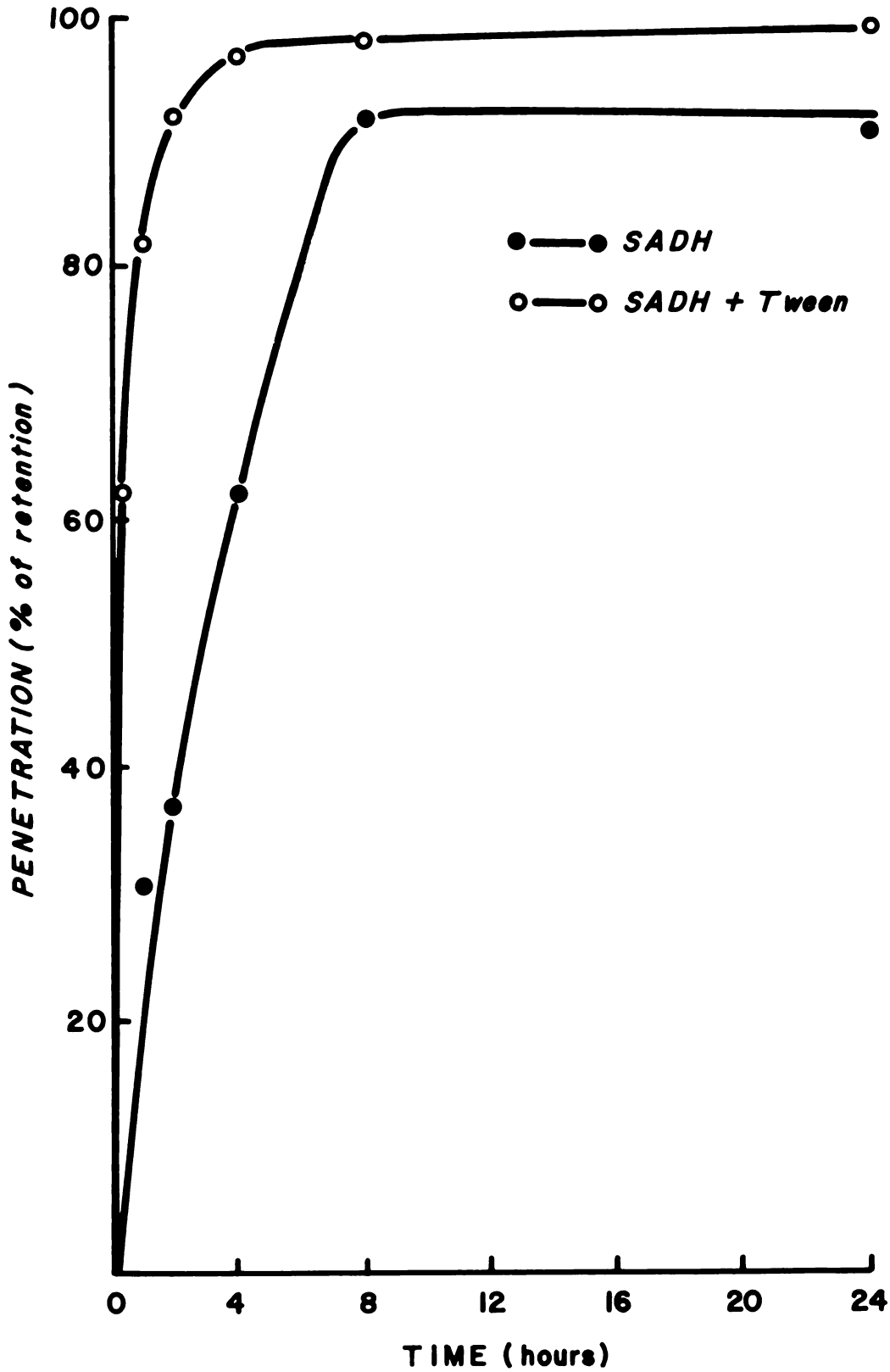

100
90
80
70
60
RETENTION (% of control)
Tween 20
Buffer x
Surfactant P303
0.01
0.05
0.1
CONCENTRATION (%)

Figure 25.--The effect of Tween 20 (0.1%) on penetration of ^{14}C-SADH into bean primary leaves.


100
80
60
40
20
PENETRATION (% of retention)
SADH
SADH + Tween
0
4
8
12
16
20
24
TIME (hours)

dipping the leaves approximately 80% of the ^{14}C-SADH retained after dipping had penetrated in the plus Tween treatment, whereas the comparable figure for the minus Tween treatment was ca. 30%. The difference between the plus and minus Tween treatments diminished with time, 8 hours after dipping ca. 90% (minus Tween) and 98% (plus Tween) of the ^{14}C-SADH had penetrated. Thus, Tween 20 affected the initial rate of penetration rather than the total amount of SADH that penetrated.

The relationship between the two effects of surfactants (reduction of retention, growth promotion) is recorded in Table 12. Tween 20 (0.1%) reduced retention by 25% and enhanced penetration of ^{14}C-SADH. Six hours after dipping, 29% of the SADH retained could still be washed off the leaves if the treating solution lacked Tween 20, whereas in the plus Tween 20 treatment only 5% could be removed. Interestingly, the total amount of SADH which penetrated within 6 hours was similar in both treatments.

All surfactants increased the internode length of bean plants, although the effect of Buffer X was statistically not significant (Table 13). At a concentration of 0.1% P 303 increased internode length by 25% and Tween 20 by 62%.

This effect of surfactants prevailed in the presence of 5×10^{-4} M SADH as the internodes of the

TABLE 12.--The effect of Tween 20 (0.1%) on retention of ^{14}C-SADH solution and penetration into bean primary leaves.

Treatment	Amount retained		Amount removed after 6 hours		Amount penetrated within 6 hours
	$cpm \cdot cm^{-2}$	%	$cpm \cdot cm^{-2}$	%	$cpm \cdot cm^{-2}$
- Tween	61.16 a	100	17.81 c	29	43.34
+ Tween	45.92 b	75	4.29 d	5	41.63

Means followed by different letters are significantly different at P = 0.05.

TABLE 13.--The effect of surfactants (0.1%) and SADH (5×10^{-4} M) on elongation of the first internode of bean plants.

Surfactant	Internode length				
	Control		SADH treated		
	mm	%[1]	mm	%[1]	%[2]
no surf.	113.0 a[3]	100	62.8 a	100	55.5[4]
Buffer X	124.6 a	110	70.8 a	112	56.8
P 303	141.5 b	125	82.8 a	132	58.0
Tween 20	182.8 c	162	80.8 a	129	43.7

[1]Per cent of no surfactant

[2]Per cent of control

[3]Means within a column followed by different letters are significantly different at P = 0.05.

[4]All SADH effects are significant at P = 0.05.

SADH treated plants were longer when the treating solution contained a surfactant (Table 13). For Buffer X and P 303 this increase corresponded closely with the increase found in controls. Statistically, however, these differences were not significant. The comparison of the internode length of SADH treated plants with the appropriate surfactant-control shows that the surfactants did not alter the growth response to SADH, since a similar inhibition was obtained whether the treating solution contained a surfactant of not.

The independent action of SADH and surfactants on the elongation of internodes is also illustrated in Table 14. In this experiment, treated leaves were detached after specified time intervals such that only those quantities of SADH and Tween 20 which had penetrated and were translocated out of the treated leaf could affect growth. With control plants one leaf was detached and the remaining one dipped in buffer or in buffer plus Tween 20.

The plus Tween 20 control again had longer internodes (31%) than the minus Tween 20 control. But even plants in which penetration and translocation were stopped after one hour had longer internodes if the treating solution contained Tween 20. The plants whose treated leaves were removed 3 or 6 hours after dipping had practically the same internode length irrespective

of the presence of Tween 20, whereas during the 12 hour penetration and translocation period sufficiently more SADH was apparently exported into the plant to counteract the Tween 20 effect and to inhibit internode elongation even further.

TABLE 14.--The effect of SADH (5×10^{-3} M) and Tween 20 (0.1%) on elongation of the first internode of bean plants.

Treated leaf detached after	Internode length		
	- Tween	+ Tween	Tween effect
hours	mm	mm	%
control[1]	60.6 a[2]	79.6 a	131
1	54.5 a	66.0 b	121
3	44.7 bc	41.1 cd	100
6	42.5 bc	42.2 d	100
12	33.7 c	26.2 e	78

[1]One leaf was detached from control plants and the remaining one being dipped into buffer or buffer plus Tween 20 respectively.

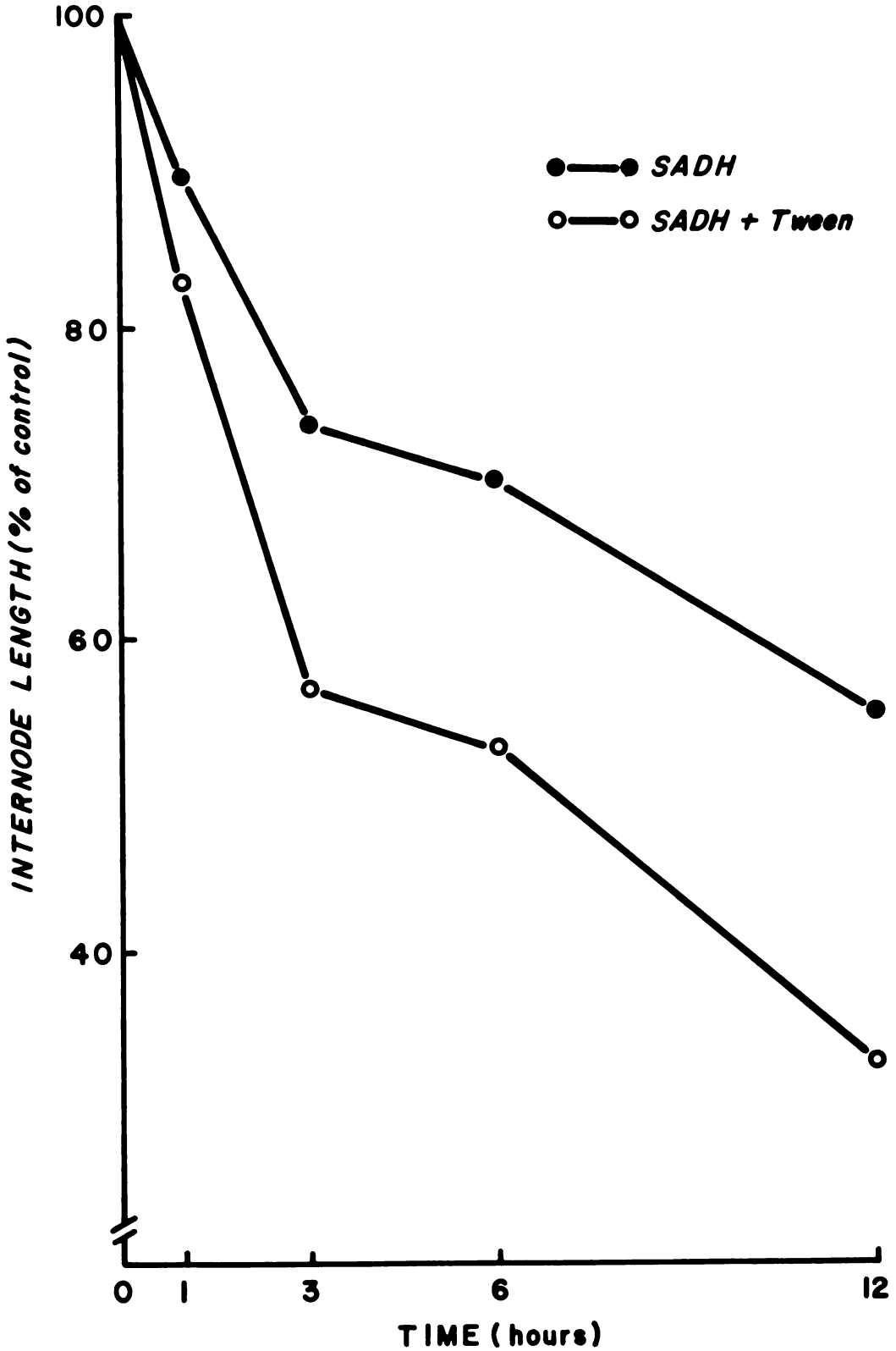
[2]Means within a column followed by different letters are significantly different at P = 0.05.

Inhibition of internode elongation was much stronger when the treating solution contained Tween 20 (Figure 26). In this figure internode length of SADH treated plants is expressed in per cent of the internode length of the

Figure 26.--The effect of Tween 20 (0.1%) on penetration of SADH into bean leaves, as indexed by inhibition of elongation of the first internode.

(Data from Table 14 plotted on a percentage basis.)

Inhibition of internode elongation was significant (P = 0.05) after 1 hour in the SADH plus Tween treatment and after 3 hours in the SADH treatment.


100
80
60
40
SADH
SADH + Tween
INTERNODE LENGTH (% of control)
0
1
3
6
12
TIME (hours)

minus or plus Tween 20 control plants respectively. A 1-hour penetration and translocation period (treated leaf detached) was sufficient to obtain a significant inhibition of internode elongation in plants treated with SADH plus Tween, whereas 3 hours were required in plants treated with SADH alone. Thus, the enhancement of foliar penetration of SADH by Tween 20 could be demonstrated using a biological response.

The Effect of pH of the Treating Solution

To test the effect of the pH of the treating solution on foliar penetration of SADH, one primary leaf per plant was dipped into 5 x 10^{-3} M SADH solution buffered at pH value of 3.0, 5.0 and 7.0. The treated leaves were detached after one hour. In control plants one leaf was detached and the remaining one was not treated.

Internode elongation was inhibited at all pH values (Table 15) and the degree of inhibition increased with decreasing pH values. The greatest inhibition was obtained with a solution of pH 3.0 which was significant for both internodes. Inhibition by solutions of pH 5.0 or 7.0 was significant only for the first internode.

Penetration of SADH Into Apple Leaves

A penetration period of more than 6 hours was needed to significantly inhibit terminal growth of apple

TABLE 15.--The effect of the pH of SADH treating solution (5×10^{-3} M) on elongation of the first and second internode of bean plants.

pH	Internode length			
	First internode		Second internode	
	mm	%	mm	%
Control	56.0 a[1]	100	189.3 a	100
7.0	45.6 b	81	168.2 a	89
5.0	40.0 c	71	164.6 a	87
3.0	29.8 d	53	116.8 b	62

[1]Means within a column followed by different letters are significantly different at P = 0.05.

seedlings (Table 16). Penetration continued even for a longer time as terminal growth of seedlings whose leaves were not washed was significantly less than that of seedlings washed 12 hours after application of Alar 85.

Discussion

Factors influencing penetration of SADH into bean leaves have been studied using excised leaf disks. Such a system proves very useful for investigating the mechanisms involved in penetration of SADH from an aqueous solution, and may also furnish some insight as to factors influencing SADH penetration under field conditions. However, while the leaf disk method is essentially a static system providing constancy of concentration of

SADH and surfactant, temperature, light and humidity, are factors effecting penetration under field conditions subject to changes, particularly the concentration of SADH and surfactant.

TABLE 16.--Penetration of SADH (applied as Alar 85) into apple leaves as indexed by inhibition of terminal growth of apple seedlings.

Leaves washed after	Terminal shoot growth	
hours	cm	%
Control[1]	33.5 a [2]	100
1/3	32.7 a	98
1	28.8 ab	86
6	26.1 ab	78
12	24.1 b	72
not washed	15.9 c	48

[1]non-treated

[2]Means followed by different letters are significantly different at P = 0.05.

Therefore, it seemed desirable to establish whether or not the effects of certain factors on penetration of SADH observed in the isolated system could be confirmed under conditions similar to those in the field, particularly when a biological response of the plants to the growth regulator is used as an index for penetration.

Bukovac (11) reported that the most pronounced growth response of bean plants to MADH was the reduction in internode length. The inhibition of elongation of the first and second internode was used as a biological response and related to penetration of SADH.

Unfortunately, the dosage response curve for the first and second internodes was sigmoidal and the effective concentration range was rather narrow. This limits this test system for quantitative evaluations but it is sufficiently sensitive to establish some general qualitative relationships.

The role of surfactants was more complex than in the leaf disk system. They were effective in three different ways: (a) surfactants reduced retention of treating solution, (b) they enhanced the initial rate of penetration and (c) promoted internode elongation.

The reduction in retention of treating solution by 15 - 25% due to surfactants, that is to say the reduction of the amount of growth regulator deposited on the leaf surface by 15 to 25%, might be of practical importance. Particularly, when there is no positive effect of the surfactant on the total amount of SADH which penetrated, as observed in this study with bean. Practically all the SADH deposited on the leaf surface penetrated irrespective of the absence or presence of Tween 20, which affected mainly the initial rate of

penetration. However, this might be different in plants whose leaves are more difficult to wet.

The increase in penetration of SADH by Tween 20 observed in the leaf disk system could be confirmed. Since the effect of Tween 20 was on the rate of penetration rather than on the total amount, it was necessary to include the time factor in the experiments by removing the SADH pool on the leaf surface after prescribed time intervals. However, by detaching the treated leaf, SADH which had penetrated but was not yet exported out of the leaf was also removed. This accounts for the relatively small differences between the plus and minus Tween 20 treatments observed in this study. Following a 3-hour penetration and translocation period an inhibition of internode elongation of 26% was observed in plants treated with SADH alone, while 48% was observed in plants treated with SADH plus Tween 20 (Table 14, Figure 28). Differences found with the leaf disk system were much greater. Nevertheless, it should be noted that the enhancement of foliar penetration of SADH by Tween 20 could be demonstrated with three different methods: the leaf disk method, by determining the non-penetrated ^{14}C-SADH following dipping of the leaves (Figure 25) and by utilizing a biological response (Table 14, Figure 28).

Enhancement of penetration of a variety of compounds by surfactants has been shown. More ^{32}P

penetrated into the lower surface of McIntosh leaves when Triton X 100 was added (29) and penetration of 3-amino-1,2,4-triazole (45) and IAA (59) into bean leaves was also increased by a variety of surfactants. In these cases, however, the first determination of the amount which had penetrated was made 24 hours after treatment. Therefore, the effect of the surfactants on penetration during the drying of the spray deposit was not determined. It was during this time period that Tween 20 had the greatest effect on SADH penetration.

Penetration of SADH into apple leaves was strikingly slower than in bean leaves. The SADH was applied as Alar 85 which contains the surfactant P 303. During drying of the spray deposit very little SADH penetrated as indicated by the lack of response. Since the inhibition of terminal growth increased with increasing time of penetration up to 12 hours and longer, penetration must have taken place from an apparently dry surface. The fact that SADH could be easily washed from the leaves is of practical importance. A rain within 12 hours after spraying may remove a large portion of the spray deposit and significantly reduce performance.

The third effect of surfactants was the promotion of internode elongation in bean plants. Growth promoting effects of surfactants have earlier been observed, although surfactant toxicity is more common (83).

Methylesters of fatty acids, Tween 20 and Tween 80, which are also esters of fatty acids, were shown to enhance the activity of GA and IAA in promoting growth of pea stem sections (116). Growth of young barley seedlings was promoted by 0.01% of fatty acid ester types of non-ionic surfactants (82). Surfactants were also found to increase growth of corn and soybean plants (62). In the present study it was shown that the growth promoting effect of surfactants and the inhibiting effect of SADH were independent from each other. The growth promotion by surfactants could be overcome by SADH. There is no indication whether surfactants will promote the growth of fruit trees as well. If they do so, some of the growth retardant will be required for compensation of the growth promoting effect of the surfactant.

From the factors affecting foliar penetration of SADH as evident from direct measurements using ^{14}C-SADH and the leaf disk system the role of surfactants and pH as determinants of penetration was confirmed using inhibition of internode elongation as index for penetration. Both Tween 20 and a low pH of the treating solution enhanced penetration. The role of light and transpiration in this biological system remain to be studied.

Translocation of Succinic Acid 2,2-Dimethylhydrazide in Apple and Peach Trees

Material and Methods

One-year-old apple (Malus sylvestris cv. "McIntosh") and peach (Prunus persica cv. "Suncling") trees, budded on seedling rootstocks, were planted in light soil in 5-gallon plastic containers on April 22, 1968. They were placed on a bench in a greenhouse equipped with a cooling device capable of maintaining a day temperature of approximately 25° C. The night temperature was controlled at 20° C. Vigorous growth of the trees was maintained by carefully watering and fertilization and by adequate control of pests and diseases.

Two shoots were allowed to develop on each tree approximately 15 cm apart oriented in opposite directions. They will subsequently be referred to as upper and lower shoots. Peach trees were treated on May 25 and again on June 8. Apple were treated on June 1. At the time of treatment shoots were ca. 25 cm long.

Treatment consisted of spraying either the upper or the lower shoot with 5,000 ppm Alar 85. During spray application the entire tree, with the exception of the shoot to be treated, was covered with a plastic sheet. To prevent spreading of the spray liquid blotting paper was wrapped around the base of the treated shoot.

This was removed once the spray dried. Control trees were not treated.

During the course of the experiment shoot growth was measured and recorded at weekly intervals. At the end of July shoot growth had ceased and the experiment was terminated. One tree, typical for each treatment, was selected and photographed. Next, leaves which were present at time of treatment were removed from each shoot. A 3.5 cm diameter disk was punched from each leaf after which the disks and the remainder of the leaves were dried at 70° C in a forced draft oven. After attaining constant dry weight, the leaf area per gram leaf dry weight was computed from the leaf disks. This factor was used to obtain an estimate of the leaf area present on each shoot at the time of treatment.

The shoots were detached from the stem and subdivided into the portion grown before treatment (pre-treatment growth) and after treatment (post-treatment growth). The length of each shoot segment was measured and after drying to constant weight at 70° C the dry weight was determined. Since in peach numerous side shoots had developed, these were included in the dry weight of the main shoots but not in the shoot length.

Leaves were taken from comparable peach and apple trees and retention of spray liquid per unit leaf surface was determined. Detached leaves were dipped into a

5,000 ppm Alar 85 solution to which a trace of ^{14}C-SADH had been added. The procedure was essentially as described for determination of retention by bean leaves.

A split plot design was utilized, each tree being considered a whole plot and the upper and lower shoots sub-plots. Each treatment was replicated five times. Analysis of variance was performed on the data and a comparison among the treatment means was made according to Tukey's w-procedure.

Results

Alar 85 at 5,000 ppm inhibited shoot elongation of the treated shoots on apple and peach trees (Figures 27, 28, 29). On apple, growth of the treated shoot ceased 2 weeks after spray application, while in peach little inhibition was observed following the first treatment. A second spray was required to terminate growth of the treated shoots in peach (Figure 28). In both, apple and peach, growth of the non-treated shoot continued at a rate similar to the shoots of the control trees. Elongation of the upper and the lower shoots was virtually identical in control trees, indicating that apical dominance was not a factor in this study.

Apple trees generally did not develop side shoots, while numerous side shoots developed on peach trees (Figure 29) on both, upper and lower shoots. The number

Figure 27.--The effect of Alar 85 (5,000 ppm) on shoot growth of apple trees (McIntosh).

Arrow indicates the date of treatment.

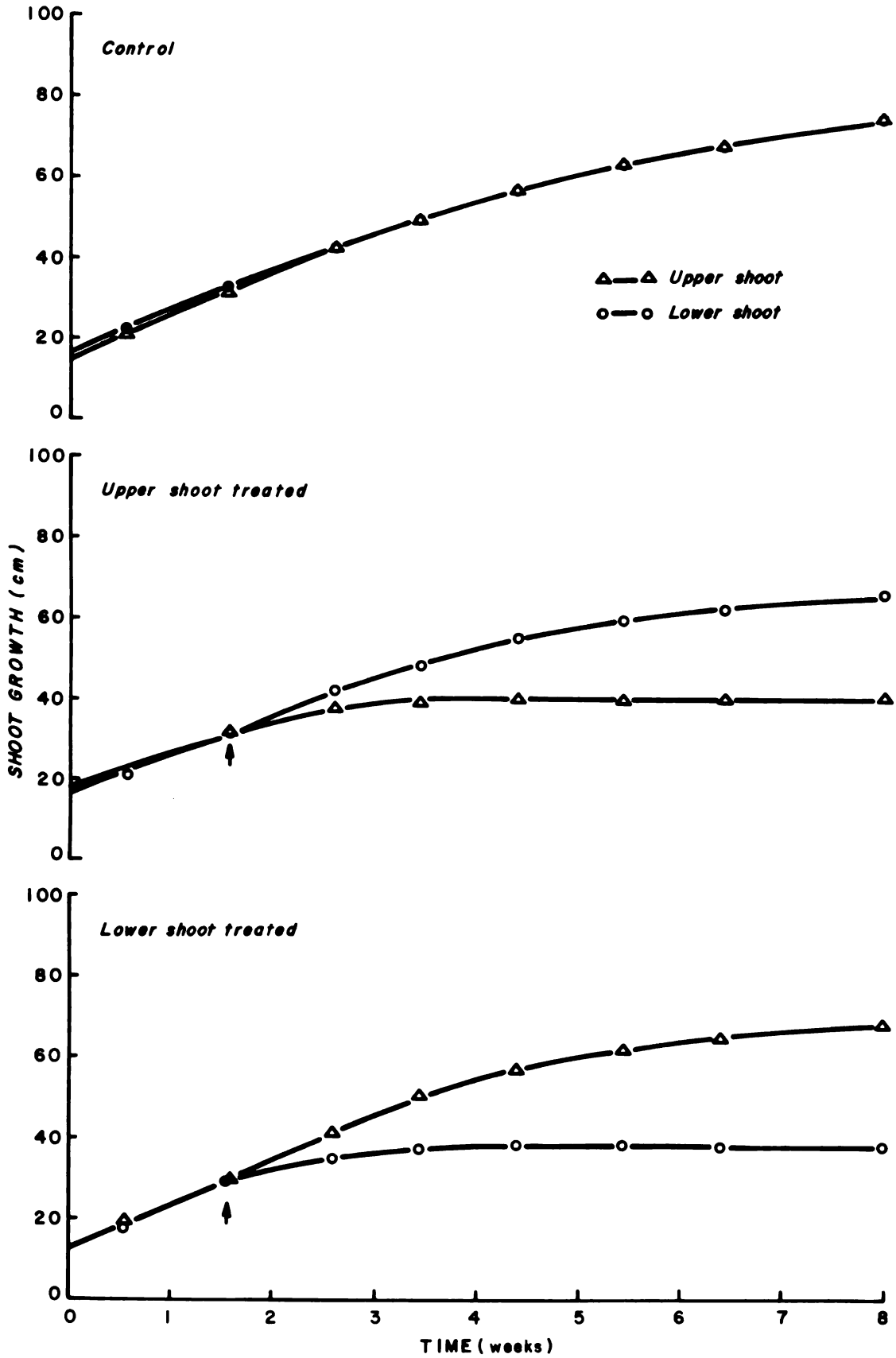

Control
Upper shoot
Lower shoot
Upper shoot treated
Lower shoot treated
SHOOT GROWTH (cm)
TIME (weeks)
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8

Figure 28.--The effect of Alar 85 (5,000 ppm) on shoot growth of peach trees (Suncling).

Arrows indicate dates of treatment.

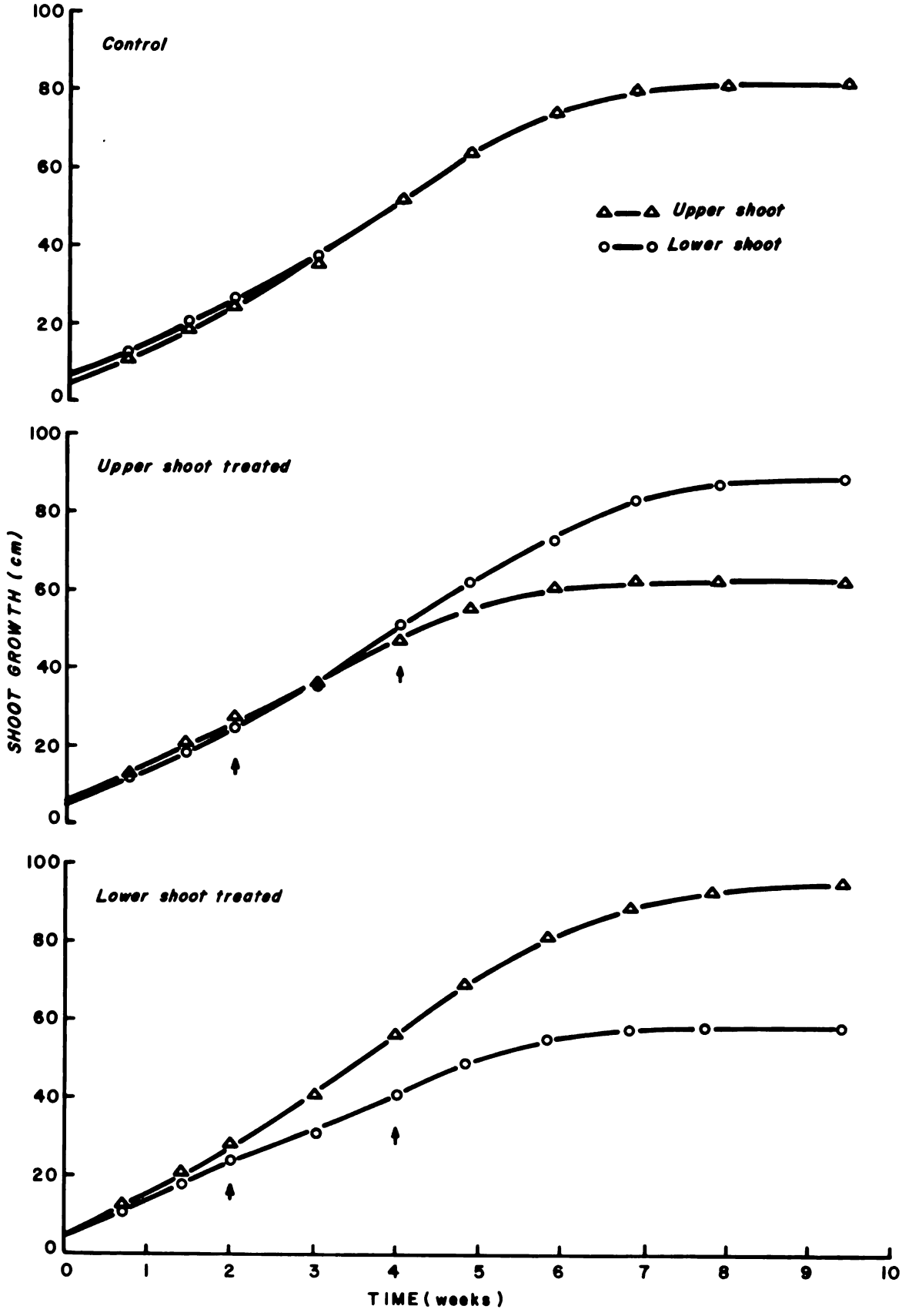

Control
Upper shoot
Lower shoot
Upper shoot treated
Lower shoot treated
SHOOT GROWTH (cm)
TIME (weeks)
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8
9
10

Figure 29.--The effect of Alar 85 (5,000 ppm) on growth of apple and peach trees.

A. Apple, control.

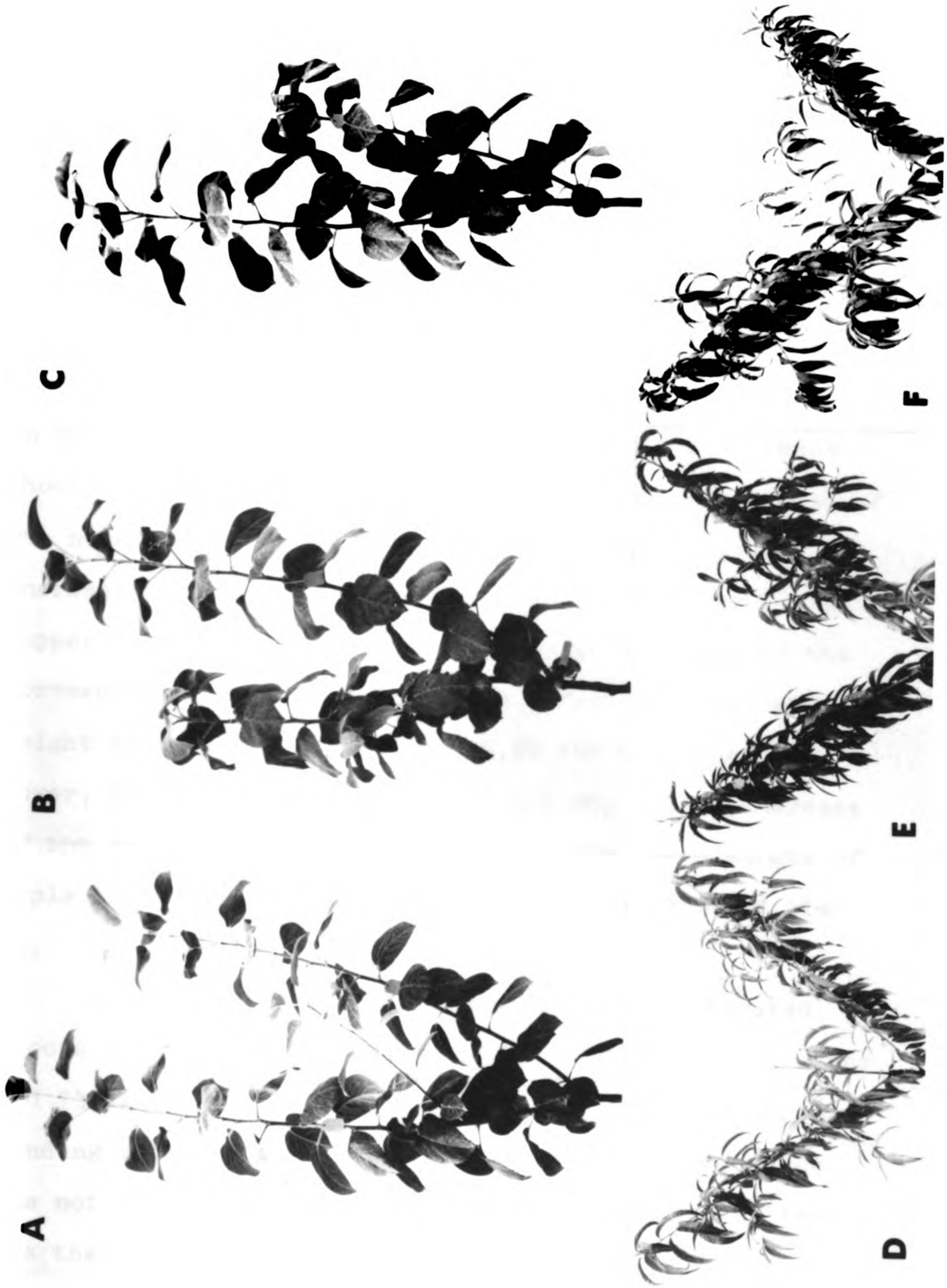
B. Apple, upper shoot treated.

C. Apple, lower shoot treated.

D. Peach, control.

E. Peach, upper shoot treated.

F. Peach, lower shoot treated.


A
B
C
D
E
F

and the length of the side shoots was reduced by Alar 85. More detailed information was obtained by analysis of post-treatment growth increments (Tables 17, 18).

Response of
Apple Trees

There was no significant difference in the growth increment between the upper and lower shoot of the control trees, although the lower shoot tended to be lower in dry weight (Table 17). The increment of the treated shoots (length and weight) was significantly less than of the non-treated shoots. Alar 85 drastically reduced the increase in shoot length of the treated shoot to 14.7% (upper) and 11.7% (lower) of the length increase of the corresponding control shoot. The effect on shoot dry weight was similar, 14.1% and 15.9% for the upper and lower, respectively, in terms of the dry weight increase of the control. Thus, the upper and the lower shoots of apple trees responded similarly to a given dose of Alar 85.

The increase in shoot length of the non-treated shoots of the treated trees was less (84.9% for the upper and 73.1% for the lower shoot) than that of the corresponding shoot of the control trees, but the difference was not statistically significant. Also non-significant was the slightly greater increment in dry weight of the non-treated shoots as compared to control shoots. Thus,

TABLE 17.--The effect of Alar 85 (5,000 ppm) on shoot growth of apple (McIntosh) trees.

Treatment	Shoot growth							
	Elongation				Dry weight			
	Upper shoot		Lower shoot		Upper shoot		Lower shoot	
	cm	%	cm	%	g	%	g	%
control	47.0 a[1]	100.0	48.0 a	100.0	4.04 a	100.0	3.15 a	100.0
upper sh. treated	6.9 b	14.7	35.1 a	73.1	.57 b	14.1	3.44 a	109.2
lower sh. treated	39.9 a	84.9	5.6 b	11.7	4.25 a	105.2	.50 b	15.9

[1]Means (cm or g) followed by different letters are significantly different at P = 0.05.

in apple trees growth of the upper shoot was not affected by 5,000 ppm Alar 85 applied to the leaves of the lower shoot and vice versa.

Response of
Peach Trees

There was no difference in shoot length increment between the upper and the lower shoot of the control trees (Table 18). The dry matter increment, however, was significantly different. The lower shoot weighing only 5.92 g as compared to 8.64 g for the upper shoot. This difference is largely due to the side shoots, which were more numerous on the upper shoots.

The length and dry weight increment of the treated shoots were in all cases significantly less than the increment of the non-treated shoots for both treatments. The treated shoots remained shorter and gained less in dry weight than the corresponding control shoots. This difference was statistically significant only for the dry weight data.

The non-treated shoots of the treated trees grew more vigorously than their respective controls. This difference was apparent both in terms of shoot elongation and shoot weight, but was statistically significant only for shoot elongation. Following treatment of the upper shoot, the lower shoot accumulated 203.5% of the weight of the lower control shoot and the upper shoot

TABLE 18.--The effect of Alar 85 (5,000 ppm) on shoot growth of peach (Suncling) trees.

Treatment	Shoot growth							
	Elongation				Dry weight			
	Upper shoot		Lower shoot		Upper shoot		Lower shoot	
	cm	%	cm	%	g	%	g	%
control	57.6 ab[1]	100.0	54.2 ab	100.0	8.64 a	100.0	5.92 b	100.0
upper sh. treated	36.8 a	63.9	71.0 bc	131.0	5.83 b	67.5	12.05 ac	203.5
lower sh. treated	68.4 ac	118.7	33.2 a	61.2	15.76 dc	182.4	2.35 e	39.7

[1]Means (cm or g) followed by different letters are significantly different at P = 0.05.

showed an increase of 182.4% over the upper control shoot when the lower shoot was treated.

Thus, in peach trees treatment with Alar 85 resulted in a growth inhibition of the treated shoots and in a growth promotion of the non-treated shoots independent of position.

Leaf Area at Time of Treatment and Spray Retention

The leaf area at the time of treatment was very similar for both apple and peach trees. Since there was little difference between the upper and lower shoots, the average of both shoots is presented (Table 19). At the time of the second treatment the leaf area in peach was almost 2.5 times larger than at the first treatment.

TABLE 19.--Leaf area and spray retention of apple and peach trees at the time of treatment.

Species	Leaf Area	Relative retention	
		Per unit area	Total
	cm^2		
Apple	471	3.5	1,648
Peach			
1st treatment	485	1.0	485
2nd treatment	1163	1.0	1,161

Apple leaves retained 3.5 times more treating solution per unit leaf area than peach leaves. This resulted in a much smaller amount of SADH deposited per shoot treated at the first treatment. The sum of the total retention from the first and second treatment of peach trees is almost identical with the total retention in apple.

Discussion

Terminal growth of the treated shoots of apple and peach trees was strongly inhibited by 5,000 ppm of Alar 85 applied as foliar spray. Growth of the non-treated shoots was not inhibited.

The need for a second spray application in peach to stop terminal growth of the treated shoot was probably the consequence of the smaller spray retention by peach leaves as compared to apple leaves (Table 19). Other factors, such as a differential foliar penetration or response between the two species, may have been involved as well.

Growth of the non-treated shoots of peach trees was promoted. The main shoots were longer and had more and longer side shoots, as compared to the comparable shoots of control trees. Although this cannot be explained physiologically, it might be of practical

significance, since Alar applications may reinforce apical dominance if the tops of trees are not covered thoroughly.

The lack of growth inhibition in the non-treated shoots suggests that SADH was not translocated from the treated shoots to the non-treated shoots in amounts sufficient to produce a biological response. The transpiration stream cannot serve for passive basipetal transport of SADH, since it is directed upward. Thus, SADH which might accumulate in the apoplast of the leaves following spray application cannot be translocated in this way. Rather, uptake of SADH into the leaf cells and translocation in the symplast is a prerequisite for basipetal movement. However, it was speculated earlier that SADH might not be readily taken up by leaf cells. This could account for the lack of translocation observed in this study.

These results are in contrast to conclusions of Martin et al. (75) that SADH is readily translocated and coverage should be of less importance in SADH applications to the foliage. This conclusion was not derived from the study of translocation of foliar applied SADH, but rather following trunk injection, root uptake or uptake through a severed petiole. In all cases acropetal movement was studied.

There is, however, good agreement between the present study and the findings of Wertheim et al. (125) who reported that there was little growth inhibition of the shoots of the lower portion of pear trees, when only the upper portion was sprayed with SADH. It would appear that complete coverage of SADH or Alar sprays is a pre-requisite for uniform growth inhibition of all shoots.

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APPENDICES

APPENDIX I

CRITICAL EVALUATION OF THE CONCEPT OF ECTODESMATA WITH REFERENCE TO FOLIAR PENETRATION OF POLAR COMPOUNDS

Introduction

Ectodesmata were observed for the first time by Schumacher and Halbsguth (98) and since have been the subject of numerous investigations, among them three Ph.D. dissertations which dealt mainly with the problem of demonstrability of ectodesmata (69, 97, 104). Later, Franke made several attempts to demonstrate that ectodesmata function in foliar penetration of polar compounds (34, 35, 39, 40, 43, 44). His concept appears to have been widely accepted, although the ideas as to how ectodesmata might be involved, are quite different.

Currier and Dybing (17) described ectodesmata as protoplasmic strands which push into and alternately withdraw from pores in the outer epidermal wall. Crafts and Foy (16) suggested that ectodesmata may desorb penetrating molecules from the cutin reservoir. That ectodesmata participate in foliar absorption of mineral nutrients was assumed by Jyung and Wittwer (65) and Hull (60) wrote that the function of ectodesmata as routes of transport between the cuticle and the living parts of the tissue had been demonstrated almost beyond question. Sargent (95) presented a more recent view of Franke on the nature of ectodesmata as pores, filled with reducing agents

excreted by the protoplast under conditions in which staining of ectodesmata is possible.

In view of the diversity of contradictory opinions concerning the nature and function of ectodesmata it was decided to critically re-examine the literature dealing with ectodesmata. A clarification was also needed with respect to a possible role of ectodesmata in foliar penetration of SADH.

The Nature of Ectodesmata

Originally ectodesmata were thought to be plasmatic in nature (69, 98, 99) but later, Franke came to favor the view of their being non-plasmatic (41, 42, 43, 44). The problem of the nature of ectodesmata is closely related to the problem of consistently demonstrating them.

All attempts to demonstrate ectodesmata with the electron microscope have failed so far. These structures could not be stained with OsO_4, the classical protoplasmic stain (104). Using $HgCl_2$ staining Schnepf (97) produced some electron micrographs of what he considered to be ectodesmata, but they were claimed to be artifacts by Bollinger (8). Further, what Huber et al. (58) interpreted as ectodesmata in cross-sections of guard cells of Helleborus niger were considered artifacts by Sievers (104) and Schnepf (97). Thus, nothing convincing can be said about the ultrastructure of ectodesmata at the present time.

The classical method for demonstrating ectodesmata is the Gilson method (98, 69, 97, 104). The Gilson fixative used by Lambertz (69) was composed of: 40 ml 30% ethanol, 10 ml formic acid, 5 ml 40% formalin, 1 ml 65% HNO_3 and 2-3 $HgCl_2$ to saturation. The presence of $HgCl_2$ is essential as ectodesmata cannot be demonstrated without it. Changes in other components affected only shape and number of ectodesmata.

Whole leaves or leaf segments were fixed in the Gilson misture from 10 minutes up to 24 hours, sectioned with a cryostat and the sections washed with 50% ethanol to remove excess $HgCl_2$. Then, sections were transferred to 20% KI solution saturated with iodine (5-10 minutes) and stained in pyoktannin acidified with H_2SO_4. Following staining the sections were washed in water and embedded in glycerin for microscopic examination.

Schnepf (97) pointed out that the fixing technique was crucial: ectodesmata could not be demonstrated when the leaves were infiltrated with the Gilson fluid under vacuum, or when cryostat sections were fixed instead of large leaf segments (10 x 10 mm) prior to sectioning. Ectodesmata could not be found along the cut edges of the leaf segments. From the failure of demonstrating ectodesmata in thin sections (20-40 um) Lambertz (69) concluded that ectodesmata might be destroyed when the cells are killed during sectioning. However, Schnepf (97) observed

that even non-injured cells close to the cut edges failed to exhibit ectodesmata. He argued that a rapid penetration of the fixative through the cut edges might destroy these structures and that they can be demonstrated only if the fixing solution penetrates the tissue slowly and in a special 'orderly' manner, probably through the cuticle.

This view is supported by the fact, that after only a short fixing period, short, incomplete ectodesmata occurred which were attached to the cuticle and which seemed to grow towards the plasma membrane (97, 34). Franke argued this kind of formation of ectodesmata would not occur if the Gilson fluid would spread by diffusion in the cellulose wall under the CM.

Three different forms of ectodesmata have been observed. Complete ectodesmata--those which extend through the entire outer wall from the CM to the plasmalemma. Shortened ectodesmata--those which are cone-shaped and attached to the CM. Their tips are oriented towards the plasma membrane of the epidermal cell but not in contact with it. This type of ectodesmata generally occurred under conditions less favorable for demonstrating ectodesmata. 'Abbauformen'--those which are also cone-shaped but attached to the plasma membrane, oriented with their tips towards the cuticular membrane without reaching it. This type was observed in old yellow leaves and in wilting leaves. The diameter of all three types of ectodesmata

varied from very thin and thread-like (0.5 µm) to broad cylindrical or cone-shaped (5 µm) ectodesmata (from figures by Lambertz (69)).

Lambertz (69) observed a distinct diurnal rhythm in the occurrence of ectodesmata. A great number of complete ectodesmata could be demonstrated during the night while during the day the same leaves showed only few and usually shortened ectodesmata. Sievers (104) established the role of temperature and light in this process. Temperatures between 20 and 30° C caused ectodesmata to disappear or to become shortened, while leaves kept at 3 - 6° C exhibited numerous complete ectodesmata. Light reduced demonstrability and dark improved it. The light and temperature effects were reversible, which was taken as an indication that ectodesmata are plasmatic extensions of the cytoplasm projecting into the wall or withdrawing from it, depending on environmental conditions (69, 97). Later it became clear that the structures were always present in the wall, only their demonstrability with the Gilson mixture changed.

Schnepf (97) succeeded in demonstrating ectodesmata with an iodine-silver method. Leaf segments were fixed in 5% KI solution saturated with iodine (8-16 hours), rinsed quickly with water and transferred to a 1% $AgNO_3$ solution (for a few hours) and then sectioned

with a cryostat. The sections require no further treatment. With this method a part of the outer wall under the CM stained a diffuse dark brown. Sometimes, the dark band was very broad and filled the entire space between CM and cytoplasm. In cases where the band remained small and confined to the zone under the CM numerous ectodesmata could be seen extending from the dark band to the plasmalemma.

There are striking differences in the demonstrability of ectodesmata between the Gilson and the iodine-silver method. Using the Gilson method, light and temperature had a pronounced effect on demonstrability and ectodesmata could not be demonstrated in killed tissue. Treating leaves with CO, HCN and ether seriously impaired demonstrability of ectodesmata. In old and yellow leaves only the 'Abbauformen' type could be demonstrated. All of these factors were without effect on demonstrability of ectodesmata using the iodine-silver method where numerous complete ectodesmata formed under all conditions and even in killed tissue.

In spite of these differences, the structures produced with the two methods were considered identical (34, 97) although the processes leading to their demonstration were quite different. Schnepf (97) suggested the formation of AgI crystallites following treatment of tissue with KI and $AgNO_3$ which lined

certain structures in the outer wall, thus making them visible with the light microscope as ectodesmata. Since AgI is not stable in light Ag^+ will be reduced and what is visible is probably not AgI. The formation of ectodesmata with the iodine-silver method requires nothing else but intermicellar spaces large enough to accommodate the crystallites. The existance of such spaces in the cellulose wall has been shown as well as the tendency of copper, silver and gold crystallites to line these spaces when cellulose fibers were submerged in the appropriate salt solution and the metal reduced with hydrazinehydrate (47).

The processes which make ectodesmata visible following fixing with the Gilson mixture must be of a different nature, as suggested by the fact that they could not be demonstrated in dead tissue, after freezing or treatment with CO, HCN and ether. The number of complete ectodesmata could be increased by administering ascorbic acid to the leaves prior to fixing them with the Gilson mixture (41, 97). Franke (41) concluded that a reducing substance might be involved in demonstrating ectodesmata with the Gilson method, and that the natural reductant might be ascorbic acid. This was postulated on the basis of the following observations: (a) the number of complete ectodesmata could be increased with ascorbic acid but not with other naturally occurring reducing

compounds such as glutathion and cystein, (b) in variegated leaves of Pelargonium zonale the ascorbic acid content was twice as high in the green than in the white portions and the number of ectodesmata was also greater in the green portions of the leaf, and (c) the ascorbic acid content of the epidermis tends to be 2-10 times higher than in other plant tissues (41).

In a subsequent paper (42) Franke discussed the nature of ectodesmata in which he favors the hypothesis of their being non-plasmatic in nature. The nature of ectodesmata is of importance with respect of their role in foliar penetration. While the function of plasmatic ectodesmata would be relatively straight forward it is more difficult to justify a postulated participation of non-plasmatic ectodesmata in foliar penetration of polar compounds.

Ectodesmata as Plasmatic Structures

The effect of environmental conditions on the demonstrability of ectodesmata was considered a strong argument for their vitality. This view was maintained when it became clear that no such changes could be observed using the iodine-silver method. Since demonstration with the Gilson mixture involved a reduction step ($Hg^{++} \longrightarrow Hg^{+}$), it was hypothesized that the reducing ability of the plasm was affected by

all factors influencing the demonstrability of ectodesmata (34, 38, 100). This, however, is in contradiction with the observed mode in which these changes take place. The shortened ectodesmata, formed under unfavorable conditions, are attached to the CM and they are not in contact with the cytoplasm. Transferring such leaves with shortened ectodesmata to conditions which promote demonstrability (dark, low temperatures) resulted in an elongation of the shortened forms and, if sufficient time was allowed, they finally became complete ectodesmata. Thus, the reducing ability was apparent first in proximity of the CM and not in proximity of the cytoplasm as one would expect if ectodesmata were plasmatic.

The formation of so-called 'Abbauformen' in wilting leaves and the reoccurrence of complete ectodesmata following re-establishment of turgescence was also considered an argument for their vitality (104).

The similarity between ectodesmata and plasmodesmata in polarized light--both appearing as dark isotropic structures in the birefringent wall--was another indication that ectodesmata might be plasmatic as are plasmodesmata. However, Buer (10) questioned the identity of structures observed in polarized light and those produced with the Gilson method, because the isotropic bands in the outer wall extended through the

entire depth of the section (30-50 μm) while ectodesmata are generally thin and thread-like. Furthermore, the isotropic bands disappeared when the outer epidermal wall was separated on one side from the anticlinal wall. This caused the outer wall to relax and become straight, while it was arched before. From this Buer concluded that the isotropic bands were folds in the outer wall.

Finally, the failure of ectodesmata to stain with OsO_4 is another indication that they are probably non-plasmatic in nature.

Ectodesmata as Non-Plasmatic Structures

If ectodesmata are not plasmatic, then the Hg^{++} has to be reduced by a substance other than the plasm. As already mentioned, Franke (41) suggested that ascorbic acid might be the natural reductant. From his research (35, 37, 39, 40) he arrived at the conviction that ectodesmata can function as transport pathways in the outer epidermal wall and suggested that the vapor pressure gradient was the moving force in transport from the cytoplasm to the CM. The movement of water during CT is thought to follow at least partially distinct pathways through the outer wall and the CM. Solutes such as ascorbic acid are thought to accumulate in these postulated pathways (pores) when the water evaporates from the surface of the CM.

The localized ascorbic acid would then result in localized precipitates of Hg^{+}-compounds, when such a leaf is fixed in the Gilson mixture. The following are the main arguments Franke places in support of his hypothesis:

(a) Under severe water stress leaves start wilting. A portion of the imbibition water of the outer wall will be lost by CT and an additional portion is thought to be drawn towards the cytoplasm carrying with it dissolved substances. Under these conditions the reducing agent will be concentrated near the protoplast and this would explain why only 'Abbauformen' type ectodesmata can be demonstrated in wilting leaves.

(b) Ectodesmata could not be demonstrated in leaves having a very thick cuticle or strongly cutinized walls. In these species CT is low and therefore the localized accumulation of reductant cannot take place.

(c) CT and excretion are affected by environmental factors. During rainy days only few shortened ectodesmata could be demonstrated. Under these conditions CT will be low and the reductant might be leached out. Dry and sunny days will result in increased CT and since no leaching takes place the reductant carried in the transpiration stream can be accumulated in the pores of the outer wall and thus explain the good demonstrability reported under these conditions (104).

In the dark, stomata are closed and stomatal transpiration is low, but CT is maintained or even increased (reference by Franke 41), hence the excretion of reductant and its accumulation in the outer wall can continue. Furthermore, ascorbic acid is more stable in the dark and at low temperatures than in light and high temperatures. With high temperatures incipient drying will reduce the degree of imbibition of the CT and thus reduce CT. Therefore, more complete ectodesmata can be demonstrated in leaves which have been subjected to low temperatures in the dark than in leaves exposed to light and high temperatures.

(d) Plants grown in greenhouses tend to have only few and shortened ectodesmata (97, 104). Franke explained this by pointing out that the high relative humidity found in greenhouses will reduce CT.

(e) The pores in which movement of transpiration water and accumulation of the reductant take place, might be brought about by pull forces and pressure exerted against the wall. Areas exposed to mechanical stress, such as guard cells, anticlinical walls, the basis of trichomes and the elevated veins of the lower leaf surface, tend to be densely populated by ectodesmata.

The argument is based on assumptions such as: (a) the occurrence of straight continuous pores in the

outer wall of the epidermis perpendicular to the orientation of the cellulose fibrils, (b) the excretion of a water soluble reductant and its accumulation in pores which occur only in plants exhibiting ectodesmata following appropriate treatment but not in plants with a thick CM, and (c) the localization of a water soluble reductant in pores. The third assumption deserves some further considerations.

The cell wall materials are very polar and the pore spaces (intermicellar and interfibrillar spaces) are generally assumed to be filled with water (94, 46, 48, 118). Rapid water movement in the cell wall system (apoplast) has been shown to occur (6, 118). Thus, it is not easy to visualize a water soluble reductant localized in certain pores and not diffusing out into the water saturated cell wall environment. In this context, Franke's argument as to why fluorescent dyes cannot be used to demonstrate ectodesmata is in conflict. Attempts to demonstrate ectodesmata with fluorochromes failed because they diffused out into the wall (40).

Since the outer epidermal wall is composed of polar materials such as cellulose and polyuronides and saturated with water (46, 48), there is no obvious need for transport channels for polar compounds. Franke (42) stated that often the outer walls do not

exist of a simple cellulose skeleton covered by a cuticle, but, within this skeleton lipoidal materials such as wax and cutin are embedded which hamper the free diffusion of hydrophilic materials taken up by the leaf. In this case pores would be required. It can be imagined that these pores could also serve as pathways for precursors of wax, cutin and suberin.

It should be pointed out, however, that cutin precursors do not appear to traverse the wall in pores (8, 48). More important, ectodesmata could not be demonstrated in plants having thick cuticles or incrusted outer walls (42, 104).

In conclusion, Franke's argument in support of his hypothesis (41) is not convincing. This does not mean to imply that ectodesmata may not be non-plasmatic--they probably are. However, the processes which lead to their demonstration are probably different from what has been suggested.

Ectodesmata and Foliar Penetration

Diverse substances such as glucose, amino acids, ascorbic acid and caffein were found to affect the demonstrability of ectodesmata when applied to the leaf surface prior to fixation with Gilson mixture (99). This observation led these authors to suggest a relationship between ectodesmata and foliar absorption.

This idea has been extensively pursued by Franke (34, 35, 36, 37, 39, 40, 43, 44).

The discussion is rendered difficult due to the fact that ectodesmata are ill-defined with respect to nature and structure. Indeed, the nature can only be negatively defined as being non-plasmatic. Virtually nothing can be said about the structure of ectodesmata. With respect to their role in foliar penetration the points of origin under the CM and near the cytoplasm are important. Originally it was reported that ectodesmata do not penetrate the cuticle (69, 97, 104) and no pores or thin areas were found in the cuticle over ectodesmata. This was confirmed by Franke (44) in writing, however. Figure 1 of his paper depicts two ectodesmata which appear to penetrate through the cuticular membrane. In accordance with the majority of reports and from examination of appropriate figures in publications cited it appears likely that ectodesmata do not extend through the cuticle.

Franke appears to be convinced that ectodesmata perform a function in foliar penetration of polar compounds. The experimental evidence presented is indirect and will be examined next.

(a) When leaf segments of Plantago major were fixed with the Gilson mixture but the subsequent steps were omitted, coarse crystals of unknown nature were

formed at the same sites where ectodesmata can be demonstrated. Since these crystals cannot be seen in fresh leaves, it was concluded that they must have formed under participation of the Gilson mixture, which is proof that it had penetrated, possibly through the cuticle over those crystals. There is little doubt that the Gilson mixture containing formic acid, ethanol and formalin can penetrate the cuticle, but there is little proof "that the exchange of materials between the leaf surface and the inner tissue took place through the ectodesmata."

(b) When strips of living epidermis of onion bulb scales (Allium cepa) were stained in neutral red and mounted in paraffin oil, the excretion of small droplets above the cuticle was observed (5, 37, 49). The droplets formed chain-like rows along the anticlinal walls of the inner epidermis, but were more randomly scattered over the outer epidermis. Ectodesmata were similarly distributed. The nature of the droplets was not determined--they were water soluble when fresh, but water solubility diminished on prolonged exposure to air (49). Occasionally droplets contained neutral red indicating that not only water but also solutes dissolved therein can be excreted. Since there was good agreement between the sites of droplet excretion and ectodesmata, Franke concluded that the excretion

occurred through the ectodesmata neglecting the cuticular membrane.

(c) Strugger (118) had shown that in Helxine soleirolii CT occurred preferentially from cuticular ledges, anticlinal walls and from the basis of glandular trichomes. Further, berberin sulphate was shown to penetrate the CM at these same sites. Using the same plant species, Franke (35) demonstrated ectodesmata in large numbers in guard cells, along anticlinal walls and around the basis of glandular trichomes. He concluded that in Strugger's experiments penetration of BS took place only at those sites corresponding to ectodesmata. He again minimized the fact that ectodesmata are structures in the cell wall and not in the cuticular membrane.

(d) Droplets of a solution containing ^{14}C-labeled sucrose, amino acids and phosphate esters were placed on isolated epidermal strips of spinach (Spinacea oleracea) and pansy (Viola tricholor) leaves mounted on glass slides. Penetration was permitted for 10 to 60 minutes. After washing the surface of the strips with distilled water microradioautograms were prepared. Comparable tissue was prepared to demonstrate ectodesmata. More silver grains were found in the film over guard cells and anticlinal walls than elsewhere. Many ectodesmata could be demonstrated in the same areas and this was considered proof that ectodesmata function as pathways

in foliar absorption (40). Unfortunately, Franke failed to make sure that the blackening of the film over the guard cells and anticlinal walls was not due to surface bound material. This is a possibility since it was shown (130) that ^{45}Ca and ^{36}Cl were preferentially bound to the cuticle (onion leaf) over anticlinal walls and guard cells.

Franke's argument that certain compounds were excreted or penetrated through ectodesmata is not convincing since ectodesmata do not seem to perforate the cuticle, which has been shown to be the prime barrier in foliar penetration of polar compounds. All that Franke's evidence shows is, that ectodesmata can be demonstrated where the CM has been shown to be permeable for polar compounds. This is not surprising, since the Hg^{++} ions essential for demonstrating ectodesmata in the wall are polar themselves and cannot partition through the lipid membrane and depend for penetration on polar pathways which had earlier been shown to occur.

From this discussion it is concluded that ectodesmata can be demonstrated in the wall where the CM permits penetration of polar compounds (including Hg^{++}) provided there is some reductant in the wall.

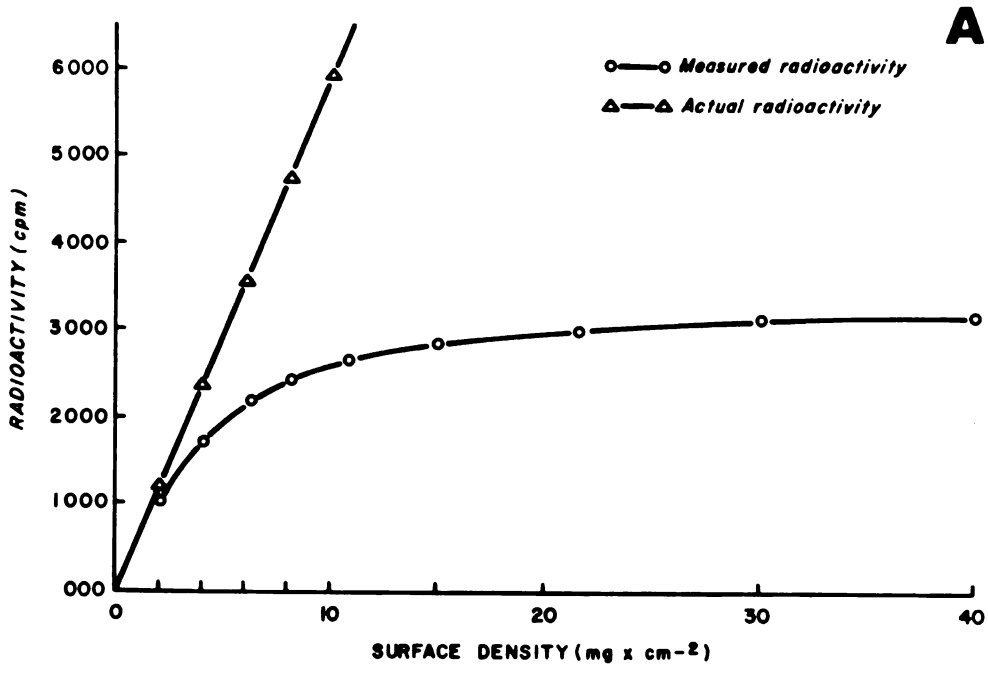
APPENDIX II

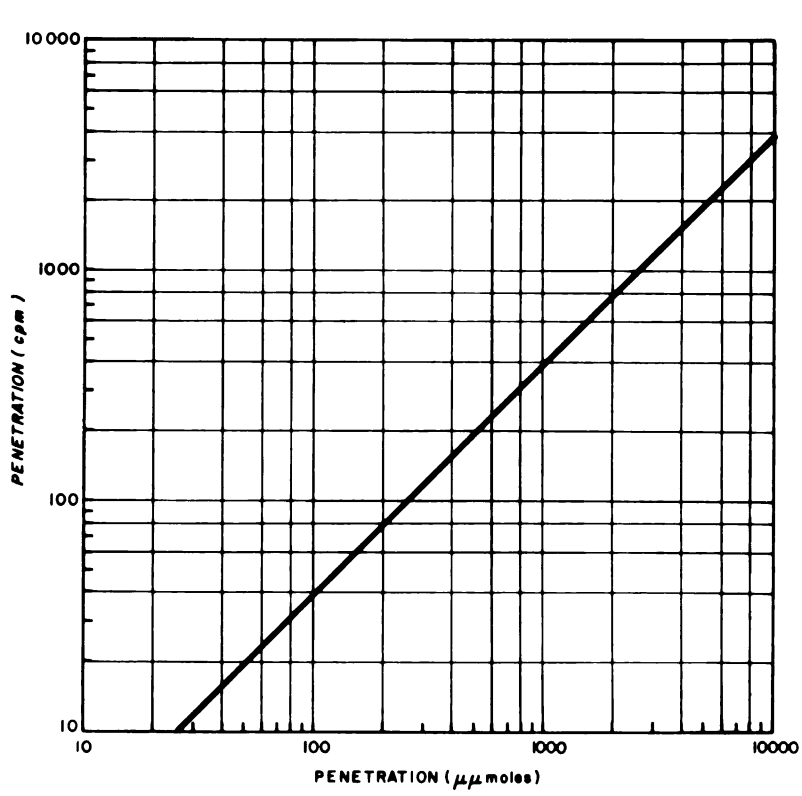
APPENDIX II A.

Plot of actual radioactivity vs. measured radioactivity for ^{14}C-SADH (self-absorption curve).

APPENDIX II B.

Plot penetration (cpm) vs. penetration (μμmoles) (conversion graph).


A
Measured radioactivity
Actual radioactivity
RADIOACTIVITY (cpm)
6000
5000
4000
3000
2000
1000
000
0
10
20
30
40
SURFACE DENSITY (mg x cm-2)


B
PENETRATION (cpm)
10000
1000
100
10
10
100
1000
10000
PENETRATION (μμ moles)