FACTORS INFLUENCING THE FOLIAR PENETRATION OF NAPHTHALENEACETIC ACID AND NAPHTHALENEACETAMIDE INTO LEAVES OF PEAR (PYRUS COMMUNIS L.)

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ABSTRACT

FACTORS INFLUENCING THE FOLIAR PENETRATION OF NAPHTHALENEACETIC ACID AND NAPHTHALENEACETAMIDE INTO LEAVES OF PEAR (PYRUS COMMUNIS L.)

By

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Factors influencing the foliar penetration of naphthaleneacetic acid (NAA) and naphthaleneacetamide (NAAm) into pear leaf disks (Pyrus Communis L. Cv. Bartlett) were studied to determine the extent to which each factor influenced the amount entering the leaf. An improved method of assessing foliar penetration is described. Small glass cylinders were attached to leaf disks with silicone rubber, placed in Petri dishes lined with filter paper moistened with water, and arranged in a water bath under a light bank. Factors affecting penetration could be more critically assessed because there was maximum control of experimental conditions. The penetration of NAA and NAAm through the upper surface was linear with time. There was rapid initial penetration through the lower surface followed by a reduced rate after 24 or 48 hours. Penetration of NAA applied in glass cylinders was similar to that applied as microdroplets, providing the droplets were prevented from drying out. Droplet drying resulted in increased penetration. Penetration was linear with increasing concentration (10^{-6} to 10^{-3} M) except for the reduced rate of NAA penetration through the lower surface at concentrations above 10^{-4} M. Increasing temperature (5-35 C) resulted in increased NAA or NAAm penetration. Temperature coefficients generally ranged between 1.51 to 5.46. Penetration of NAA and NAAm through the lower

surface increased with increasing light intensity from 0 up to about 600 ft-c. Increased light intensity above this resulted in no additional penetration. Light enhanced penetration through the upper surface could only be demonstrated where the cuticle did not greatly restrict diffusion. The light effect was independent of stomatal opening. Inhibitors of the Hill reaction (Atrazine, Monuron, Terbicil) and oxidative phosphorylation (DNP, m-Cl-CCP, p-F-CCP), phenylmercuric acetate, and nitrogen all significantly reduced penetration of NAA. It was concluded that penetration into leaf disks is an active process requiring oxygen and products of photosynthesis. No surfactant studied increased NAA or NAAm penetration through the upper surface. At 0.01 and 0.1% concentration Tween 20 and Tergitol 15-S-9 increased NAA penetration through the lower surface. Tween 20, Triton B-1956, and X-77 significantly increased NAAm penetration through the lower surface at a 0.1% concentration. X-77 was found to be the most effective. Surfactants increased NAAm penetration to a greater extent than for NAA. Stomatal penetration has been established under conditions where a surfactant in the treating solution sufficiently lowered (42 dynes/cm^2) the surface tension. However, entry via stomata is not considered to be a major portal of entry. Penetration of NAA was always greater through the lower surface than through the upper surface. Uptake of NAAm was greater through the upper surface of young leaves but greater penetration occurred through the lower surface after leaves had fully expanded. Penetration was greater in younger leaves than in older leaves with the exception of NAA penetration through the upper surface. Silver nitrate was shown to preferentially pass through the lower cuticle above veins. No preferential pathways of NAA through isolated upper pear leaf cuticle were observed.

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INTRODUCTION

The use of foliar applied plant growth regulators, pesticides and nutrients has increased exponentially in the past 25 years. Contributing factors have been a declining labor supply, increased cost of production, and a shift toward complete mechanization.

Numerous contact, preemergence, general and selective herbicides have virtually eliminated the need of hand weed removal and have allowed weed control to an extent never before possible. New plant growth regulation compounds and uses are found regularly. Foliar applications of these compounds are used to effect flowering and fruiting, control plant growth, retard or accelerate fruit abscission, to increase market quality, and reduce labor to mention a few.

There is a diverse nature of compounds, a wide spectrum of applications and variety of climatic conditions under which these compounds are applied. Results are often not reproducible from year to year. Spray recommendations vary with location and time of application. Penetration information for a class of compounds for a particular set of conditions often is not applicable to other compounds and conditions.

All aerial portions of a plant are covered by a cuticle. This cuticle is lipoidal in nature and thus may act as a sink for lipophilic compounds. Other foliar applied compounds may be absorbed, translocated and accumulated in edible portions of the plant making spray residues a problem for present and future generations. These problems involving foliar penetration of compounds must be investigated to eliminate or at least alleviate health problems and difficulties involved in their use.

In spite of the considerable amount of work devoted to the study of foliar penetration, large gaps still exist in our knowledge of how a compound in a spray droplet migrates from a position on the surface of a leaf to a place in the plant where it elicits a response. Many questions remain to be answered concerning factors influencing foliar penetration and the exact pathway or pathways a compound follows on entering a leaf.

Applications in early foliar penetration studies were made in the form of sprays or droplets. Changes in concentration, buffering capacity and area during droplet drying and a lack of environmental control limited the kind of data that could be obtained. Precise methods of determining foliar penetration and pathways of foliar penetration are now available, especially through the use of radioactive compounds and improved methods of application. The leaf has been taken apart and its component parts studied; the isolated cuticle and isolated cells.

Much information has been gathered in this manner, which has been an invaluable asset contributing to the body of knowledge of foliar penetration. However, until critical studies are carried out on an intact system under controlled conditions, voids will still exist in our knowledge of the process of foliar penetration.

LITERATURE REVIEW

The ever increasing use of foliar applied plant growth regulators, pesticides, and nutrients has resulted in a large volume of literature pertinent to this subject. A number of reviews have appeared in recent years in an attempt to organize the existing body of information. Reviews stressing foliar penetration of herbicides have been written by Blackman et al. (1951), Crafts (1953), Crafts and Foy (1962), Currier and Dybing (1959), Foy (1964), and Woodford et al. (1958). Information specifically involving foliar penetration of plant growth regulators has been discussed by Sargent (1965). Reviews by Boynton (1954), Jyung and Wittwer (1965), and Wittwer and Teubner (1959), dealt with penetration of nutrients. A more general discussion of foliar penetration of organic compounds has been presented by Franke (1967), Hull (1964), and Van Overbeek (1956).

The Cuticle: Structure, Function, and Composition

All plant surfaces exposed to the air are covered by a thin lipoidal membrane, the cuticle (Van Overbeek, 1956). This covering over the epidermal cells is generally considered to be lamellar in form (Crafts and Foy, 1962), although demarcation between layers is often unclear (Martin, 1965). Waxes varying in form and composition are present on the surface of the cuticle (Juniper and Bradley, 1958; Eglinton and Hamilton, 1967). Cutin is the major constituent of the cuticle and is formed by polymerization of long-chain fatty acids and alcohols, (Crafts and Foy, 1962; Frey-Wyssling and Muhlethaler, 1959). Highly oriented wax is embedded in the cutin matrix (Sitte and Rennier, 1963; Norris and Bukovac, 1968). Pectic substances

and cellulose are interspersed with the cutin, in varying amounts, especially in the region where the cuticle merges into the underlying epidermal cell wall (Baker et al., 1962; Martin, 1965, 1966). Franke (1964), Norris and Bukovac (1968) and Roelofsen (1952) have presented schematic diagrams of the cuticle and the underlying epidermal cell wall.

Waxes

Composition of the surface wax may be one of the important factors regulating penetration. Identification of waxes from a number of plants has revealed that they are composed of long-chain paraffins, alcohols, ketones, fatty acids, hydroxy fatty acids, and esters of the alcohols and acids (Martin, 1966; Purdy and Truter, 1963). The relative proportion of the various wax components may vary with species, age, and position within the cuticle (Baker et al., 1964; Kurtz, 1950; Silva-Fernandes et al., 1964). Chain length of the wax components can vary with the species (Eglinton and Hamilton, 1967) and the particular components (Martin, 1966).

During expansion of the leaf there is continual extrusion and deposition of surface waxes (Bystrom et al., 1968; Martin, 1960), however, deposition of these usually ceases with leaf expansion but there is a continual deposition of cutin waxes (Schieferstein, 1957). The embedded waxes are usually deposited in lamellae or pockets deeply seated in the cuticle (Roelofsen, 1952; Meyer, 1938) or in lamellae near the cuticle surface (Norris and Bukovac, 1968).

Conflicting reports appear in the literature concerning the existence of pores, which could allow wax or wax precursors to migrate from the epidermal cells through the cuticle to the surface. Schieferstein and Loomis (1956) and Juniper (1960) were unable to show wax canals in the cuticle. Using a modified gold-palladium replica method, Hallum (1964)

confirmed these findings. It was suggested that wax migrates between the cuticular lamellae to the surface of the leaf rather than through pores extending directly from the epidermal cell wall to the exterior. Hall and Donaldson (1962) reported wax channels were beneath each wax platelet in Trifolium repens and Brassica oleracea. Using a modified freeze-etch technique, Hall (1967) was able to confirm these observations in the case of Trifolium repens. The significance of wax pores as possible pathways of penetration for nonpolar compounds has been pointed out by Mitchell et al. (1960). However, until a technique is developed providing control of experimental variables on a number of species, the existence of wax canals will likely remain a moot question (Hull, 1964).

Before entering the plant a spray droplet must come in contact with the cuticle. The chemical groups exposed and the physical configuration of the surface wax influence the degree with which a droplet can come in contact with the cuticle (Fogg, 1948; Silva-Fernandes, 1965; Holly, 1964). Silva-Fernandes (1955) considered the physical configuration of the waxes to be most influential in allowing a spray droplet to come in contact with the surface. Holly (1964) has pointed out that droplets may balance on wax projections and dry out without coming into contact with the cuticle proper. Hall and Jones (1961) have shown that surface wax removal by brushing increases wettability and cuticular transpiration.

Increased absorption fo 3-chlorophenoxy-α-propionic acid by peach leaves following brushing has been demonstrated (Bukovac, 1965). Adam (1948, 1958) explored the effect of functional groups on contact angles. Water on paraffin waxes can give a contact angle of 105-110° because only methyl groups are exposed at the surface. Polyethylene has a smaller contact angle, 94°, because more hydrophilic $-CH_2$ groups are exposed.

Cutin

Cutin constitutes the structural framework of the cuticle. Procutin has been shown by Frey-Wyssling and Muhlethaler)1959) and Muhlethaler (1961) to migrate through the epidermal wall to the cellulose-cutin interface in submicroscopic droplets. Hydrolysis of cutin has shown these cutin precursors to be fatty acids and hydroxylated fatty acids in the approximate range of C_{12} to C_{22} length carbon chains (Baker et al., 1964). Matic (1956) and Baker and Martin (1963) have found the major cutin constituents in Agave americana and cutin from a number of fruits and leaves to be hydroxy-octadecanoic acids containing from one to three hydroxyl groups in the carbon chain. The lipophilic hydrocarbon chains are oriented toward the outside and the carbonyl groups oriented toward the more hydrophilic epidermal cell wall (Foy, 1964; Frey-Wyssling, 1953). Polymerization occurs at the surface by esterification of the hydroxyl and carboxyl groups (Matic, 1956; Martin, 1966). These groups impart hydrophilic properties and $-CH_2$ and $-CH_3$ groups give lipophilic properties to the cuticle. Oxidation of cutin at the surface makes the outer surface more lipophilic (Hull, 1964). Cutin is negatively charged when the pH of an applied solution is above the pK for dissociable carboxyl groups present (Crafts and Foy, 1962).

Pectin

Separating the cuticle from the cell wall is a layer of pectic substances, composed of long-chain polygalacturonic acid molecules having side carboxyl groups (Foy, 1964). Pectins are highly hydrophilic, thus, their potential importance as a pathway for the entrance of water soluble compounds must be recognized (Crafts, 1964). Roberts et al. (1948) have examined in cross-section leaves of Malus domestica and have shown a continuous path of pectic substances between the surface of the leaf and the vein

extensions or bundle sheaths. Norris and Bukovac (196) found no evidence of the extension of pectic substances to the surface in the pear leaf (Pyrus communis L.).

Cellulose

Cellulose, a β 1,4-glucose polymer, is a fourth major component of the cutucle. It is arranged in micelles, about 10 A^{o} apart allowing penetration of water and halogens (Frey-Wyssling, 1953). Micelles are further organized into larger units, microfibrils. The space between these is about 100 A^{o}, large enough to allow penetration of larger molecules such as dyes (Frey-Wyssling, 1953). Like pectins, cellulose is hydrophilic in nature and is no obstacle to the penetration of water soluble compounds (Foy, 1964).

Methods of Studying Foliar Penetration

Types of Plant Systems Used

Various approaches have been taken in the study of foliar penetration. Among these are: the whole plant (Allen, 1964; Hauser, 1955), a leaf attached to the intact plant (Allgren and Sudia, 1967; Jyung and Wittwer, 1964), excised leaves (Kamimura and Goodman, 1964; Luckwill and Lloyd-Jones, 1962), leaf disks (Sargent and Blackman, 1962, 1965), isolated cuticle (Yamada et al., 1964, 1965), isolated cells (Jacoby and Dagan, 1967; Jyung et al., 1965) and leaf strips (Rains, 1967; Smith and Epstein, 1964). Information gained using these methods is invaluable in determining the relative importance of the cuticle and cells in the whole process of foliar penetration. Since the cuticle and cells are associated in the leaf in an interacting system, penetration of one influences the other. Therefore, it would appear that the most suitable way to study

foliar penetration, as it actually occurs when a spray droplet impinges upon the surface of a leaf, is by the use of the leaf disk method. Sargent (1965) has discussed the advantages of studying foliar penetration using this type of method.

Methods of Application

Numerous methods have been devised to apply substances for penetration study. The most commonly used methods are (a) foliar sprays (Westwood and Batjer, 1960; Edgerton and Haeseler, 1959), (b) leaf immersion (Jyung and Wittwer, 1964; Ahlgren and Sudia, 1967) and (c) microdroplets (Luckwill and Lloyd-Jones, 1962; Prasad et al., 1967). A more unique approach by Hughes and Freed (1961) and Webster (1962) used a lanolin ring to contain a larger amount of liquid on a defined area. Improvements on this method have been made by Kamimura and Goodman (1964) and Sargent and Blackman (1962, 1965) by securing glass tubes to excised leaves or leaf disks and maintaining them in a covered Petri dish. The advantages of this method are: (a) the solution is not allowed to dry out, thus, maintaining a constant external concentration, (b) a defined area is maintained, (c) buffering capacity is not lost due to drying and (d) the tissue is removed from the influence of the rest of the plant, e.g. translocation.

Method of Detection

Determining the amounts of foliar applied substances entering a plant is often difficult because of the small amount entering the plant or insensitivity of the method chosen to measure penetration. Recording injury caused to a plant may be adequate (Davis, 1956; Hauser, 1955; Westwood and Batjer, 1958). Biological responses (Currier et al., 1964; Westwood and Batjer 1958) are more sensitive but require movement to a part of the leaf or plant where they elicit a response. Fluorescent

dyes (Dybing and Currier, 1959; Hull, 1964) provide a quantitative estimate of penetration. The use of radioactive tracers provides the most sensitive determination of the amount of a substance entering a plant (Sargent, 1965). If sufficiently high specific activities are used, the sensitivity of this method exceeds that of colormetric (Freed, 1964) or spectrophotometric (Holly, 1956) determinations.

Portals of Entry

The cuticle is generally considered a barrier for foliar penetration. However, pesticides, plant growth regulators and nutrients have been demonstrated to permeate the cuticle. Preferential pathways of penetration into leaf cited in the literature are: anticlinal walls, trichomes and epidermal hairs, areas over veins and stomata, guard cells and accessory cells.

Anticlinal Walls

Preferential penetration has been shown to occur over anticlinal walls (Fogg, 1948; Dybing and Currier, 1961). Increased penetration over anticlinal walls has been attributed to a thinner cuticle. However, Baker and Martin (1963) and Norris and Bukovac (1968) have shown the cuticle to be actually thicker over anticlinal walls. Frey-Wyssling and Hauserman (1941) have shown a decrease in birefringence over anticlinal walls. This would indicate either a reduced amount of embedded waxes or a less highly oriented wax layer; both conditions may result in increased permeability. Eglinton and Hamilton (1967) show electronmicrographs providing evidence that more wax is present on the surface over periclinal walls than over the anticlinal walls. This is attributed to an easier diffusion pathway of waxes

from the cell through the periclinal region than the anticlinal region between cells. Bystrom et al. (1968) have shown surface replicas of Beta vulgaris which indicate a wax-coated central plaque-like area of coalesced wax rodlets. The surface over anticlinal walls was nearly devoid of wax.

Trichomes

Trichomes or epidermal hairs have been cited as pathways of penetration of 2,4-D (Ennis and Boyd, 1964). Hull (1944) has extensively reviewed the literature concerning the preferential absorption of dyes. Mitchell et al. (1960) have emphasized the importance of these structures as possible portals of entry of exogenously applied substances. Hairs which persist most often contain protoplasm (Esau, 1953) and as such provide the shortest pathway into the protoplasm of the plant. Since the cuticle is thinner (Martin, 1966), less wax is present (Bystrom et al., 1968). Because of size and geometry they rise above the water repellent epicuticular waxes. Trichomes appear physically suited as preferential areas of penetration.

Veins

Preferential absorption of dyes (Currier and Dybing, 1961; Hull, 1964), 2,4-D (Crafts, 1966; Leonard, 1958; Pickering, 1965) and potassium ferrocyanide (Van Overbeek, 1956) have been reported over veins. Anatomical differences of the vein area offer a possible reason for increased penetration. Thin-walled parenchyma cells, the bundle sheath cells, are present over the surface of veins (Esau, 1953). These often extend to the upper and lower epidermis (Van Overbeek, 1956). There is no reduction in cuticle thickness over the veins of pear but discontinuity of the wax layer may be a significant factor influencing penetration (Norris and Bukovac, 1968).

Water lacking a surfactant is capable of penetration over veinal areas (Van Overbeek, 1956).

Guard Cells and Accessory Cells

Stomatal pores, which penetrate the epidermis, are regulated by very specialized epidermal cells, the guard cells. Morphologically and physiologically they are quite different from the ground epidermal cells. They are the only epidermal cells containing chloroplasts (Esau, 1953; Ketellapper, 1963; Zuker, 1963). There is evidence that guard cells may preferentially absorb exogeneously applied compounds. Sargent and Blackman (1962) concluded that penetration of 2,4-D takes place preferentially through guard cells and accessory cells since a close relationship was found between stomatal density and entrance of 2,4-D into the abaxial and adaxial surfaces of Phaseolus vulgaris and Coleus blumei. Jyung et al. (1965) found a correlation between stomatal frequency and rate of absorption of Rb in bean and tomato leaves. Guard cells have been shown to preferentially reduce $AgNO_3$ (Hofler, 1939). Microradioautograms of Spinacea oleracea and Viola tricolor treated with radioactive sucrose showed the highest density of reduced silver grains above cuticular edges of the guard cells (Franke, 1964b). Under specific conditions Pallas (1966) was able to show neutral red accumulation in guard cells of Vicia faba.

Pores, Cracks and Fissures

There are reports both supporting and refuting the existence of pores in the cuticle. These have been discussed in the section describing deposition of epicuticular waxes. If wax canals or pores traverse the cuticle, then there is a direct pathway from the surface of the leaf to the cell for lipophilic substances. Fissures, punctures and insect punctures undoubtedly allow mass flow of foliar applied substances (Currier

and Dybing, 1959; Harley et al., 1956; Orgell, 1957; Scott and Baker, 1947).

Ectodesmata

Anticlinal walls, trichomes, veins and guard cells have been cited as preferential areas of penetration. Franke (1961, 1964a) has shown, using a mercury-chloride method, that ectodesmata are especially plentiful in these areas of favored foliar penetration. Ectodesmata are considered to be interfibrillar spaces extending through the cell wall to the cuticle (Franke, 1967). The similarity between the distribution of reduced silver grains of microradioautograms over guard cells (Franke, 1964b), anticlinal walls (Franke, 1964c) and veins (Franke, 1964a) has led Franke to conclude that ectodesmata function as pathways of transport in foliar absorption. However, definitive proof is still lacking. One should not disregard the possibility that the cuticle above these areas of preferential absorption is more permeable and thus ectodesmata may be demonstrated only when the cuticle allows sufficient entrance of the foliar applied substance.

Stomatal Penetration

Stomata are present on all leaves and when open provide an avenue to circumvent the external cuticular barrier. They must be considered as a potentially important pathway for foliar penetration. However, the question of whether a foliar applied solution can pass through the stomatal pore and enter the substomatal chamber has been in controversy for an number of years. There is general agreement that aqueous solutions in the absence of a surfactant are unable to penetrate into the substomatal chamber (Fogg, 1948b; Teubner et al., 1957; Weaver and DeRose, 1946). However, with a surfactant present, the occurrence of stomatal penetration is a debated

question. Westwood et al. (1960b) concluded that stomatal penetration of dinitro-o-cresol by apple leaves is unimportant since the major portion of the DNOC was absorbed after the spray dried. Middleton and Sanderson (1965) discount mass flow of inorganic ions through the stomatal pores because (a) absorption was linear with time, (b) there was not a linear relationship with applied concentration and (c) per cent uptake fell as the concentration increased. Muzik et al. (1954) and Wallihan and Heymann-Herschberg (1956) considered stomatal entry unimportant because an equal amount or less of CMU or zinc, respectively, penetrated through the lower surface as compared with the upper surface.

Stomata have been indicated as major sites for entry of urea (Cook and Boynton, 1952), NAA (Harley et al., 1957), and Co^{60} (Gustafson, 1956) since more penetration occurred through the lower surface where stomata are more numerous. Fluorescent dyes have been used to study stomatal penetration in a number of plants. From surface observations Dybing (1958), Dybing and Currier (1961) and Hull (1964) have concluded that stomatal penetration can occur provided that efficient surfactants were used at the proper concentration. Stomatal penetration also varied with the species tested. The most definitive evidence offered in proof of stomatal penetration has been presented by Pickering (1965). Microradioautograms of Zebrina leaf cross sections clearly show C^{14}-2,4-D to have moved into the substomatal chamber.

There are several reasons why conflicting reports of stomatal penetration may appear in the literature. There is still no general agreement on the process of stomatal movement. However, a number of environmental factors are known to influence stomatal opening and closing; among the more important are light, carbon dioxide concentration, temperature, and

water stress on the leaf. The effect of these environmental factors on stomatal movement have been discussed by Ketellaper (1963), Meidner and Mansfield (1965), Pallas (1966) and Zelich (1965). Stomata are closed in the dark except for succulents (Levitt, 1967). Opening depends on light intensity and wavelength. The higher the light intensity the wider the opening. Low CO_2 concentrations cause stomatal opening and high CO_2 concentrations tend to close the stomata. Intermediate concentrations partially close stomata. Temperature (5-25 C) may or may not influence stomatal movement. However, temperatures over 30 - 35 C generally cause closing. When the water deficit reaches a certain value, characteristic for a species, stomatal aper ture tend to become smaller, and if severe become completely closed. Therefore, stomatal aperture widths are highly dependent upon a combination of environmental factors. Changes in any one of these factors may have a profound effect on stomatal width and thus the amount of a solution that potentially can enter through a stomatal pore. Currier et al. (1964) have pointed out the difficulties involved in controlling environmental conditions in the greenhouse sufficiently to consistently demonstrate stomatal penetration.

The size of the stomatal pore varies according to the plant species. Phaseolus vulgaris was found to have a maximum stomatal aperture of 7 x 3μm and Zebrina pendula 31 x 12μm (Eckerson, 1908). The size of stomata on a plant and the number is influenced by the environmental conditions under which the leaves develop (Meyer et al., 1960).

Surfactants differ in their ability to reduce surface tension (Foy and Smith, 1965). Dybing and Currier (1961) have found that stomatal penetration is dependent upon concentration and surfactant used and the

species being treated. In general, the more effective surfactants caused the greatest reduction in surface tension and the species showing greatest stomatal penetration had large stomatal aperture.

If stomatal penetration is a reality then its importance in foliar penetration must be determined. Skoss (1955) considered stomatal penetration the major portal of entry into the leaf. Cook and Boynton (1952) and Foy (1962) regarded stomatal penetration important for initial entry into the leaf but over a longer period of time cuticular penetration was thought to be the most important pathway of entry. Although stomatal penetration may occur (Crafts, 1964), it cannot be considered an important pathway of entry because it is impossible to predict when stomata will be open since opening and closing is a complex process involving many uncontrollable environmental conditions (Crafts, 1961). Further, lining the substomatal chamber is an internal cuticle (Norris and Bukovac, 1968; Scott, 1950; Yamada et al., 1966). Even if a solution can enter the substomatal chamber, it still must traverse this internal cuticle. However, the internal cuticle is hydrated much thinner and increases the absorbing surface (Wittwer et al., 1967).

Factors Influencing Foliar Penetration

Environmental Factors

Environmental factors such as light, temperature and humidity prior to spraying, have been implicated as factors that may determine the subsequent performance of a spray application (Edgerton and Haeseler, 1959; Westwood and Batjer, 1960). High light intensity has been shown to increase cuticle thickness (Orgell, 1954), thus increasing the diffusion pathway of a penetrating molecule. Juniper (1959) has demonstrated a

direct correlation between wettability and surface waxes and light intensity under which Pisum sativum plants were grown. Low contact angles were associated with low light intensities. The lower the contact angle the larger the area of a spray droplet in contact with the leaf surface. The influence of temperature on foliar penetration may be made manifest through its effect on growth. Apple and peach leaves absorbed more NAA-C^{14} when grown under cool air temperatures (60 F) than those developed under warmer temperatures (70 F) (Donoho et al., 1961). It would seem that growth is slowed down, cuticle development is slower and wax production retarded at lower temperatures. Immature ivy leaves have thinner cuticle and less wax than mature leaves (Schieferstein, 1957). Foliar absorption of 3-CP (Bukovac, 1965) and 2,4-D (Sargent and Blackman, 1962) has been shown to be greater in immature than in mature fully expanded leaves. Donoho et al. (1961) have shown that peach leaves developed under a high humidity environment absorbed more NAA-C^{14} than those developed under low relative humidity conditions.

Time-Course

Following the rate of foliar penetration over a period of time has been one method used by investigators to determine uptake patterns of foliar applied compounds. Ehlig and Bernstein (1959), Jyung and Wittwer (1964) and Sargent and Blackman (1962) have reported foliar penetration of sodium and chloride, phosphate and rubidium, and 2,4-D respectively, to be linear with time. Other workers have shown two phases (Prasad and Blackman, 1962), three phases (Vickery and Mercer, 1964), and four phases (Allen, 1964) of foliar uptake. These phases differ in length of time and relative importance in the whole process. However, the first phase is generally the shortest and most rapid. Care must be taken in

evaluating time-course studies in the literature since application has often been made by spraying. Interpretation is made difficult by changing concentration and buffering capacities as a result of droplet drying.

Concentration

The influence of external concentration of the applied solution has been determined for a number of foliar applied compounds. Penetration into leaves has been found to be directly correlated with concentration for 2,4-D (Sargent and Blackman, 1962; Thimann, 1948), NAA (Westwood and Batjer, 1958), urea (Kuykendall and Wallace, 1954), and salts (Eaton and Harding, 1959; Middleton and Sanderson, 1965). Bukovac and Norris (1966) showed a linear relationship between applied concentration and the amount of NAA and NAAm bound to the upper surface of pear leaves. Deviations from linearity with applied concentration are seldom observed. However, Jyung and Wittwer (1964) have shown uptake of Rb to deviate from linearity at higher concentrations.

Temperature

As temperature increases, it is generally agreed that there is an increase in foliar penetration. Currier and Dybing (1959) indicated that the complexity of the overall penetration process makes it difficult to determine where temperature exerts its effect. It is becoming eminently clear that temperature influences both accumulation by the cells and penetration through the cuticle. Cuticular penetration is considered to be a physical diffusion process (Franke, 1967). The temperature coefficient (Q_{10}) for ion diffusion through water is about 1.2 (Briggs et al., 1961). Lipid membranes may exhibit high Q_{10} values (Sutcliffe, 1962). Van Overbeek (1956) has pointed out that wax lamellae at low temperatures are near solidification and have low permeability. As the temperature is raised

these fatty substances become less viscous and more permeable. Briggs et al. (1961) indicated that molecules moving through lipid containing membranes may have to acquire a relatively large amount of kinetic energy to break the number of bonds between the lipid molecules. Norris and Bukovac (1969) have shown that the Q_{10} for penetration between 15-25 C of NAA through enzymatically isolated upper pear leaf cuticle is 5.5-6.0. This is particularly significant since this high Q_{10} value was derived over a physiological temperature range.

Temperature coefficients of 2.0 and greater have been considered evidence suggestive of metabolically mediated uptake. Temperature coefficients for foliar penetration in excess of 2.0 led Goodman and Goldberg (1960), Rice (1948), and Sargent and Blackman (1962) to suggest that absorption is governed by metabolic processes. Since cuticular penetration has been shown to be profoundly influenced by temperature and Q_{10s} for active accumulation by cells are known to be 2.0 or greater, it is reasonable to assume that the temperature coefficient for foliar penetration is a result of temperature influence on both metabolic processes and the cuticle. Therefore, temperature coefficients for foliar penetration should be rejected as a basic criteria for active uptake.

pH

The influence of pH on regulating the amount of a plant growth substance entering a leaf is well documented. This has been especially true for weak acids such as 2,4-D and NAA. In general, there is an inverse relationship between pH of an applied solution and amount entering the leaf (Currier and Dybing, 1959). Crafts (1953, 1956) and Sargent and Blackman (1962) have found the greatest 2,4-D penetration at low pH values (below 6.0). Bukovac and Norris (1966) reported that sorption of NAA into the

pear leaf was pH dependent, being greater below the pK (4.2) than above. Giese (1962) has discussed the influence of pH on the dissociation of weak acids and bases in relation to their ability to pass through lipoidal cell membrances. Above the pK (the pH at which there is an equal number of charged and uncharged molecules) most weak acid molecules are dissociated, have a charge and are not lipid soluble. Below the pK more molecules are in the undissociated form, have no charge, and are more lipid soluble.

Cutin has a large number of free COOH groups (Foy, 1964; Franke, 1967). The pH and the hydrogen ion concentration of the applied solution may influence the charge on the cuticle. Van Overbeek (1956) has reported a pK of over 5 for the cutin and Bukovac and Norris (1966) have shown the pK for the surface of pear cuticle to be between 2.8-3.2. At low pH values the carboxyl groups would be largely undissociated and thus more permeable to anions than at higher pH values where more carboxyl groups would be dissociated and, thus, negatively charged (Hull, 1964). Orgell (1957) observed that acid substances were absorbed to the greatest extent at acid pH values. Hull (1964) has attributed this to the probable neutralization of negatively charged acid residues.

Light

The influence of light on foliar penetration is less clear than for other factors. Although light has generally been found to increase foliar penetration, there are a number of instances where equal or greater absorption occurred at lower light intensities or in the dark (Herrett and Linck, 1961; Smith et al., 1959; Weintraub et al., 1954). The energy required for active absorption can be derived in green leaves from photosynthetic processes (Franke, 1967). Increases in foliar penetration under the influence of increasing light have been attributed to the production of

photosynthetic reserves (Arisz and Sol, 1956; Kamimura and Goodman, 1964). Ahlgren and Sudia (1967) and Van Lookeren Campagne (1957) attributed light dependent foliar penetration to energy derived from light directly; presumably photophosphorylation and not a translocatable product. Sargent and Blackman (1962, 1965) have shown that the light effect on increased absorption of 2,4-D in bean leaf disks is very complex. 2,4-D passes through the cuticle and cell wall by diffusion. The steepness of the diffusion gradient is determined by light-stimulated uptake into the underlying cells and by a conversion into some 2,4-D metabolite. It was concluded that both physical and metabolic factors influenced 2,4-D penetration.

Currier and Dybing (1959) and Dybing (1958) considered that light may influence penetration by causing stomata to open. Light must be regarded as a prerequisite for foliar penetration to occur via stomata because other factors which may open stomata, i.e. low CO_2 concentrations, would not occur under normal treating conditions.

Surfactants

The physical arrangement of epicuticular waxes often prevents spray droplets from coming in contact with the leaf surface (Eglington and Hamilton, 1967). Epidermal hairs covering leaves may also prevent spray droplets from coming in contact with the surface (Holly, 1964). To overcome the problem of incomplete wetting of foliar surfaces a number of surfactants have been used to reduce the surface tension of aqueous solutions.

Physical and Chemical Properties of a Surfactant

The term surfactant is a general one referring to a molecule containing two opposing characteristics (Behrens, 1964). One portion of the molecule has an affinity for the solvent sufficient to bring the whole molecule into

solution. The other portion of the molecule which has a low affinity for the solvent tends to accumulate at an interface (Osipow, 1964). Surfactants are commonly classified as anionic, cationic, non-ionic or amphoteric, depending on the nature of the electrical charge or absence of ionization on the hydrophilic portion of the molecule (Parr and Norman, 1965). Aqueous solutions of surfactants exhibit a rather abrupt change in their micelle concentration, osmotic pressure, electrical conductance, and freezing and boiling points over a narrow concentration range. This point is referred to as the critical micelle concentration (cmc). At concentrations greater than the cmc value, the surface tension of the solution does not decrease with an increase in surfactant concentration (Osipow, 1964).

Surfactant Effects on Cuticular Penetration

Surfactants, in most cases, have been shown to greatly enhance foliar uptake. Bryan et al. (1950) and Hauser (1955) attributed enhanced penetration to a reduction of surface tension, resulting in an increase in the area of spray contact (Stanforth and Loomis, 1949). It is becoming increasingly apparent and well documented that other factors in addition to reduction in surface tension are involved in surfactant effects on penetration (Bayer and Drever, 1965; Freed and Montgomery, 1958; Hughes and Freed, 1961). Jansen (1964) and Westwood and Batjer (1958) have shown that there is a relationship among surfactants, chemical, and species involved in determining the extent to which a chemical can penetrate. Some surfactants inhibit or are ineffective in increasing absorption (Jansen et al., 1961; Westwood and Batjer, 1960). The amount of spray on a leaf may be reduced by the use of surfactants (Koontz and Biddulph, 1957). Surfactants may be effective only when conditions, created by the environment,

are unfavorable for absorption (Thompson et al., 1958; Westwood and Batjer, 1958).

The critical micelle concentration for most surfactants is usually found in the concentration range between 0.01 and 0.1 per cent (Jansen, 1961). Maximum reduction in surface tension occurs at the cmc (Parr and Norman, 1965). However, since many surfactants increase penetration at concentrations far above the cmc, factors other than reduction in surface tension appear to be important (Colwell and Rixon, 1961; Freed and Montgomery, 1958; Holly, 1964). Surfactant effects on herbicidal entry probably correlate best with the colloidal state of the surfactant system (Jansen et al., 1961). The ability of surfactant solutions to dissolve or solubilize water-insoluble materials starts at the cmc and increases with the concentration of micelles (Osipow, 1964). Micelles can apparently dissolve oils and waxes and remove large areas of wax from leaf surfaces, thus enhancing surfactant penetration of the cuticle, resulting in extended phytotoxic effects (Parr and Norman, 1965).

Surfactant Effects on Stomatal Penetration

The influence of surfactants has already been discussed in reference to stomatal penetration. It may be concluded from the work of Dybing (1958) and Dybing and Currier (1961) that surfactants most effective in reducing surface tension allow greatest stomatal penetration of aqueous solutions.

Active Uptake

In order for a growth substance to induce a response it must enter the living protoplasm of the plant. There are a chain of events which take place from the time a spray droplet impinges upon the surface of a

leaf until it reaches its site of action in the living continuum of the plant. A molecule must first pass through the cuticle and cell wall. This portion of absorption is considered to be by diffusion, a physical process (Franke, 1967). Once inside the leaf a molecule may be taken up actively by a cell or move in the plant in the cell walls and xylem (Crafts, 1961b). Jyung et al. (1964, 1965) have shown foliar absorption by isolated leaf cells to be an active process. Because uptake by cells in an intact leaf may be modified by the cuticle that covers them, the mechanism of absorption may be ill-defined (Wittwer et al., 1967).

Ahlgren and Sudia (1967), Arisz and Sol (1956), Jyung and Wittwer (1964) and Van Lookeren Campagne (1957) have concluded that uptake of nutrients is metabolic. Evidence has been given for metabolic uptake of streptomycin by apple leaves (Kamimura and Goodman, 1964), 2,4-D by bean leaves (Sargent and Blackman, 1965), and sucrose by bean leaves (Vickery and Mercer, 19 4).

Criteria often used for active uptake include: time-course analysis, temperature, accumulation against a concentration gradient, irreversibility, oxygen, energy dependence and sensitivity to metabolic inhibitors (Giese, 1962; Jyung and Wittwer, 1965). Because a molecule must pass through a cuticle present on the intact leaf or leaf disk, it may be necessary to modify some criteria. Temperature is a questionable criterion because penetration through the plant cuticle has been shown to have high temperature coefficients (Norris and Bukovac, 1969). Active uptake by cells is generally considered to be two phases; a rapid initial phase and a slower linear metabolic phase (Jyung and Wittwer, 1965). This criterion may also have to be modified.

Naphthaneneacetic Acid and Naphthaleneacetamide

Investigations have been carried out to evaluate the effectiveness of naphthaleneacetic acid (NAA) and naphthaleneacetamide (NAAm) as abscission promoting and preventing compounds since Gardner et al. (1939) reported NAA and NAAm as active in delaying abscission in ripening apples. Southwick et al. (1953) found NAA to be effective as a fast action stop-drop compound on McIntosh apples, but other compounds were superior. In recent years the use of NAA and NAAm has been restricted to post-bloom sprays as a thinning agent (Harley et al., 1957, 1958; Hoffman et al, 1955; Southwick and Weeks, 1957; Thompson et al., 1958; Westwood and Batjer, 1958). Considerable variation in thinning responses from year to year and location to location has stimulated investigations to determine the factors that may influence the absorption of NAA and NAAm and thus lead to the variable thinning results observed in the field (Donoho et al., 1961; Edgerton and Haeseler, 1959; Thompson et al., 1958; Westwood and Batjer, 1958, 1960).

Murneek and Teubner (1953) have attributed the thinning responses of NAA to a relationship between (a) an inhibition of embryo development and (b) a temporary retardation in separation of cells along the abscission zone. Donoho et al. (1961) have shown that ring-labeled C^{14} NAA was detectable in fruits often after one hour. Distribution throughout the fruit was found after 96 hours with some accumulation in vascular areas and the seeds. Luckwill and Lloyd-Jones (1962) reported rapid conversion of NAA in detached apple leaves to a water-soluble addition Compound I, lacking auxin activity. This addition compound was slowly converted into addition Compound II. Zenk (1962) has demonstrated that a large proportion of the

NAA entering etiolated pea stems was converted to the glucose or aspartate conjugates. Sudi (1967) has confirmed the formation of 1-naphthaleneacetyl-L-aspartate from NAA in the etiolated pea stems and has shown this conjugate formation to be accomplished by an inducible enzyme.

MATERIALS AND METHODS

Pear trees, Pyrus communis L. Cv. Bartlett, (Hawley Nursery, Hart, Michigan) were selected as the test plant. A description of the pear leaf in relation to foliar penetration (Norris and Bukovac, 1968a), binding to pear leaf cuticle (Bukovac and Norris, 1966), and penetration through the isolated pear leaf (Norris and Bukovac 1968b, 1969) have established a basis for the use of the pear as the test plant. α-Naphthaleneacetic acid (NAA) carboxyl ^{14}C and α-naphthaleneacetamide (NAAm) carboxyl ^{14}C (Tracerlab, Waltham, Mass.) were selected as the model compounds. ^{3}H-NAA for microradioautography was obtained from Nuclear Chicago Corp., Des Plaines, Ill.

Growing Plants

During the growing season leaves were collected from established trees on the Michigan State University Horticulture Farm. When leaves were not available from orchard trees, 2-year-old trees were grown in the greenhouse. The general method for growing was to plant the trees in plastic containers in a mixture of 2/3 sandy loam and 1/3 peat. Holes were punched in the bottom of the containers for water drainage. Initially all trees were headed back to about 18 inches and then 2 lateral shoots allowed to grow. All other shoots were removed as they appeared. Leaves were selected from experimentation when fully expanded and a deep green color had developed.

Selection of a Method to Determine Foliar Penetration

Four methods for determining foliar penetration were evaluated on the basis of variability of results and ease and rapidity of carrying out the procedure. A leaf disk procedure was modified after the method of Sargent and Blackman (1962). This method allowed the greatest control of experimental variables and environmental conditions during the absorption period. Twelve 1.5-cm leaf disks, with attached glass cylinders, were placed in each of 10 Petri dishes. Into each glass cylinder was pipetted 0.25 ml of a 5 X 10^{-4} M NAA solution buffered at pH 4.2 with 10^{-2} M phosphate-citrate and containing 0.5 μc/ml ^{14}C. After a 12-hour treatment time the radioactive treating solution was removed, the leaf disks washed with a stream of water from a wash bottle and wiped with a piece of cotton moistened with xylene. Three disks were sampled at random from each Petri dish for each of the four methods tested and radioactivity determined by the following procedures.

(1) Leaf Disk-Planchet Counting

Leaf disks were placed treated side down in 2.5-cm stainless steel planchets lined with double sticky tape and then dried in a drying oven for 12-hr at 60 C. Radioactivity was then determined in a Beckman Low Beta II proportion counter. Corrections were made for background and efficiency.

(2) CO_2 Combustion - Scintillation Counting

Leaf disks were blotted dry then placed in the drying oven at 60 C for 12 hr. CO_2 combustion flasks (Arthur Thomas Co., Philadelphia, Pa.) were purged with oxygen. The dried leaf disks, wrapped in black combustion paper, were placed in a platinum holder attached to the top

then placed in the flask. Combustion was carried out using a Thomas-Ogg Infrared Igniter (Arthur Thomas Co., Philadelphia, Pa.). Combustion time was approximately 45 sec. Ten ml of ethanol-ethanolamine (2:1) was pipetted into the flask before cooling for 30 min in an ice bath. A 5-ml aliquot was removed and placed in a glass scintillation vial with 10 ml of Cab-O-Sil scintillation cocktail (Appendix Table 1). Samples were then counted in a Packard Tri-Carb liquid scintillation spectrometer Model 574. Corrections were made for background and efficiency. A BBOT-toluene scintillation cocktail was first used but was found to be unsatisfactory because the small amount of residual water in the leaf disks caused considerable quenching. The Cab-O-Sil cocktail was able to accommodate at least 0.5 ml of water without appreciable quenching.

(3) Ethanol Extract-Scintillation Counting

Leaf disks were placed in a hand homogenizer with 0.5 ml of ethanol and macerated. The macerated leaf disks were washed twice with 3.5 ml of ethanol to make a total volume of 7.5 ml. A 0.5 ml aliquot was taken and pipetted into a vial with 15 ml of Cab-O-Sil cocktail. Samples were counted in the liquid scintillation counter. Corrections were made for quenching, background and efficiency.

(4) Leaf Disk-Scintillation Counting

Leaf disks were placed treating side down in scintillation vials containing 15 ml of Cab-O-Sil cocktail and counted in the scintillation counter. Corrections were again made for background and efficiency.

The results of this evaluation are shown in Table 1. The leaf disk method counted on the Low Beta II proportional counter was chosen as the method of determining foliar penetration. This was chosen because

Table 1. Comparison of four methods for determining foliar penetration.

Method of detection	Activity (cpm)	Standard deviation	Coefficient of variation
Leaf disk- Planchet counting	3721	379	10.2
CO_2 combustion- Scintillation counting	5388	1106	20.5
Ethanol extract- Scintillation counting	5151	2145	41.6
Leaf disk- Scintillation counting	3830	517	13.5

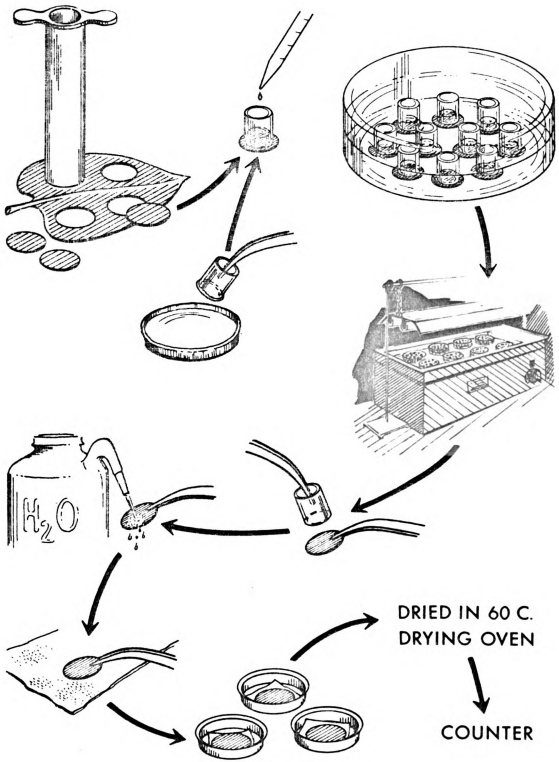
it had the lowest coefficient of variation and the least amount of manipulation of the tissue.

General Methods

Initially the method of Sargent and Blackman (1962) was chosen for application of the treating solution. However, for several reasons it was desirable to modify this procedure: (a) The melting range of the petroleum jelly is 36-41 C. At temperatures even as low as 30 C, leaks at the bottom of the glass cylinders became a problem. (b) Glass cylinders fixed to the leaf surface with petroleum jelly would not retain solutions containing surfactants. (c) Browning of the leaf disk below the petroleum jelly became apparent after 36 hr or more.

A diagramatic representation of the modified method used to apply the treating solution is shown in Fig. 1. Glass cylinders, 1-cm internal diameter, were secured to the leaf by Dow Corning Silastic 68-110-RTV silicone rubber (Dow Corning Corp., Midland, Michigan) and hardened with T-11 catalyst (Wacker Company, Munich, Germany). General Electric RTV-11 silicone rubber (General Electric Co., Waterford, New York) was also found to be an acceptable adherent. To approximately 7 g of silicone rubber was added about 0.30 ml of catalyst and thoroughly mixed with a glass stirring rod. Glass cylinders 8 mm in height and 1 cm in diameter were touched to the silicone rubber catalyst mixture so that the bottom edge of the cylinder was coated. The cylinders with the adhering silicone rubber were placed on 1.5-cm leaf disks cut with a cork borer from a leaf. Approximately 30 to 45 min was required for the silicone rubber to harden. Leaf disks were placed in a 15.0 X 2.0 cm Petri dish lined with filter paper and moistened with distilled

Figure 1.--Schematic description of the method used in studying leaf disk penetration.


H_2O
DRIED IN 60 C.
DRYING OVEN
COUNTER

water; usually 10 leaf disks per dish. The radioactive treating solution, 0.25 ml, was pipetted into each glass cylinder, the top placed on the Petri dish and then arranged in a water bath maintained at 25 C and illuminated with 1000 ft-c of light from a light bank of fluorescent tubes. At the end of the treatment period, the glass cylinders containing the radioactive treating solution were removed and the leaf disks thoroughly washed with a stream of water from a wash bottle. The leaf disks were then blotted dry on a paper towel, placed treating side down in a 2.5-cm planchet lined with double sticky tape, and dried in a drying oven for 12 hr at 60 C. Leaf disks were then counted on a Low Beta II proportional counter with correction made for background.

The standard NAA treating solution was 6.0×10^{-5} M, 1.0 µc/ml and a specific activity of 16 µc/µmole. The NAAm treating solution was 3.5×10^{-4} M NAAm, 0.5 µc/ml and a specific activity of 2.0 µc/µmole. The ^{3}H-NAA treating solution used in the microradioautographic study was 9.3×10^{-4} M NAA, 50 µc/ml and a specific activity of 468 µc/µmole. All were buffered at pH 3.0 with 1×10^{-2} M solution of citric acid and dibasic sodium phosphate. All solutions contained 2% ethanol.

Pretreatment Time

Results of Sargent and Blackman (1962) indicated that light or dark pretreatment could have a profound effect on subsequent penetration of 2,4-D. To evaluate the effect of light-dark pretreatment in the pear leaf system, leaf disks were cut and prepared according to the method described. All Petri dishes were placed in the water bath and either illuminated with 1000 ft-c of light or kept in the dark by

placing aluminum foil over the dish. NAA treating solution was applied at the end of the designated pretreatment times of 0, 4, 8, and 12 hrs. The penetration time was 12 hr. The results of this experiment are shown in Table 3. A 12-hr dark pretreatment was accepted as the standard pretreatment time.

Self Absorption

^{14}Carbon is a relatively low energy beta emitter (0.156 Mev) and as such may be absorbed by the tissue quite easily (Wang and Willis, 1965). An experiment was performed to establish to what extent self-absorption was involved in the detection of the radioactivity in pear leaf tissue. Dried pear leaves were ground to 80 mesh in a Wiley mill. Samples were weighed out in planchets on a Metler Gram-Atic Balance to provide weights of 0.5 to 24 mg/cm^2. ^{14}Carbon NAA of 0.1 μc/ml activity was pipetted into each planchet, 8880 dpm/mg ground leaf tissue. Ethanol was added to fill the planchet to one-half its height. The ground leaf tissue, ethanol and treating solution were mixed in each planchet to give a uniform coverage of the bottom of the planchet and then air dried. Samples were counted on the Low Beta II proportional counter.

The procedure used above deviates from methods more frequently used (Wang and Willis, 1965) but it was found to be most reliable for two reasons. (a) If ground tissue and a radioactive solution were mixed together and then appropriate amounts pipetted into each planchet, it was difficult to get an accurate amount in the planchet because the tissue continued to sediment to the bottom even with continuous agitation. (b) If ethanol did not comprise the major volume of solution

pipetted, the ground tissue, when dried, cracked and did not provide a continuous layer of tissue which is necessary for self absorption determinations.

Leaf Anatomy

Tranverse sections were cut on an International-Harris cryostat and stained with Sudan III and Sudan IV according to Norris and Bukovac (1968). Photomicrographs were taken with a Wild M20 research microscope using a 35mm film holder and exposure time determined by a Wild Photo-automat exposure meter. Photomicrographs of the leaf surfaces were taken with a Wild M5 Stereomicroscope fitted with a Polaroid Camera.

Time-Course of Penetration

Experiments were conducted according to the general procedure to determine the influence of time on penetration of NAA and NAAm by the upper and lower leaf surfaces. Samples were taken at 12, 24, 48, 72, 96, 120 and 144 hr for the upper surface and at 6, 12, 24, 48, 72, and 96 hr for the lower surface. For long term experiments, it was necessary to add water to the filter paper to maintain a saturated atmosphere and prevent evaporation of the treating solution.

The time-course of penetration was followed in one experiment where the treating solution was applied to the leaf as small droplets. Leaf disks were prepared and placed in the Petri dishes. A 20 µl droplet of treating solution was applied to each leaf disk. After 6, 12, 24, 48, 72, 96, and 120 hr the droplet was removed from the leaf disk with a pipette, then the leaf disk was removed, washed and prepared

according to the general method. The filter paper was kept moist at all times to prevent drying of the droplet.

Effect of Concentration on Penetration

The influence of treating solution concentration was evaluated. Concentrations below 6 X 10^{-5} M were achieved by dilution of the labeled NAA or NAAm with buffer and an appropriate amount of ethanol. Concentrations above 6 X 10^{-5} M were prepared by the addition of nonlabeled NAA or NAAm. The time of penetration for these experiments was 24 hr because of the low activity of the lower concentrations employed.

Effect of Temperature on Penetration

Experiments assessing the effect of temperature on penetration were carried out in Percival growth chambers. Water baths were placed in growth chambers maintained at 15, 25 and 35 C and illuminated with 600 ft-c of light with both fluorescent and incandescent lamps. The 5 C temperature was achieved by cooling the water bath in the growth chamber set at 15 C.

Effect of pH on Penetration

Treating solutions between pH 3.0 and 7.0 were prepared using citrate-phosphate buffer. One of the main advantages of this buffer was that it could be used over a wide range of pH values.

Effect of Light on Penetration

Experiments determining the influence of light intensity on penetration were carried out in a Sherer-Gillett growth chamber Model 37-14.

Light intensities between 0 and 1200 ft-c were achieved by varying the distance of the Petri dishes from the light source and by shading with cheese cloth. Honeywell recorder thermocouples were placed in sample leaf disks in each Petri dish and the temperature was monitored and recorded every 4 min during the course of the experiment.

Effect of Light and Dark Treatment on Penetration

Several experiments were performed to determine the influence of alternate light and dark periods on penetration. In these experiments light (1000 ft-c) was provided by fluorescent tubes above the water bath and the dark regime was achieved by covering Petri dishes with aluminum foil.

Effect of Inhibitors on Penetration

Anaerobiosis

The influence of anaerobiosis on penetration was established by following penetration of NAA into leaf disks held in specific atmospheres in desiccators partially filled with water and placed in water baths. Nitrogen and air were continually introduced into the different desiccators through a tube attached to a hollow glass tube in the top. To maintain a continuous flow of nitrogen or air, the outlet tubes were immersed 1/2 in in a beaker of water. Petri dishes were placed in water in the desiccators to minimize any changes in temperature. Leaf disks were pretreated for 12 hr in the dark in an atmosphere of nitrogen or air before the treating solution was applied. Penetration time was 12 hr.

Inhibitors Applied in Solution

Inhibitors were supplied to the lower surface of leaf disks via

glass cylinders and on saturated filter paper in Petri dishes for specific time periods (Table 2). Inhibitor solutions were removed from the glass cylinders with a small polyethylene tube attached to a syringe. Treating solution was then added.

Effect of Surfactants on Penetration

Surfactant treating solutions were prepared on a v/v basis with dilutions being made with buffer solutions. A series of dilutions, from the initial surfactant solution, was necessary because the high viscosity of the undiluted surfactants made accurate pipetting of small volumes difficult.

Effect of Surfactants on Surface Tension

Several concentrations of selected surfactants were prepared in an identical manner to the radioactive treating solution used except where nonlabeled NAA was used instead of ^{14}C-NAA. Surface tension determinations were made using a Cenco-Du Nouy tensiometer. Clean, detergent free, aluminum weighing dishes were used to hold the solution for surface tension determinations. A platinum ring was dipped in ethanol then placed in a flame and heated until red. The platinum ring was placed on the tensiometer and lowered into the solution. The force required to pull the ring from the surface was read directly, in dynes/cm^2, from the tensiometer. Three determinations were made for each concentration. The ring was cleaned after each determination.

Table 2. Inhibitors used in studying penetration of NAA by the lower surface of pear leaf disks.

Inhibitor	Concentration (M)	Pretreatment (hr)
DNP[1]	1×10^{-3}	4 1/2
NaN_3[2]	1×10^{-3}	4 1/2
PMA[3]	1×10^{-4}	4 1/2
Atrazine [4]	1×10^{-5}	12
m-Cl-CCP[5]	1×10^{-5}	12
p-F-CCP[6]	1×10^{-5}	12
Terbicil[7]	1×10^{-4}	12
Monuron[8]	1×10^{-4}	12

[1] 2,4 dinitrophenol
[2] Sodium azide
[3] Phenylmercuric acetate
[4] 2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine
[5] Carbonylcyanide m-chlorophenylhydrazone
[6] Carbonylcyanide p-trifluoromethoxyphenylhydrazone
[7] 3-tert-butyl-5-chloro-6-methyluracil
[8] 3-(p-chlorophenyl)-1, dimethylurea

Stomatal Penetration

Stomatal Aperture Width Measurements

Measurements of stomatal aperture width were made using two methods. Rhoplex AC-33 (Rohm and Haas Co., Philadelphia, Pa.) was used initially according to the method of Horanic and Gardner (1967). With this method the dried Rhoplex was removed from the leaf and the imprints measured directly. However, since the Rhoplex often failed to make good contact with the leaf surface, resulting in incomplete or inadequate coverage, the silicone rubber and cellulose acetate method was more often used (Sampson, 1961; Zelitch, 1961). There appeared to be little difference between the quality of the imprints of these two methods.

Influence of Surfactants on Stomatal Penetration when Added Subsequent to Treating Solution Application

The treating solution was added to glass cylinders, then Petri dishes were placed in the water bath and illuminated with 1000 ft-c of light for 3 hr. Twenty five µl of a concentrated (1.0%) surfactant solution was applied directly to each glass cylinder containing treating solution to give a final volume of 0.25 ml and a 0.1% surfactant solution. In cylinders receiving no surfactant 25 µl of buffered solution were added.

Effect of CO_2 Pretreatment on Stomatal Penetration

Leaf disks were prepared according to the general method. The lights were turned on over the water bath. CO_2 application was made at this time by placing a small hollow piece of Styrofoam in a Petri dish in which was placed about 100 mg of dry ice snow. Petri dish

covers were secured in place. Dry ice was added twice at 1-hr intervals. After 3 hr of pretreatment with CO_2, the treating solution containing 0.1% surfactant was added to control leaf disks and those exposed to CO_2. The treating solution was not washed off with a stream of water. Instead, the disks were blotted dry with paper towels. Omitting the water wash eliminated the possibility of forcing treating solution through the stomatal pore by hydrostatic pressure.

Stomatal Penetration of Silver Nitrate

Leaf disks were prepared according to the general method and then placed in the water bath under lights for 3 hr to allow stomata to open completely. A 0.1 M $AgNO_3$ solution containing 0.1% Vatsol OT was added. Penetration was permitted to proceed for 4 min. Leaf disks were infiltrated, fixed in Craf III solution, dehydrated in TBA, embedded in tissuemat, and cut on a rotary microtome as described by Saas (1958). Ten μm sections were cut. Photomicrographs were taken on a Wild M20 research microscope as previously described.

Effect of Droplet Drying on Penetration

Leaf disks were prepared and droplets applied according to the method described for the time-course of penetration of droplets. At the determined time when droplets were to be dried, both Petri dishes containing the control-wet droplets and those to be dried were removed from the water bath and air dried. The top was removed from the dish where the droplets were to be dried. After about 40 min the droplets had dried. The cover was replaced and both were returned to the water bath until samples were to be taken. Droplets applied to the lower

surface were dried after 12 hr and those applied to the upper surface were dried after 24 hr.

Effect of Leaf Age on Penetration

Trees used in the study of NAA penetration as influenced by leaf age were planted and grown in the greenhouse as previously described. When 17 leaves were present on the terminal shoots, the 3rd (apical), 5th, 7th, 9th, 11th, and 13th leaves were removed, leaf disks cut and penetration determined for the upper and lower surfaces according to the general method.

Trees used in the study of NAAm penetration as influenced by leaf age were planted and grown in a Percival walk-in growth chamber. Trees were illuminated with 1800 ft-c of light with a 14-hr day. Night temperature was 20 C and the day temperature 24 C. When 17 leaves appeared the 3rd (apical), 5th, 7th, 9th, 11th, and 13th leaves were removed and penetration through the upper and lower surface determined.

Statistical Analysis

Data were analyzed using analysis of variance. Where means were compared, the Tukey w procedure was used (Steel and Torrie, 1960). Standard errors of the mean are given where data is presented in graphical form.

Midroradioautography

Pear leaf cuticle was isolated from the leaf according to Norris and Bukovac (1968). Cuticle disks were cut, mounted on glass tubes

and treating solution applied as described by Norris and Bukovac (1969). One ml of 50 μc/ml ^{3}H-NAA was placed in each tube. Cuticle transverse sections were prepared according to Norris and Bukovac (1968). Kodak AR-10 fine grain stripping film (Eastman Kodak Co., Rochester, New York) was applied, exposed for 17 days and developed as described by Jensen (1962). Photomicrographs were taken with a Wild M-20 research microscope as previously described.

RESULTS

Structure of the Pear Leaf

The structure of the pear leaf in cross-section is depicted in Figure 2 A, C, E. The cuticle is lipoidal in nature and is readily stained with Sudan III and Sudan IV, Figure 2 A, C, E. A transverse section of a pear leaf is shown in Figure 2A. The lower surface is more irregular than the upper. Undulations in the cuticular surface are apparent over veins; these being more pronounced on the lower surface. The cuticle in these areas is thicker because of extensions down between the anticlinal walls of epidermal cells. Figure 2E demonstrates the rougher nature of the lower surface and extension of the lower cuticle through the stomatal pore onto the epidermal cell wall exposed to the substomatal chamber.

An epidermal hair from the lower surface is depicted in Figure 2B. Staining with Sudan III and Sudan IV suggests that epidermal hairs also have a well defined cuticle. Hairs are most frequently found on the lower surface but some may be seen on the midrib and outer edges of the upper surface. Surface photomicrographs of the upper and lower surface are illustrated in Figure 2D and F, respectively.

Effect of Light and Dark Pretreatment on Penetration

The effect of light and dark pretreatment for 0, 4, 8, and 12 hr was studied to determine the effect on subsequent NAA penetration through the lower surface (Table 3). There was no difference between pretreatment in the light and dark. However, as pretreatment time increased, NAA

Figure 2. -- Photomicrographs illustrating the structure and upper and lower pear leaf surfaces.

A. Transverse section of a pear leaf
B. Epidermal hair
C. Transverse section of upper surface
D. Upper surface
E. Transverse section of lower surface
F. Lower surface

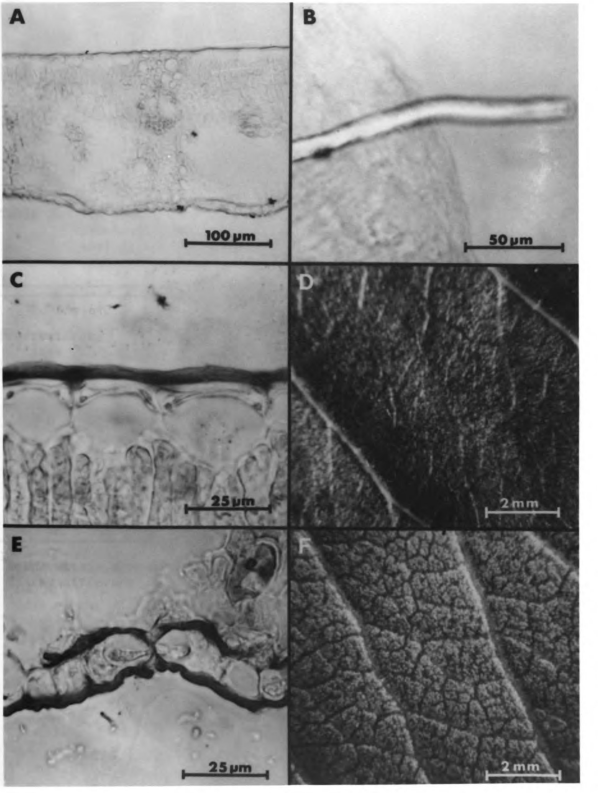
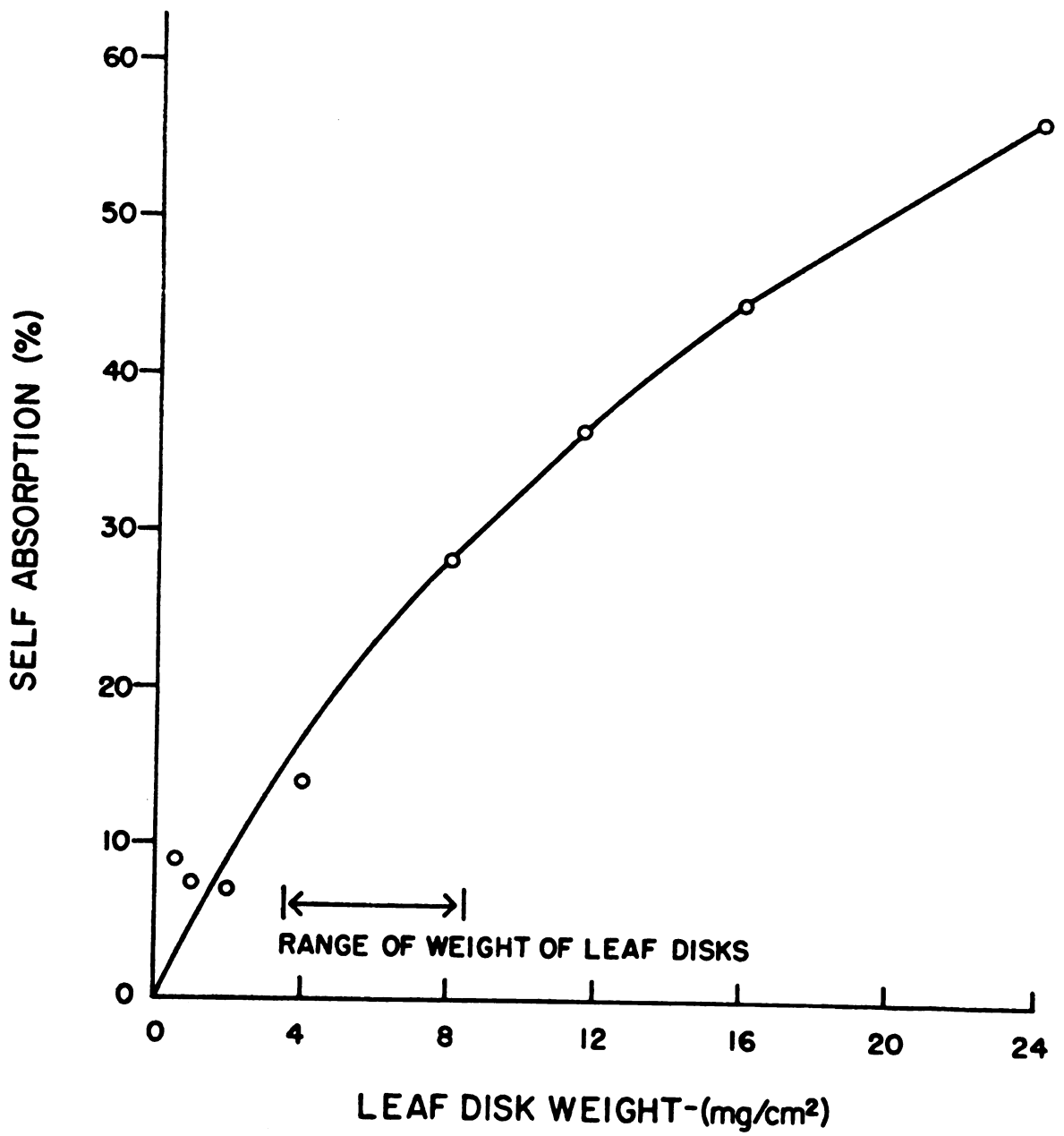

A
100 µm
B
50 µm
C
25 µm
D
2 mm
E
25 µm
F
2 mm

Table 3. The effect of light and dark pretreatment on subsequent penetration of NAA through the lower surface of pear leaf disks in light.

Pretreatment time	Penetration Pretreatment[1] Light	Dark	Mean
	(cpm)		
0	719	743	731a
4	873	718	796ab
8	945	799	872 b
12	909	901	905 b

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

Figure 3. -- Influence of leaf disk weight on self absorption of ^{14}C-NAA.


SELF ABSORPTION (%)
60
50
40
30
20
10
0
RANGE OF WEIGHT OF LEAF DISKS
0
4
8
12
16
20
24
LEAF DISK WEIGHT-(mg/cm²)

penetration also increased. A 12-hr dark pretreatment was accepted as the standard pretreatment time. It was found that if the light pretreatment exceeded 4 hr, stomata were less responsive to subsequent light treatments.

Self Absorption

The effect of leaf disk weight on detection of NAA is shown in Figure 3. The maximum and minimum weight of the leaf disks used was 8.4 and 3.6 mg/cm^2, respectively. Therefore, data presented in cpm represents between 70.5 and 86.5% of the total ^{14}C present in the leaf disk.

Effect of Time on Penetration

Penetration Following Glass Cylinder Application

The time-course of penetration of NAA and NAAm through the upper and lower surfaces of pear leaf disks was followed (Figure 4). Penetration for both surfaces increased with time. There was a linear relationship between time and penetration of NAA and NAAm by the upper surface. Penetration proceeded at a much greater rate and accumulation occurred to a larger extent for both compounds when administered to the lower surface. Penetration was linear for about the first 24 hr for NAA, and 48 hr for NAAm, after which entrance was linear and occurred at a reduced rate for the duration of the experiment.

Penetration Following Microdroplet Application

Penetration was linear with time when NAA was applied as microdroplets to the upper surface, whereas movement of NAA through the lower surface was initially rapid for the first 12 to 24 hr followed by a slower second linear phase for the duration of the experiment (Figure 5). It

Figure 4. -- Time-course of penetration of NAA and NAAm through the upper and lower surfaces of pear leaf disks.

A. NAA upper surface
B. NAAm upper surface
C. NAA lower surface
D. NAAm lower surface

For comparison NAA penetration equals NAAm penetration x 1.48.

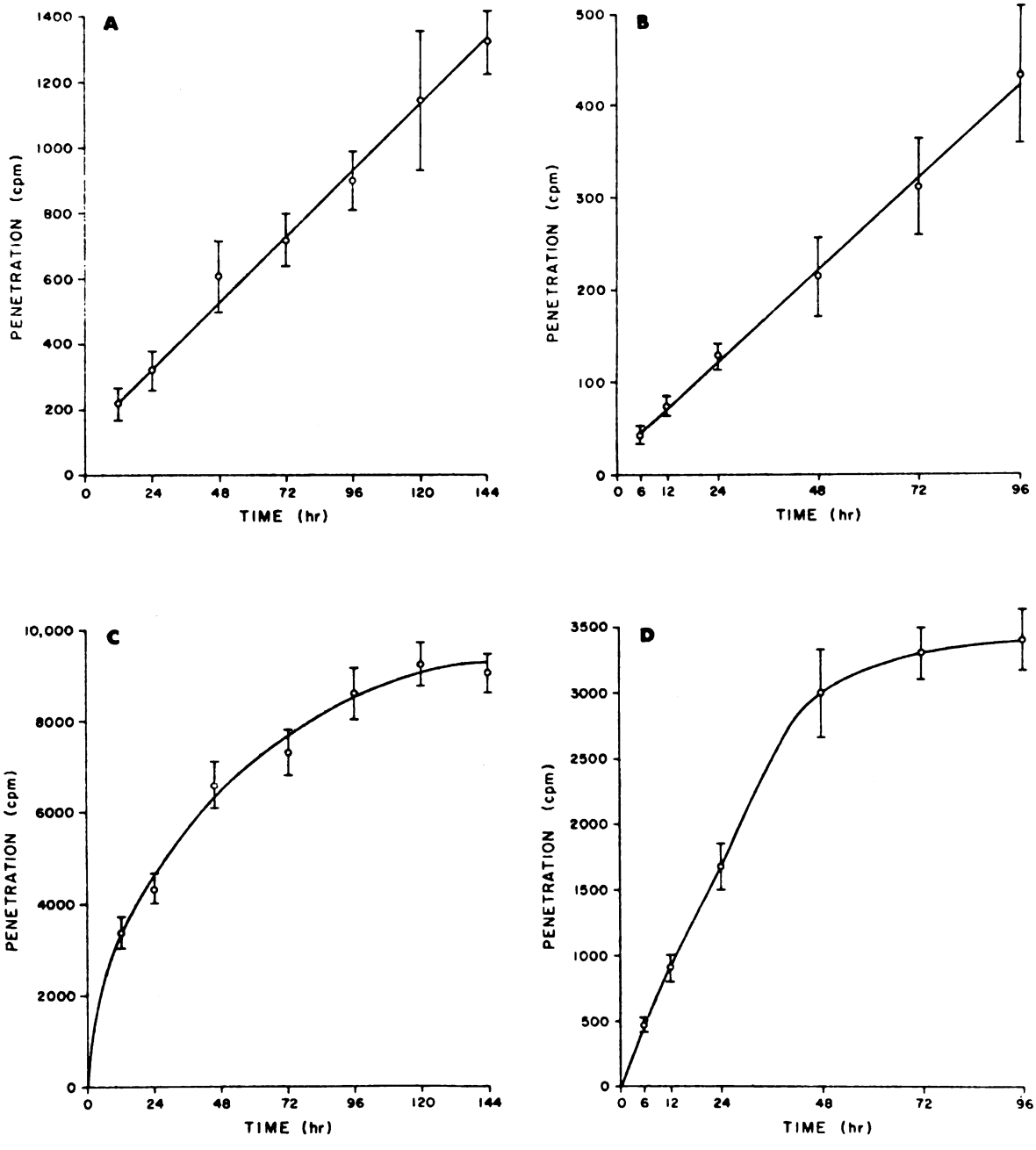
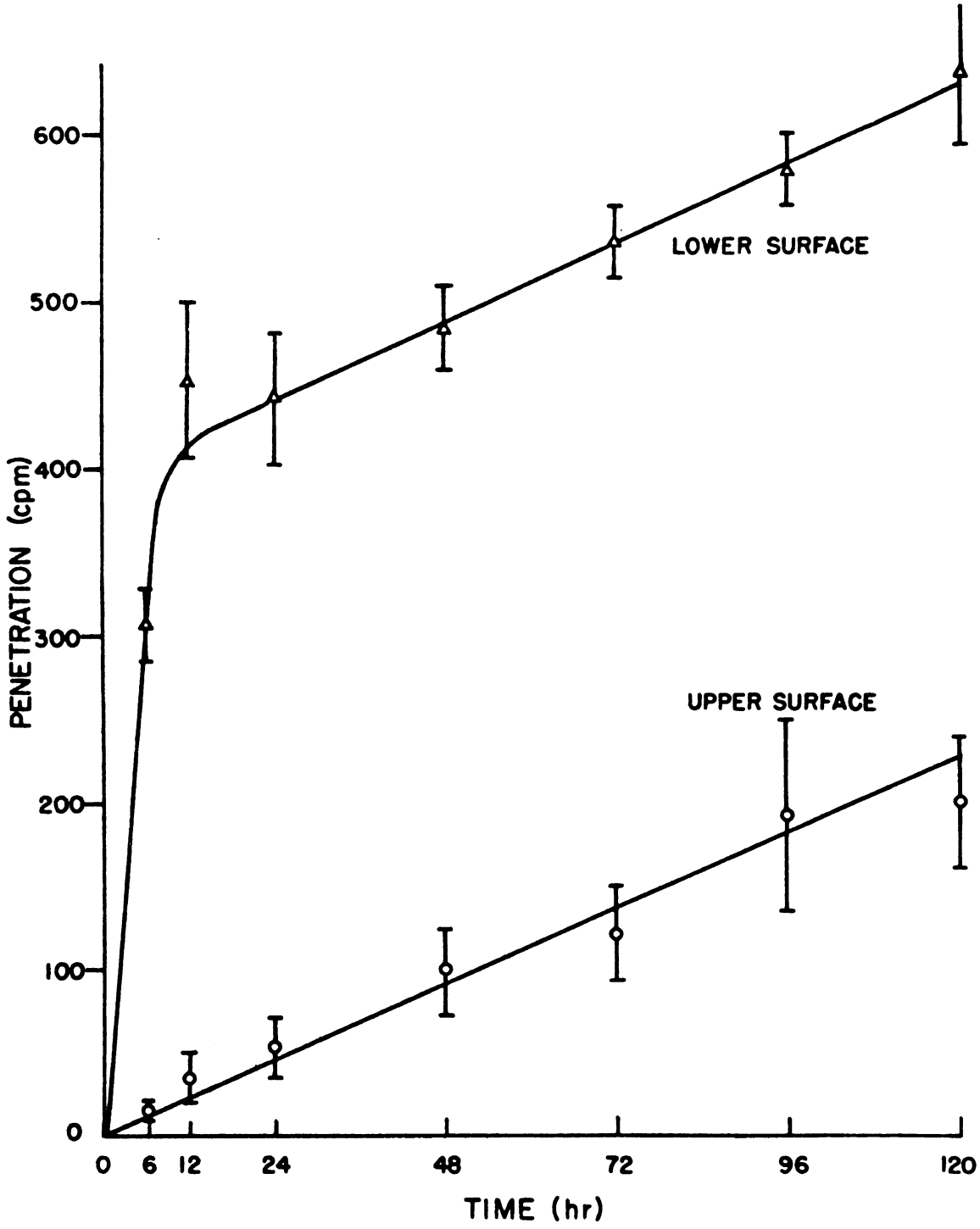

A
PENETRATION (cpm)
1400
1200
1000
800
600
400
200
0
0
24
48
72
96
120
144
TIME (hr)
B
PENETRATION (cpm)
500
400
300
200
100
0
0
6
12
24
48
72
96
TIME (hr)
C
PENETRATION (cpm)
10,000
8000
6000
4000
2000
0
0
24
48
72
96
120
144
TIME (hr)
D
PENETRATION (cpm)
3500
3000
2500
2000
1500
1000
500
0
0
6
12
24
48
72
96
TIME (hr)

Figure 5. -- Time-course of NAA penetration through the upper and lower surfaces of pear leaf disks following application in droplet form.


600
500
400
300
200
100
0
PENETRATION (cpm)
LOWER SURFACE
UPPER SURFACE
0
6
12
24
48
72
96
120
TIME (hr)

appeared that the general penetration pattern of NAA when applied either in microdroplet form or via glass cylinders was similar.

Effect of Concentration on Penetration

As the treating solution concentration was increased there was a corresponding increase in penetration of NAA and NAAm through the upper and lower surfaces (Figure 6). In general, for each 10-fold increase in concentration there was a corresponding 10-fold increase in penetration. This does not hold true for NAA penetration into the lower surface at concentrations above 10^{-4} M. It would appear that saturation has occurred at these higher concentrations.

Effect of Temperature on Penetration

NAA and NAAm penetration through the upper and lower surfaces was generally linear with increasing temperatures between 5 and 25 C and between 25 and 35 C there was a rather sharp increase (Figure 7). Temperature coefficients (Q_{10}) for data presented in Figure 7 are shown in Table 4. Q_{10} values for NAA penetration range between 1.51 to 3.03 and for NAAm between 1.59 to 5.46. Temperature coefficients of NAAm penetration are generally higher than those for NAA. In most cases, the greatest response of penetration to increasing temperature was between 25 and 35 C (Table 4).

The time-course of NAA penetration through the upper surface was linear with time for all temperatures studied; 2, 17, and 27 C (Figure 8). A penetration response to increasing temperature was more dramatic, possibly reflecting an influence of leaves 6 weeks older than those used in the previous temperature studies.

Figure 6. -- Influence of treating solution concentration on penetration of NAA and NAAm through the upper and lower surfaces of pear leaf disks.

A. NAA upper surface
B. NAAm upper surface
C. NAA lower surface
D. NAAm lower surface

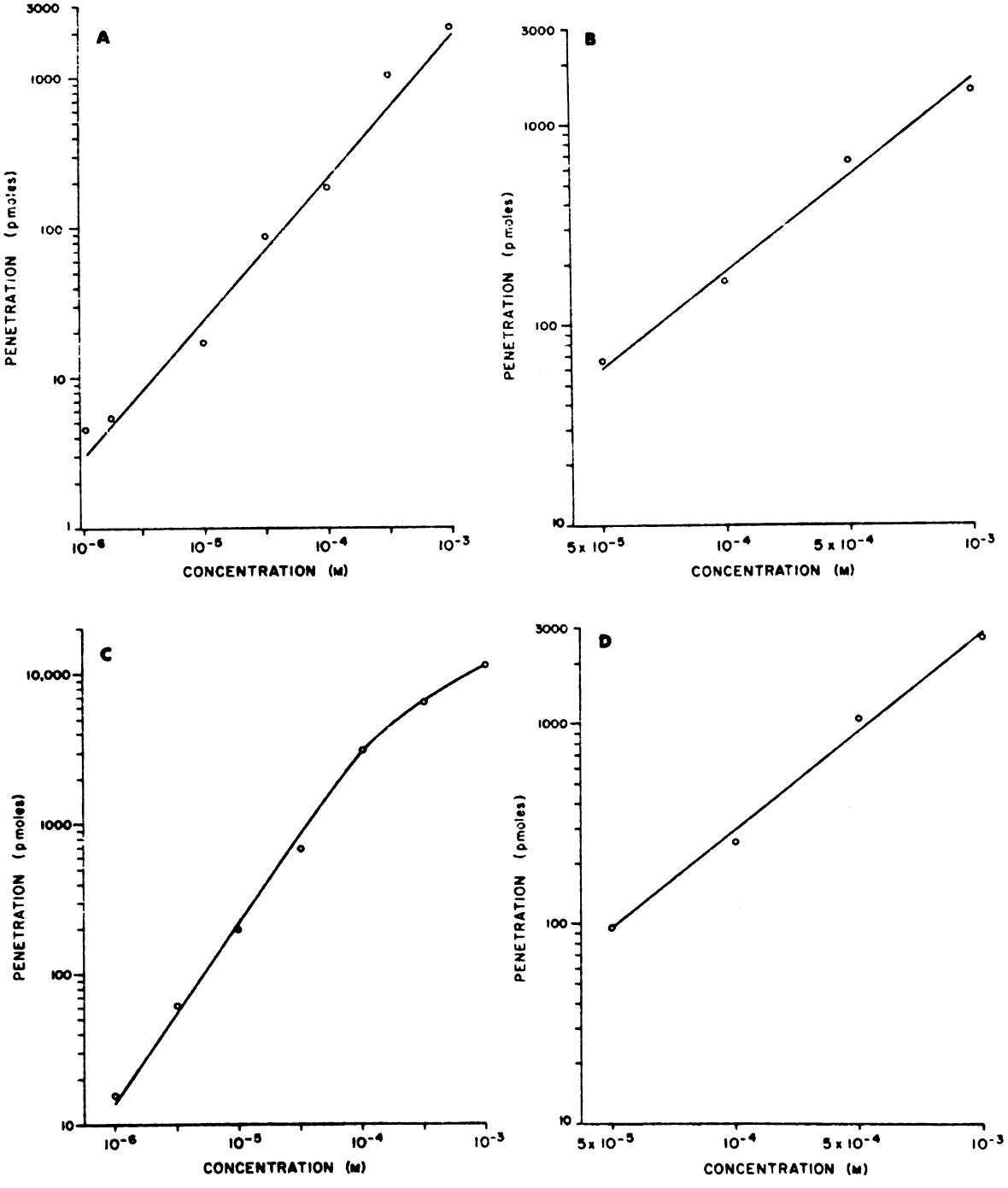

A
B
C
D
PENETRATION (pmoles)
CONCENTRATION (M)
3000
1000
100
10
1
10,000
10^{-6}
10^{-5}
10^{-4}
10^{-3}
5×10^{-5}
5×10^{-4}

Figure 7. -- Effect of temperature on penetration of NAA and NAAm through the upper and lower surfaces.

A. NAA upper surface
B. NAAm upper surface
C. NAA lower surface
D. NAAm lower surface

For comparison, NAA penetration equals NAAm penetration x 1.04.

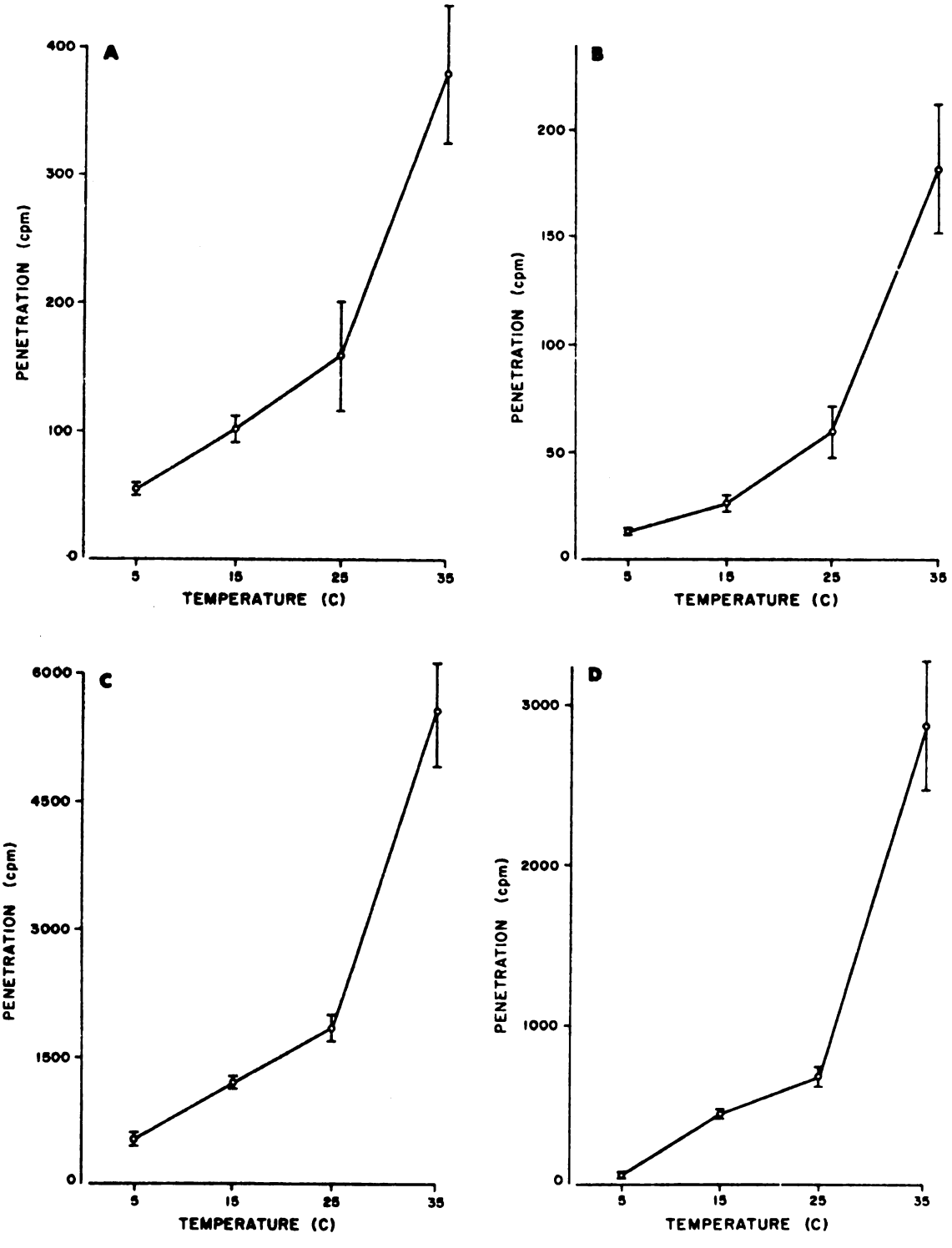
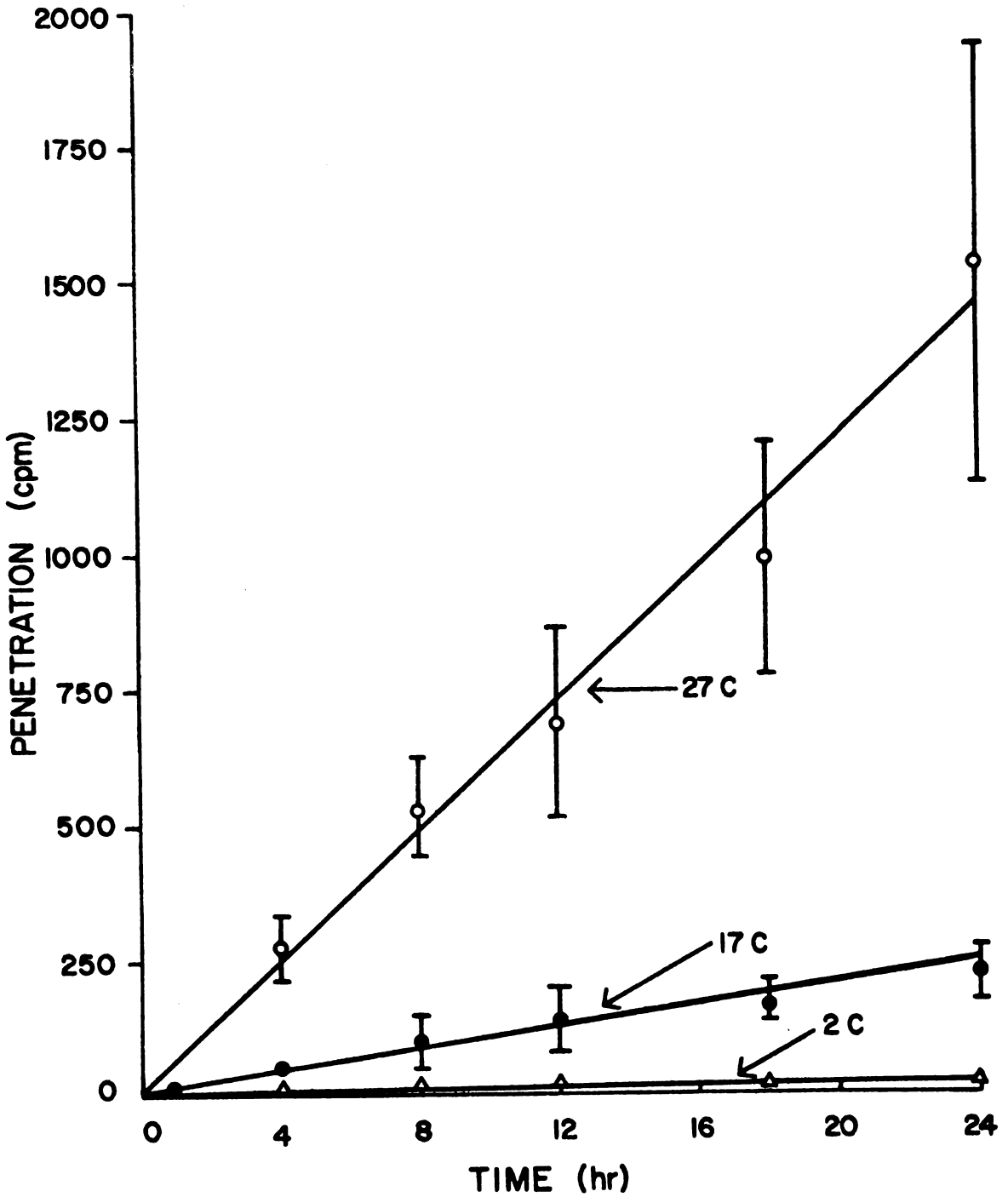

A
400
300
200
100
0
PENETRATION (cpm)
5
15
25
35
TEMPERATURE (C)
B
200
150
100
50
0
PENETRATION (cpm)
5
15
25
35
TEMPERATURE (C)
C
6000
4500
3000
1500
0
PENETRATION (cpm)
5
15
25
35
TEMPERATURE (C)
D
3000
2000
1000
0
PENETRATION (cpm)
5
15
25
35
TEMPERATURE (C)

Table 4. Temperature coefficients (Q_{10}) for penetration of NAA and NAAm into pear leaf disks through the upper and lower surfaces.

Temperature range	Temperature coefficients			
	NAA		NAAm	
	Upper	Lower	Upper	Lower
(C)				
5-15	1.87	2.35	1.86	5.46
15-25	1.62	1.51	2.23	1.59
25-35	2.95	3.03	3.14	4.43

Figure 8. -- Time-course of penetration of NAA through the upper surface of pear leaf disks at 2, 17, and 27 C.


2000
1750
1500
1250
1000
750
500
250
0
PENETRATION (cpm)
27 C
17 C
2 C
0
4
8
12
16
20
24
TIME (hr)

Effect of pH on Penetration

The pH of the treating solution greatly influenced NAA penetration through the upper surface (Figure 9). As the pH was raised from pH 3.0 to 6.0 there was a progressively lesser amount of NAA entering the leaf disk. The pK of NAA is 4.2. At this pH half of the molecules are dissociated, have a negative charge and are polar and half of the molecules are nondissociated, have no charge and are nonpolar. The more nonpolar a molecule, the more lipid soluble it is. In general, as the pH of the treating solution is lowered more NAA molecules occur in the nondissociated form, and there is a corresponding tendency for NAA to penetrate into the leaf.

The response of NAA penetration to an increase in treating solution pH through the lower surface was very similar (Figure 10). The most notable difference between the upper and lower surface was that NAA at pH 6.0, when almost completely dissociated, penetrated to a much greater extent into the lower surface as compared to the upper.

Effect of pH on Penetration of NAAm

Treating solution pH had no significant influence on penetration of NAAm into either the upper or lower surface (Table 5). Since the pK of NAAm is about 14.0, there would be no change in the charge on the NAAm molecule over the pH range studied (3.0 to 7.0). Significantly more NAAm penetrated through the lower surface than through the upper.

Effect of Light on Penetration

Light Intensity

Increasing light at low intensities resulted in a significant increase

Figure 9. — Effect of treating solution pH on penetration of NAA into pear leaf disks through the upper surface and on the dissociation of the NAA molecule.

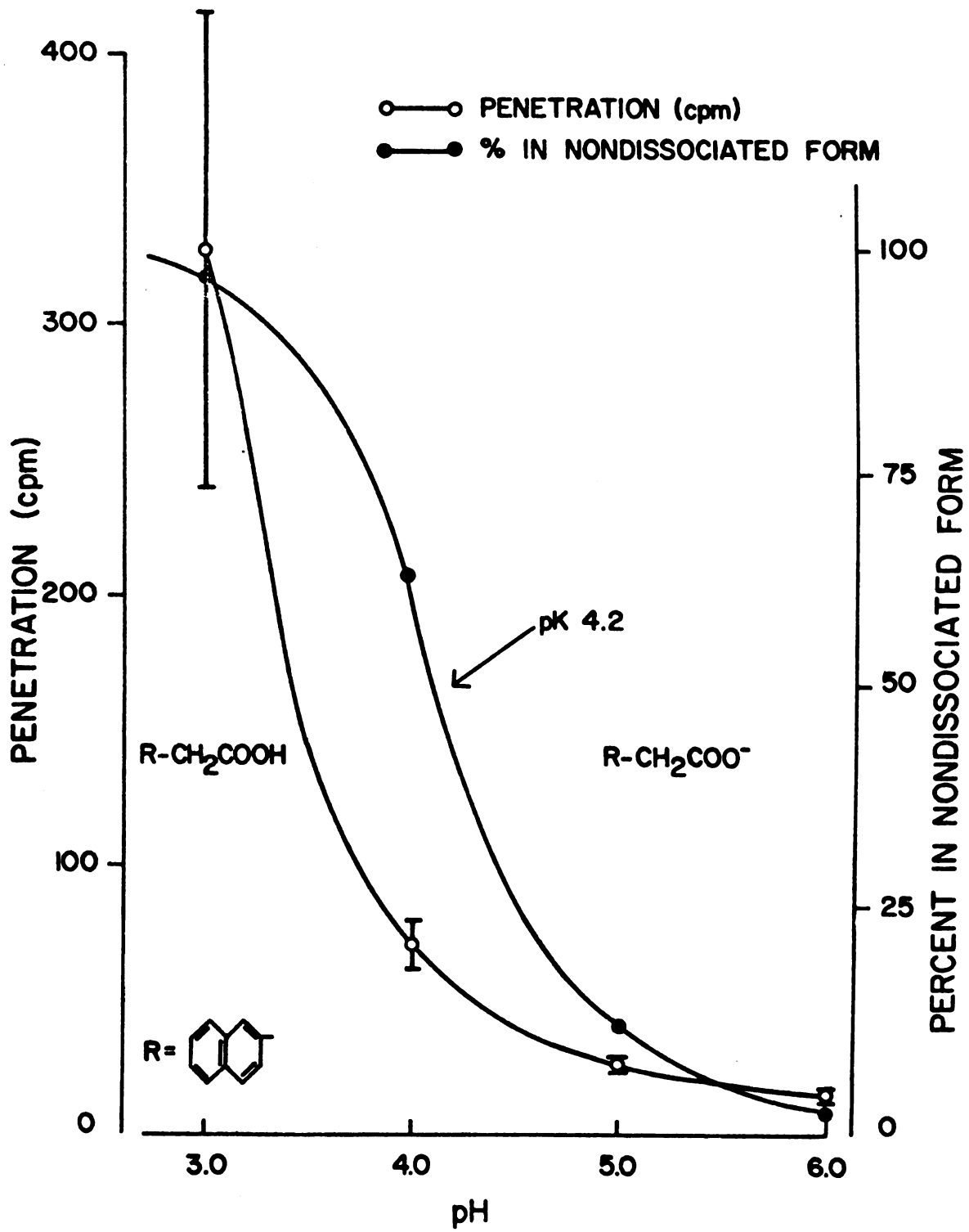

PENETRATION (cpm)
% IN NONDISSOCIATED FORM
400
300
200
100
0
PENETRATION (cpm)
100
75
50
25
0
PERCENT IN NONDISSOCIATED FORM
pK 4.2
R-CH2COOH
R-CH2COO-
R=
3.0
4.0
5.0
6.0
pH

Figure 10. -- The effect of treating solution pH on penetration of NAA into pear leaf disks through the lower surface.

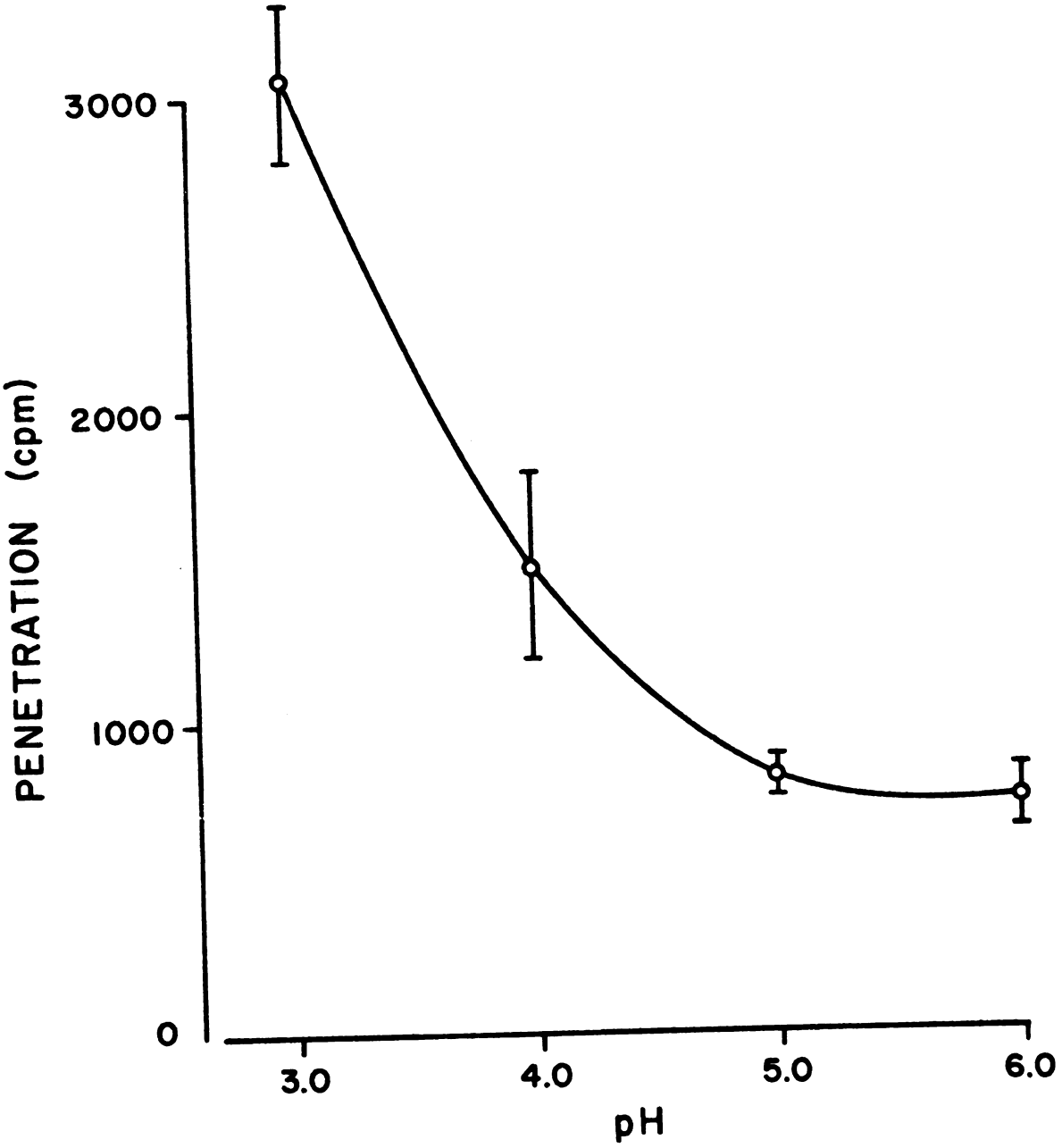

3000
2000
1000
0
PENETRATION (cpm)
3.0
4.0
5.0
6.0
pH

Table 5. The effect of pH on penetration of NAAm into pear leaf disks through the upper and lower surfaces.

pH	Penetration[1]	
	Upper	Lower
	(cpm)	
3	21a	2638 b
4	21a	3269 b
5	24a	3424 b
6	19a	3380 b
7	22a	3149 b

[1]Means within a column or within a row followed by a different letter are significantly different at P = 0.01.

in penetration of NAA (Figure 11) and NAAm (Figure 12) through the lower surface. As the light intensity was increased from 0 up to about 600 ft-c there was a 2.75-fold increase in penetration for NAA and a 4.8-fold increase for NAAm penetration through the lower surface. Above 600 ft-c little additional penetration occurred with a further increase in light intensity. The influence of increasing light intensity on penetration through the upper surface was not statistically significant for either NAA or NAAm.

Effect of Leaf Age, and Surface on NAA Penetration in Light and Dark

Leaf disks used in the light intensity studies were taken from field grown leaves. Since leaves grown in the field were found to be much less permeable than greenhouse grown leaves, an experiment was also performed using greenhouse grown leaves to determine if a light effect could be demonstrated for the upper surface. Two leaf ages with different permeability characteristics were used. With leaves of both ages, light increased penetration through the upper and lower surfaces (Table 6). Light had a much more profound effect on penetration through the lower surface. Although penetration into young leaves was twice as great as that in the older, the largest increase from the dark control was in the older leaves. The young leaves had not fully matured and were light green in color indicating incomplete chlorophyll development.

Effect of pH on Penetration in the Light and Dark

Greater penetration of NAA through the lower surface occurred in the light and from a pH 3.2 treating solution (Table 7). The most important factor to note is that pH of the treating solution or charge on the NAA molecule can influence the penetration response in light and dark. Light had a more profound effect on increasing penetration of NAA at pH 5.2 than at 3.2.

Figure 11. -- Effect of increasing light intensity on penetration of NAA into pear leaf disks through the upper and lower surfaces.

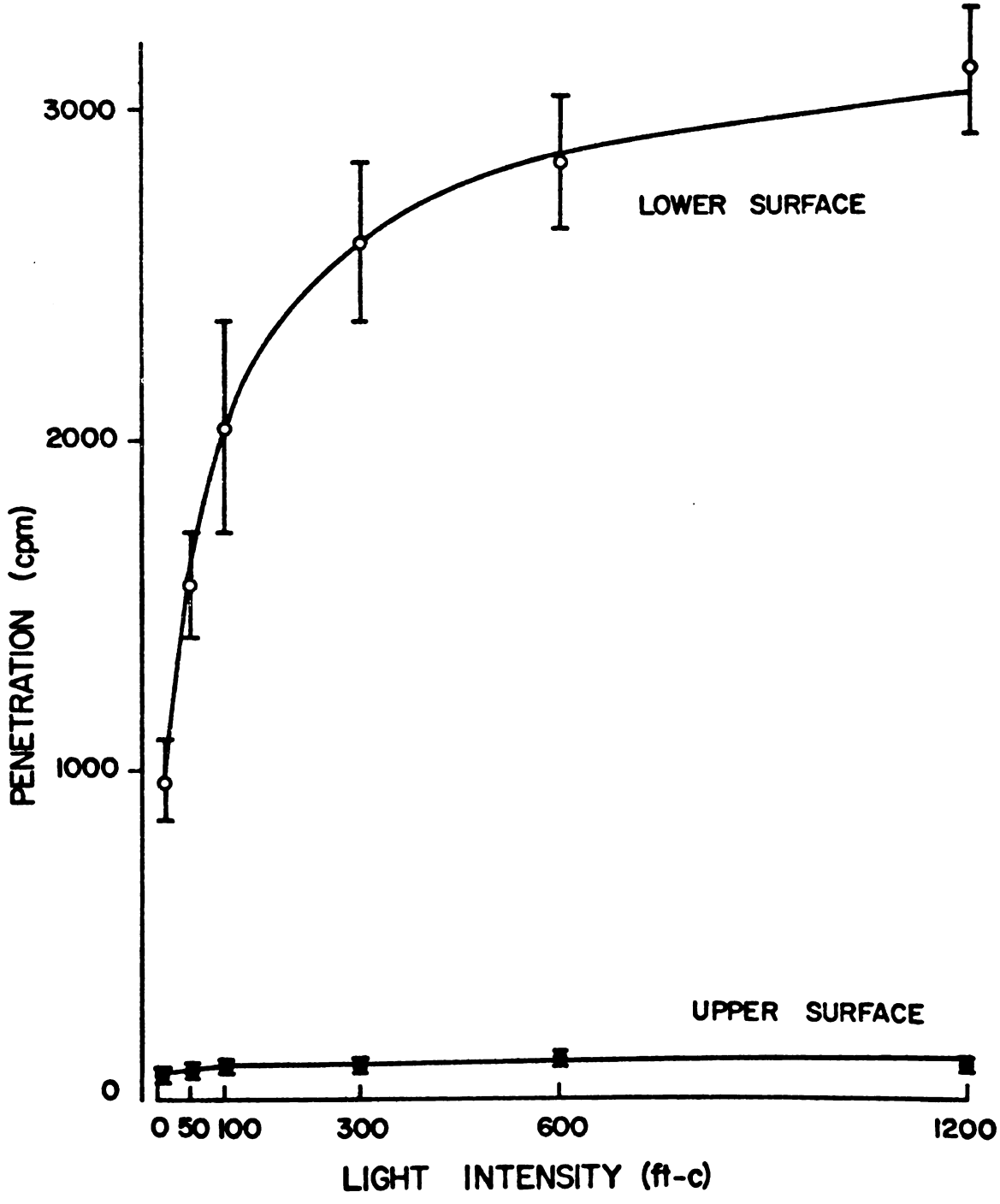

3000
2000
1000
0
PENETRATION (cpm)
LOWER SURFACE
UPPER SURFACE
0 50 100
300
600
1200
LIGHT INTENSITY (ft-c)

Figure 12. -- Effect of increasing light intensity on penetration of NAAm into pear leaf disks through the upper and lower surfaces.

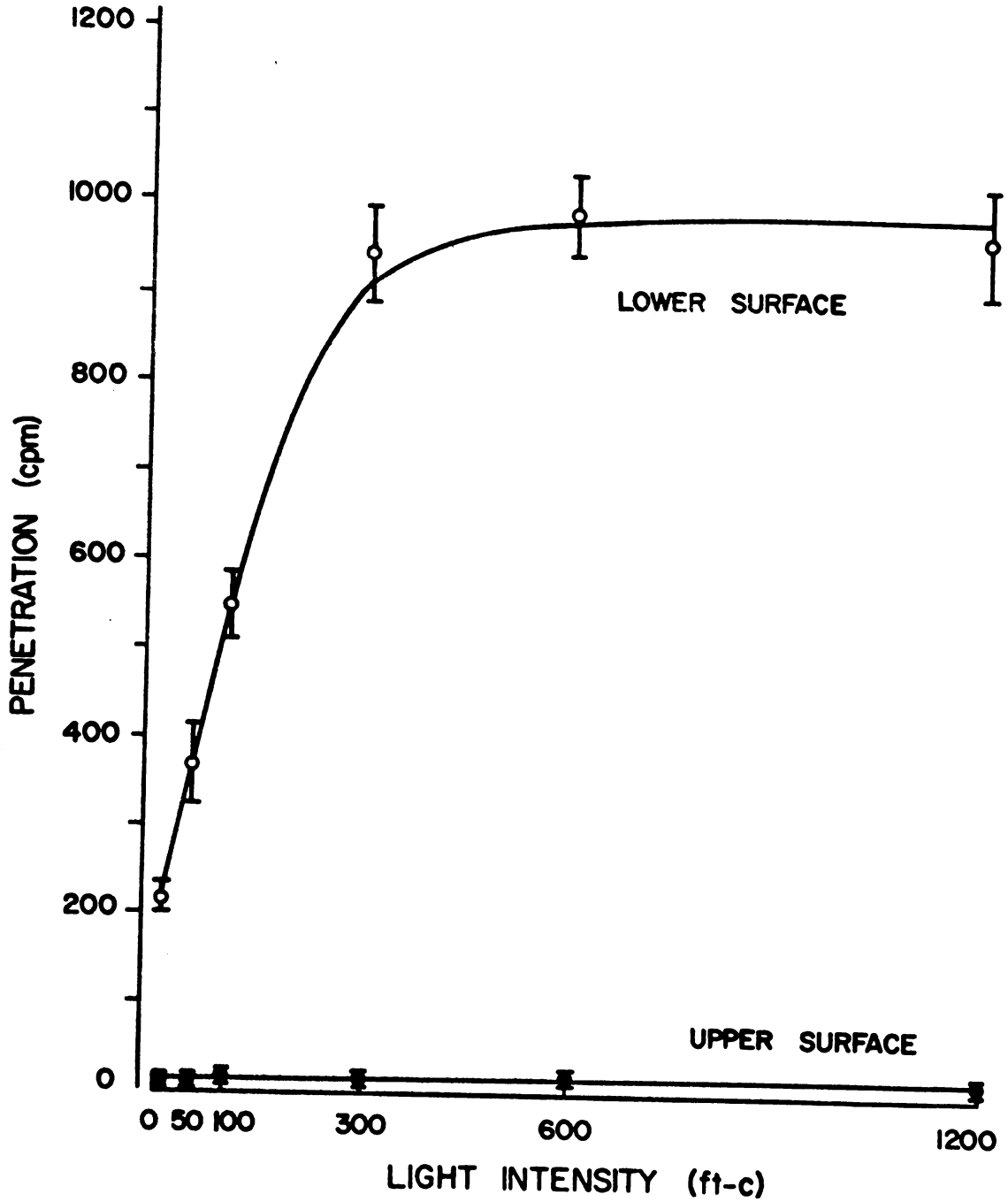

1200
1000
800
600
400
200
0
PENETRATION (cpm)
LOWER SURFACE
UPPER SURFACE
0 50 100 300 600 1200
LIGHT INTENSITY (ft-c)

Table 6. Effect of light on penetration of NAA through the upper and lower surfaces of disks taken from the first and seventh leaf of a terminal shoot.

Leaf surface	Leaf number	Penetration		Mean
		Light	Dark	
		(cpm)		
Upper	1st	1844	1134	1054**
Upper	7th	763	474	
Lower	1st	6507	2788	3523
Lower	7th	4161	636	
Mean		3319**	1258	---[2]

[1]The 1st leaf is from the apical portion of the shoot and the 7th from the basal portion.

[2]Means for the 1st and 7th leaf are significantly different at P = 0.01.

** Significant at P = 0.01.

Table 7. Effect of treating solution pH on penetration of NAA through the lower surface in light and dark.

pH	Penetration		Mean
	Light	Dark	
	(cpm)		
3.2	3471	506	1989**
5.2	912	77	495
Mean	2192**	292	---[1]

** Significant at P = 0.01.

[1] Interaction of light x pH significant at P = 0.01.

Effect of Cycling Light and Dark on Penetration

When the leaf disks were illuminated there was an almost immediate increase in penetration in response to light (Figure 13). Conversely, when the lights were turned off, the stimulating effect of light was rapidly lost. Regardless of the order in which light and dark were administered, penetration after 8 hr was the same. A similar experiment was carried out which showed disks were continually and quantitatively responsive to cycling of light and dark treatments (Figure 14).

Effect of Light and Stomatal Aperture Width on Penetration

An experiment was performed to evaluate the influence of stomatal aperture width on the response of leaf disks to penetration of NAA in the light (Figure 15). Penetration was followed for 3 hr in light. Acetate replica photomicrographs of the stomata at various stages in the experiment are shown in Figure 16. When present in sufficient amount, NAA prevented the opening of stomata following closure during a dark period. It was previously determined that a 3-hr treatment in light with NAA was sufficient to prevent the opening of stomata once closed. From Figure 15 it may be seen that penetration was actually greater into leaf disks during the second light treatment even though the stomata were essentially all closed.

Effect of Inhibitors on Penetration

Effect of Anaerobiosis on Penetration

The least noxious method of inhibiting metabolic processes in the leaf disks was considered to be by pretreatment in nitrogen and then conducting the experiment in nitrogen. The influence of a complete nitrogen atmosphere on penetration of NAA through the upper and lower

Figure 13. -- Time-course of NAA penetration into pear leaf disks through the lower surface as influenced by light and dark treatments.

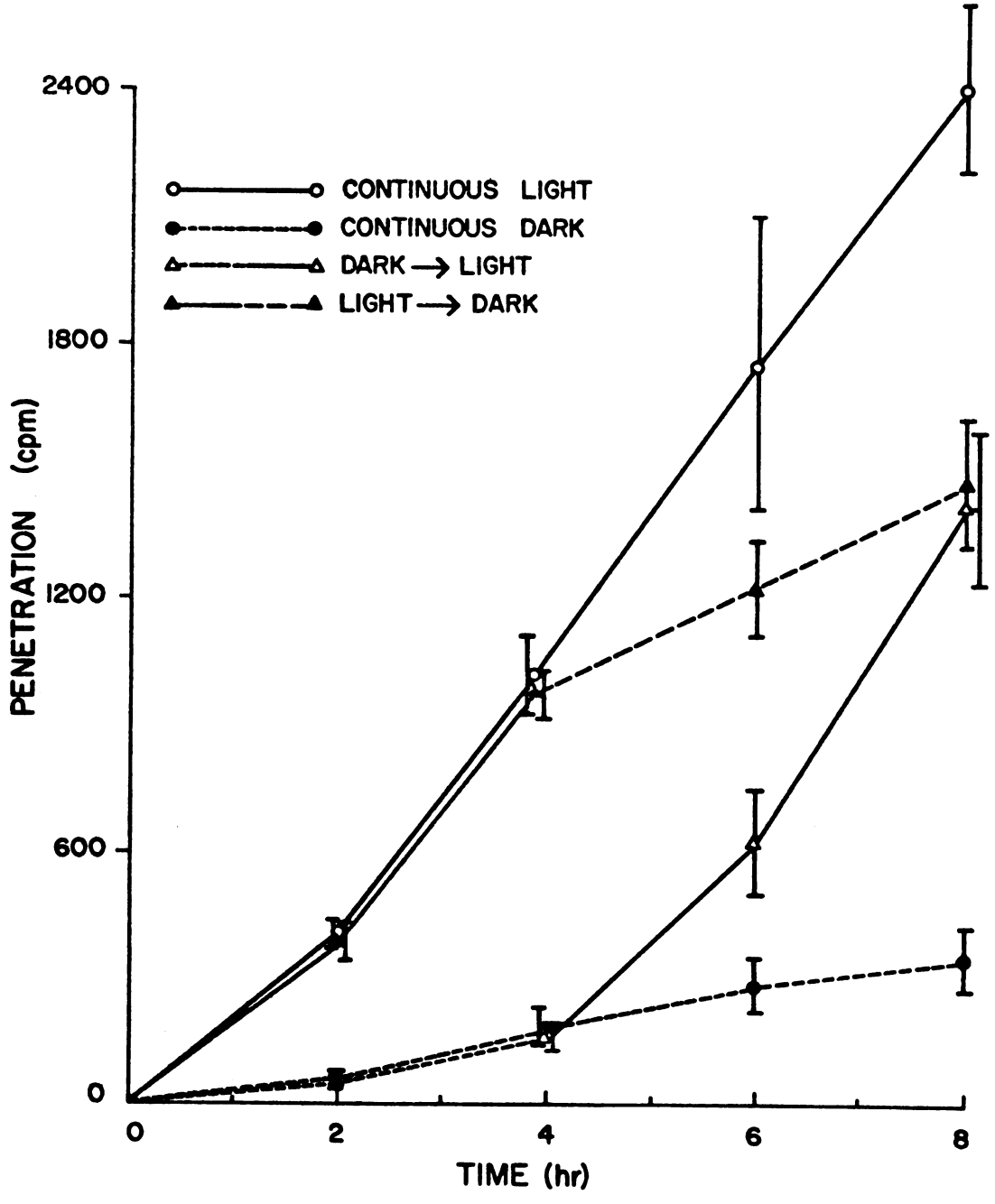

2400
1800
1200
600
0
PENETRATION (cpm)
CONTINUOUS LIGHT
CONTINUOUS DARK
DARK → LIGHT
LIGHT → DARK
0
2
4
6
8
TIME (hr)

Figure 14. — Time-course of penetration of NAA into pear leaf disks through the lower surface as influenced by cycling light and dark.

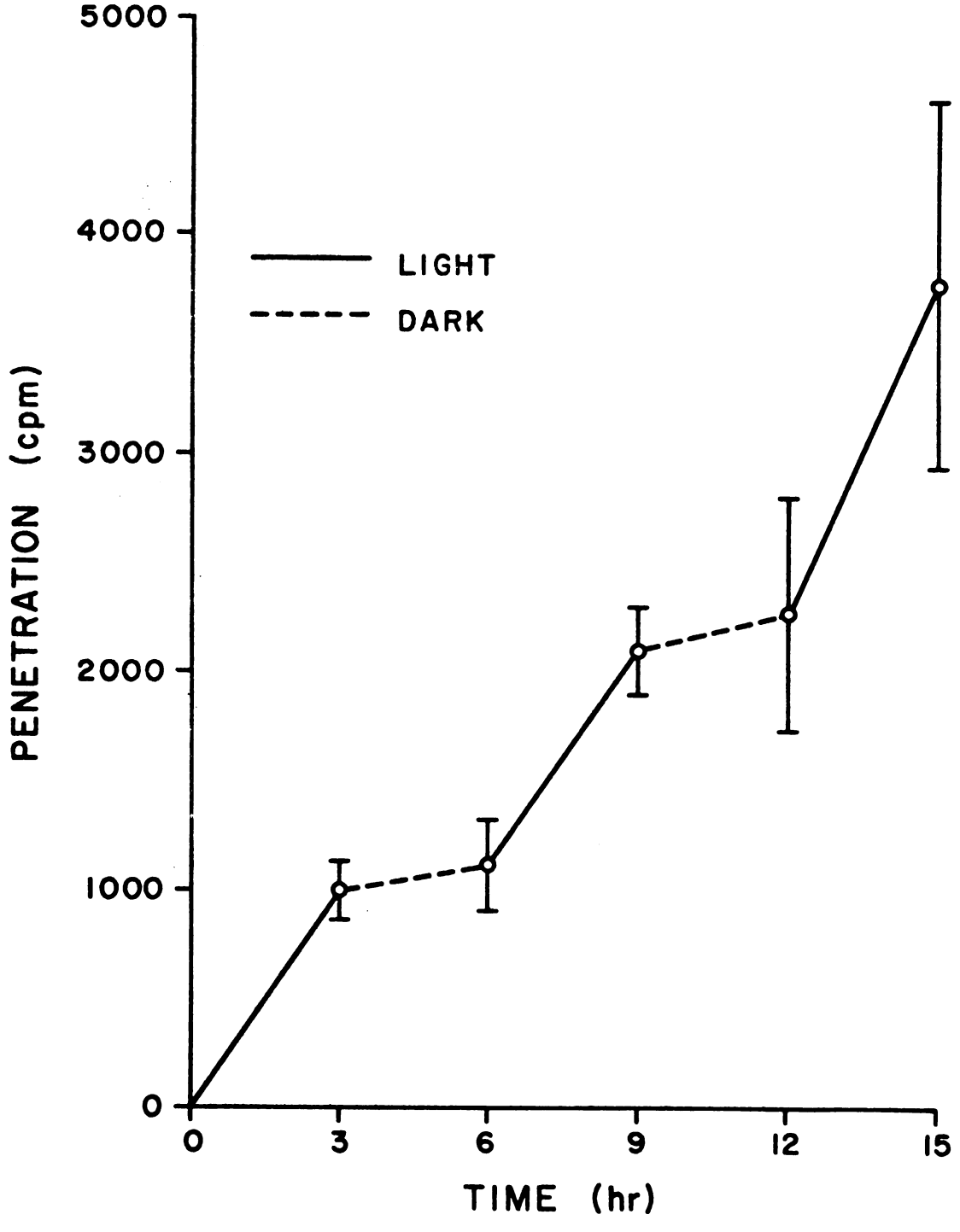

5000
4000
3000
2000
1000
0
PENETRATION (cpm)
LIGHT
DARK
0
3
6
9
12
15
TIME (hr)

Figure 15. -- Effect of light and stomatal aperture width on penetration of NAA into pear leaf disks through the lower surface.

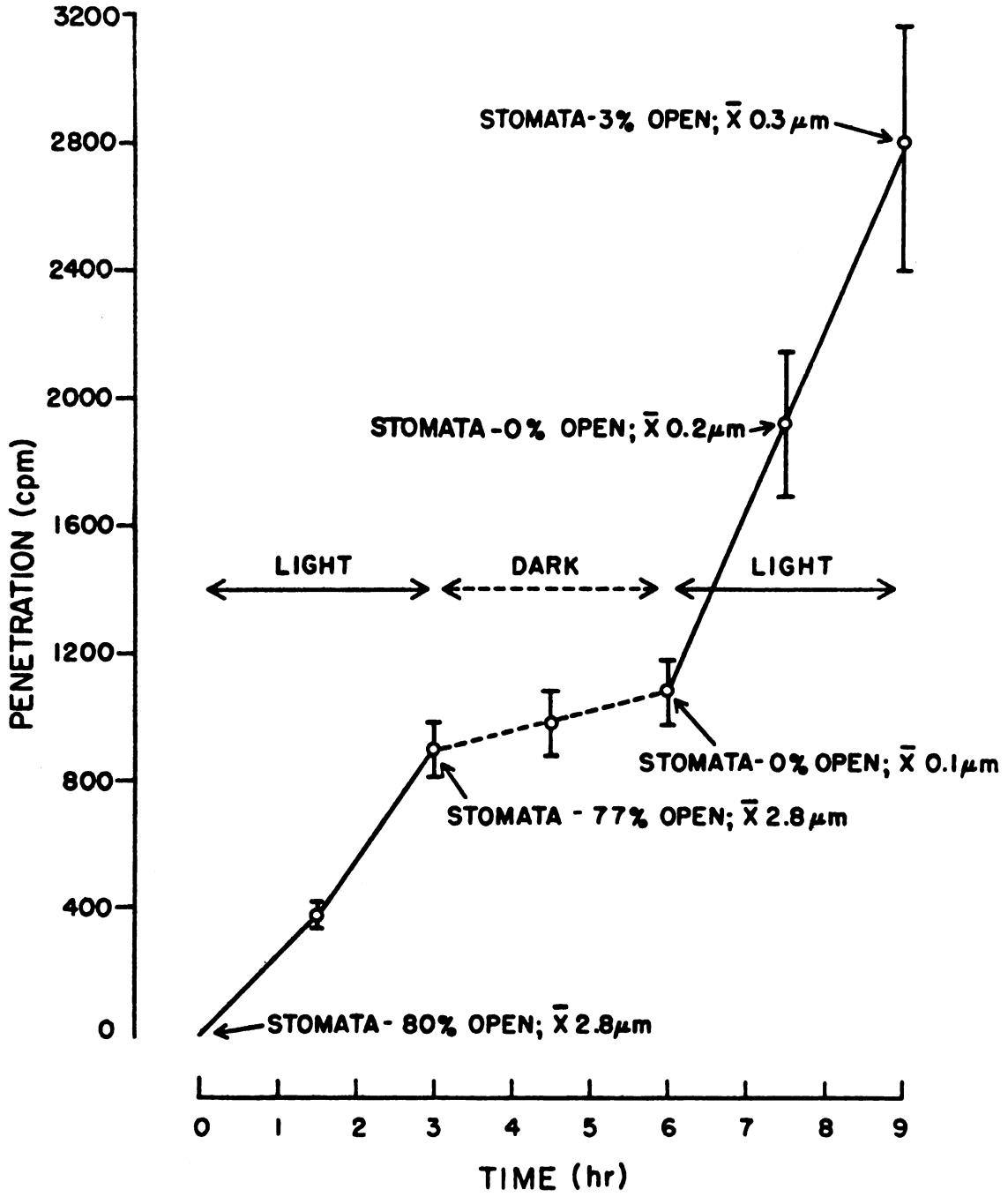
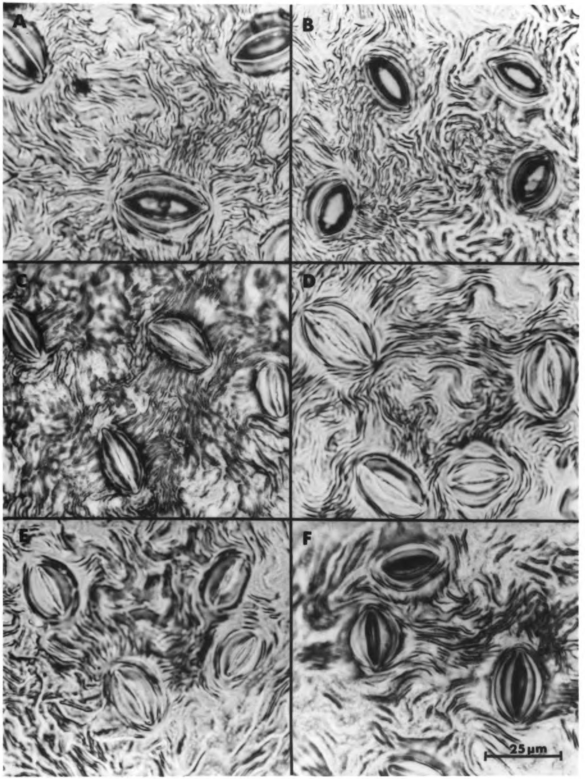

3200
2800
2400
2000
1600
1200
800
400
0
PENETRATION (cpm)
STOMATA-3% OPEN; X̄ 0.3 μm
STOMATA-0% OPEN; X̄ 0.2μm
LIGHT
DARK
LIGHT
STOMATA-0% OPEN; X̄ 0.1μm
STOMATA - 77% OPEN; X̄ 2.8μm
STOMATA - 80% OPEN; X̄ 2.8μm
0 1 2 3 4 5 6 7 8 9
TIME (hr)

Figure 16. — Photomicrographs of acetate replicas showing stomata at various periods during the course of NAA penetration with different light regimes.

A. After 1 1/2 hr light pretreatment
B. After 3 hr light treatment
C. After 3 hr light, 1 1/2 hr dark
D. After 3 hr light, 3 hr dark
E. After 3 hr light, 3 hr dark, 1 1/2 hr light
F. After 3 hr light, 3 hr dark, 3 hr light


A
B
C
D
E
F
25 µm

surfaces is shown in Table 8. Nitrogen was found to have no effect on decreasing penetration through the upper surface. In two instances there was an increase in penetration into leaf disks maintained in the nitrogen atmosphere. However, penetration of NAA through the lower surface was inhibited when leaf disks were kept in an atmosphere of nitrogen prior to and during the course of penetration. In both instances the difference in penetration between nitrogen and air control leaf disks were highly significant.

Effect of Inhibitors of the Hill Reaction on Penetration

The responsiveness of NAA penetration to light indicated that energy required for this process may be supplied directly by photosynthesis. Atrazine was the first inhibitor chosen because of its relatively high water solubility and since it was known to be taken up more readily by the leaves than other inhibitors. The influence of atrazine on penetration of NAA by the lower surface is shown in Table 9. In the dark there was no difference in penetration between control and atrazine treated plants. At a low light intensity (200 ft-c) penetration was stimulated. This stimulation was partially reversed (21%) by atrazine. At the highest light intensity (1000 ft-c), light stimulation was reversed to an even greater extent (42%).

The effect of two additional inhibitors of the Hill reaction, Terbicil and Monuron, are presented in Table 10. Terbicil inhibited NAA penetration by 16% and Monuron inhibited to an even greater extent, 33%.

Effect of Various Metabolic Inhibitors on NAA Penetration

Penetration of NAA was significantly inhibited by uncouplers of oxidative phosphorylation (Table 11 and 12): carboxylcyanide m-chloropheny-

Table 8. Penetration of NAA into pear leaf disks through the upper and lower surfaces in atmospheres of nitrogen and air.

Surface	Expt.	Penetration[1]	
		Nitrogen	Air
		(cpm)	
	I	554a	195 b
Upper	II	349a	341a
	III	357a	213 b
Lower	I	1017a	4327 b
	II	1512a	3799 b

[1] Means within an experiment followed by a different letter are significantly different at P = 0.01.

Table 9. The effect of light and an inhibitor of the Hill reaction (Atrazine)[1] on penetration of NAA into pear leaf disks through the lower surface.

Light intensity	Control	Atrazine	Inhibition
(ft-c)	(cpm)		(%)
0 (dark)	614	614	0
200	1139*	896	21.3
1000	3099**	1799	41.9

[1] 2-chloro-4-ethylamino-6-isopropyl-amino-s-triaxine (1×10^{-5} M)
* Significant at P = 0.05
** Significant at P = 0.01

Table 10. Effect of Hill reaction inhibitors on penetration of NAA into pear leaf disks through the lower surface and illuminated with 1000 ft-c of light.

Treatment	Penetration	Inhibition
	(cpm)	(%)
Control	6491	
Terbicil[1]	5455	16.0*
Monuron[2]	4346	33.0**

[1] 3-tert-butyl-5-chloro-6-methyluracil (1×10^{-4} M).
[2] 3-(p-chlorophenyl)-1, dimethylurea (1×10^{-4} M).
* Significant at P = 0.05.
** Significant at P = 0.01.

Table 11. The effect of inhibitors on penetration of NAA into pear leaf disks through the lower surface.

Treatment	Penetration[3]	Inhibition
	(cpm)	(%)
Control	4527a	
m-Cl-CCP[1]	3585 b	20.8
p-F-CCP[2]	2428 b	47.4

[1] Carbonylcyanide m-chlorophenylhydrazone (1 x 10^{-5} M).
[2] Carbonylcyanide p-trifluoromethoxyphenylhydrazone (1 x 10^{-5} M).
[3] Means within a column followed by a different letter are significantly different at P = 0.05.

Table 12. The effect of inhibitors on penetration of NAA into pear leaf disks through the lower surface.

Treatment	Penetration[1]	Inhibition
	(cpm)	(%)
Control	5267a	
DNP[2]	1219 b	76.9
PMA[3]	3422 c	35.0
NaN_3[4]	5230a	0.7

[1] Means within a column followed by a different letter are significantly different at P = 0.01.
[2] 2,4 dinitrophenol (1 x 10^{-3} M).
[3] Phenylmercuric acetate (1 x 10^{-4} M).
[4] Sodium azide (1 x 10^{-3} M).

hydrazone (20.8%), Carboxylcyanide p-trifluoromethoxyphenylhydrazone (47.4%), and 2,4 dinitrophenol (76.9%).

Phenylmercuric acetate reduced NAA penetration by 35.0% while sodium azide was found to have no effect (Table 12).

Effect of Surfactants on Penetration

Tween 20 and Tergitol 15-S-9 at 0.001, 0.01 and 0.1% concentrations were found to be ineffective in increasing NAA penetration through the upper surface (Table 13 and 14). However, penetration through the lower surface was significantly increased when treating solutions containing 0.01 or 0.1% surfactant were used.

Tween 20, Triton B-1956, and X-77, differing in ability to reduce surface tension, were evaluated on penetration of NAAm through the upper and lower furfaces. A 0.1% concentration was chosen because the critical micelle concentration for all three surfactants occurred at or slightly below 1.01%. No surfactant significantly increased NAAm penetration through the upper surface (Table 15. Tween 20 and Triton B-1956 were effective in increasing penetration through the lower surface. However, the greatest increase in penetration through the lower surface occurred with X-77.

It would appear that surfactant stimulation of NAA and NAAm penetration through the lower surface did not occur to the same extent. Tween 20 (0.01%), the surfactant used for both compounds, increased NAAm penetration to a much greater extent than it did for NAA. This may be seen by comparing data reported in Table 13 and Table 15. Although no other surface active agents were used common to both, surfactants tended to increase penetration of NAAm by the lower surface more than of NAA.

Table 13. Effect of Tween 20 concentration on penetration of NAA into pear leaf disks through the upper and lower surfaces.

Concentration	Penetration[1]	
	Upper	Lower
(%)	(cpm)	
0	1323a	2408a
0.001	937a	3129a
0.01	756a	4570 b
0.1	1038a	4375 b

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

Table 14. Effect of Tergitol 15-S-9 concentration on penetration of NAA into pear leaf disks through the upper and lower surfaces.

Concentration	Penetration[1]	
	Upper	Lower
(%)	(cpm)	
0	530a	4316a
0.001	421a	4467a
0.01	678a	7021 b
0.1	722a	8067 b

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

Table 15. Effect of surfactants (0.1%) on penetration NAAm into pear leaf disks through the upper and lower surfaces.

Treatment	Penetration[1]	
	Upper	Lower
	(cpm)	
Control	263a	329a
Tween 20	220a	1941 b
Triton B-1956	297a	1917 b
X-77	393a	2833 c

[1]Means within a column followed by a different letter are significantly different at P = 0.01.

Stomatal Penetration

Effect of Increasing Light Intensity on Penetration and Opening of Stomata

There was a close relationship between penetration and stomatal opening (aperture greater than 2μm), as light intensity was increased (Figure 17). From this, it would appear that the stomata, in some way, may be involved in bringing about the light stimulated NAA penetration.

Frequency Distribution of Stomatal Aperture Width in the Light

It was mentioned previously that stomatal aperture widths are influenced by a variety of environmental factors including quality and duration of light, temperature, CO_2 conc., water stress, etc. After measuring a large number of stomatal aperture widths it was observed that there was also a wide variation in the width of stomatal pores, even within a particular leaf disk. Results of an experiment show aperture widths in leaf disks varied between 0 to > 8 μm (Figure 18). The largest number of stomata had aperture widths in the range of 2 to 5 μm and some stomata were closed or had aperture width in excess of 6 μm. Therefore, it is difficult to accurately specify stomatal aperture width even within a given leaf disk.

Effect of Surfactants on Surface Tension

The reduction in surface tension of a treating solution is necessary if penetration through a stomatal pore is to take place. The influence of surfactant concentrations on surface tension is presented in Figure 19. As the surfactant concentration was increased the surface tension of the solution decreased. Surfactants differed in their ability to reduce surface tension. Tween 20 was least effective, Vatsol OT the

Figure 17. -- Comparative effects on increasing light intensity on penetration of NAA into pear leaf disks through the lower surface and the per cent of stomata open.

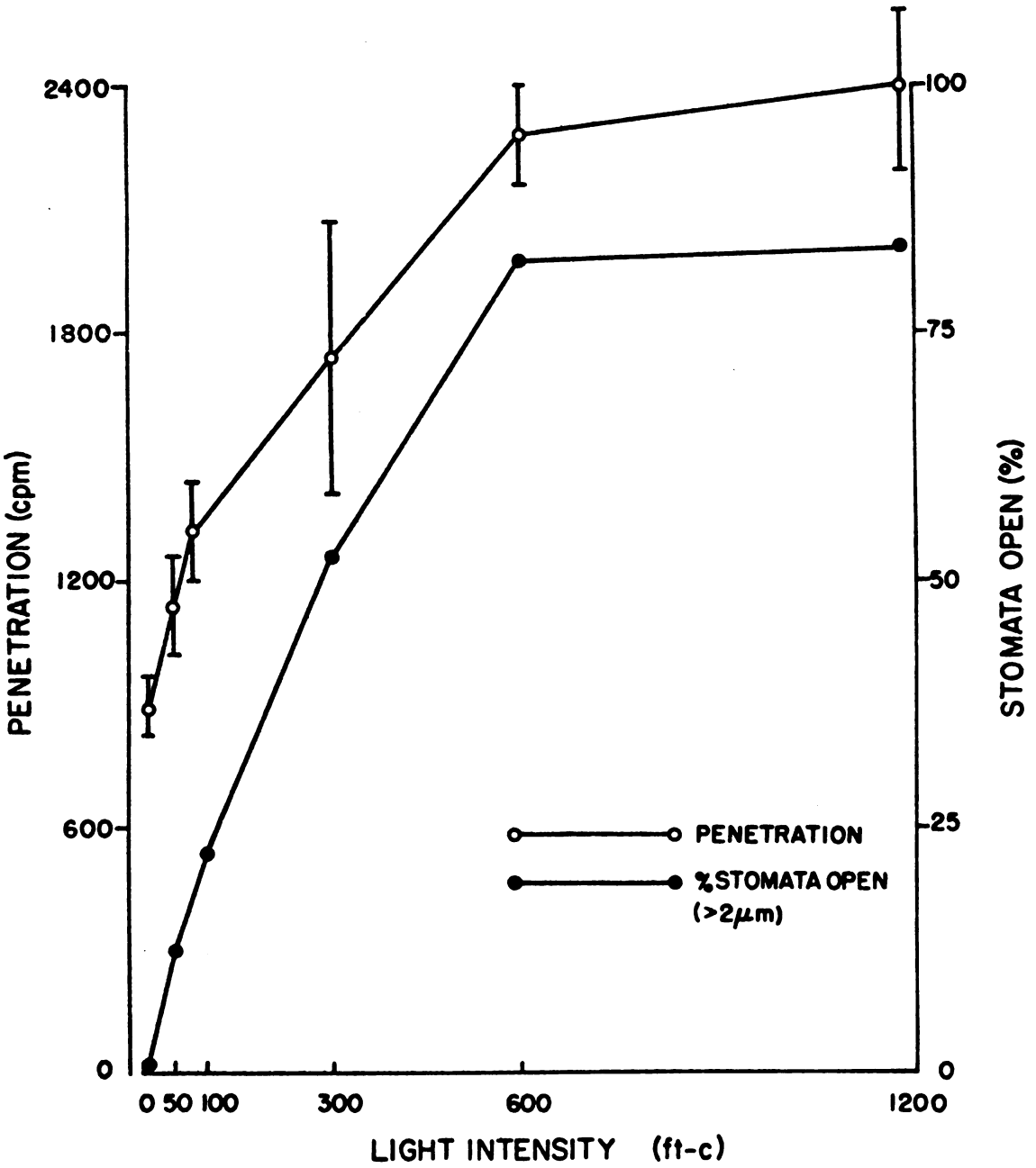

2400
1800
1200
600
0
PENETRATION (cpm)
100
75
50
25
0
STOMATA OPEN (%)
0 50 100
300
600
1200
LIGHT INTENSITY (ft-c)
PENETRATION
%STOMATA OPEN
(>2μm)

Figure 18. -- Frequency distribution showing per cent stomata with a particular stomatal aperture width falling within a given size class.

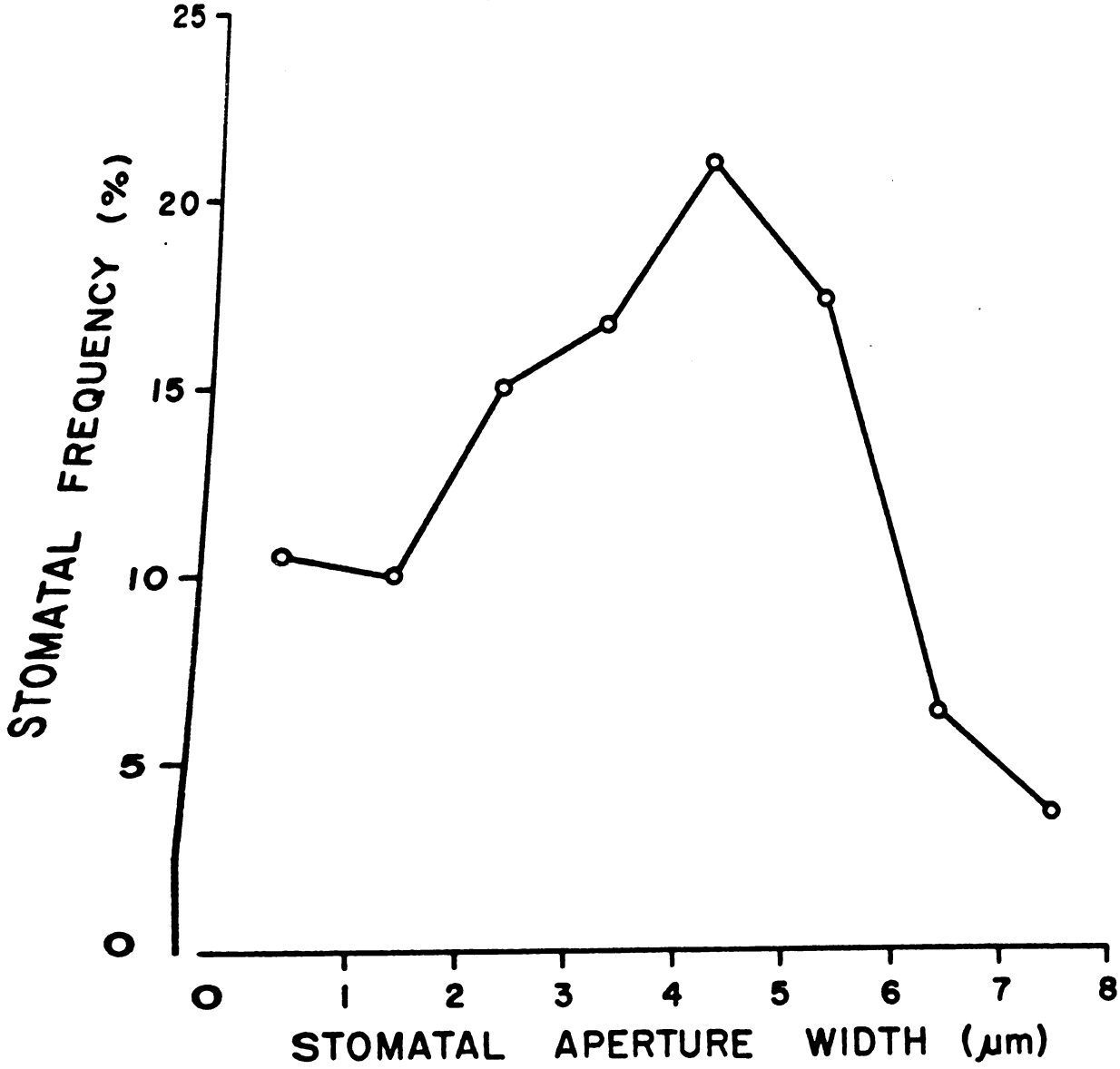
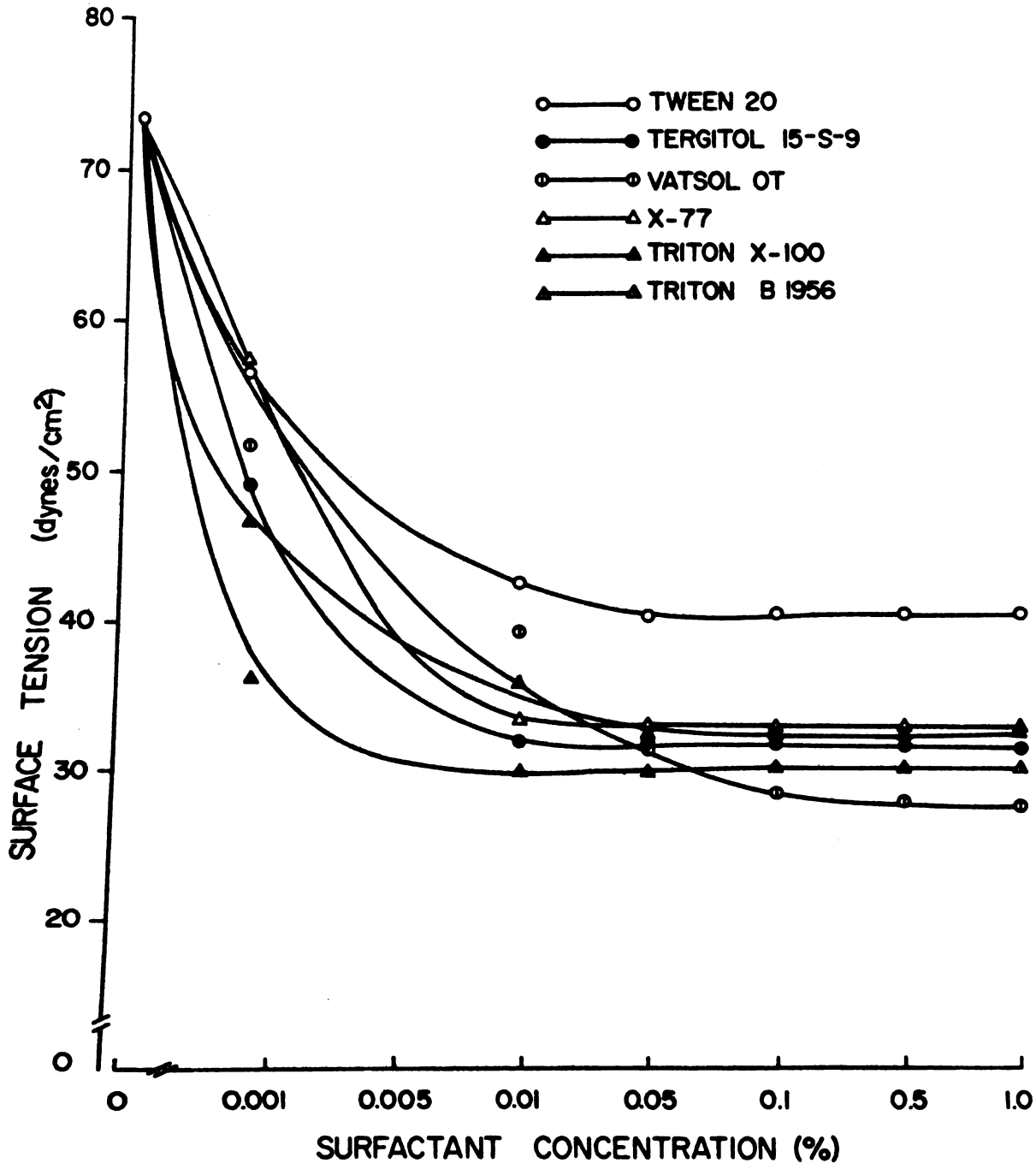

25
20
15
10
5
0
STOMATAL FREQUENCY (%)
0
1
2
3
4
5
6
7
8
STOMATAL APERTURE WIDTH (μm)

Figure 19. -- Effect of varying concentrations of selected surfactants on surface tension of buffered NAA solutions.


TWEEN 20
TERGITOL 15-S-9
VATSOL OT
X-77
TRITON X-100
TRITON B 1956
SURFACE TENSION (dynes/cm²)
80
70
60
50
40
30
20
0
0
0.001
0.005
0.01
0.05
0.1
0.5
1.0
SURFACTANT CONCENTRATION (%)

most effective and X-77, Triton B-1956, Triton X-100 and Tergitol 15-5-9 were intermediate in reducing surface tension at the higher surfactant concentrations. Triton B-1956 was most effective in bringing about a reduction in surface tension at the lowest concentration (0.001%). The critical micelle concentration (cmc) was defined earlier as the concentration at which additional surfactant would no longer reduce the surface tension. The cmc for all surfactants studied was between 0.01 and 0.1%.

Effect of Tween 20 on Penetration with Stomata Either Open or Closed

A time-course study showing the effect of Tween 20 on penetration of NAA through the lower surface with stomata either open or closed is presented in Figure 20. Surfactant-containing solution (0.1%) was added initially to one group of leaf disks and penetration was observed to be linear. The sequence of light-dark-light treatments for the second group was given to provide leaf disks having stomata closed and to provide a similar light treatment. After Tween 20 was added, the penetration response with leaf disks having stomata closed and in light was linear and quantitatively similar to leaf disks with the stomata open.

Effect of Vatsol OT on Penetration

Surface tension data presented in Figure 19 showed that Vatsol OT was the most effective surfactant at 0.1% in reducing surface tension. The addition of Vatsol OT to the treating solution at 3 hr after initiation of the experiment caused a sharp increase in NAA penetration (Figure 21). This increase was greater than that caused by the addition of Tween 20 (Figure 20).

A number of small water soaked areas appeared over the surface of the leaf disk within 1 min after the addition of the Vatsol OT. The water-soaked

Figure 20. -- Effect of Tween 20 on penetration of NAA into pear leaf disks through the lower surface with stomata open and closed. Stomata were opened initially by pretreatment with 1 1/2 hr of light.

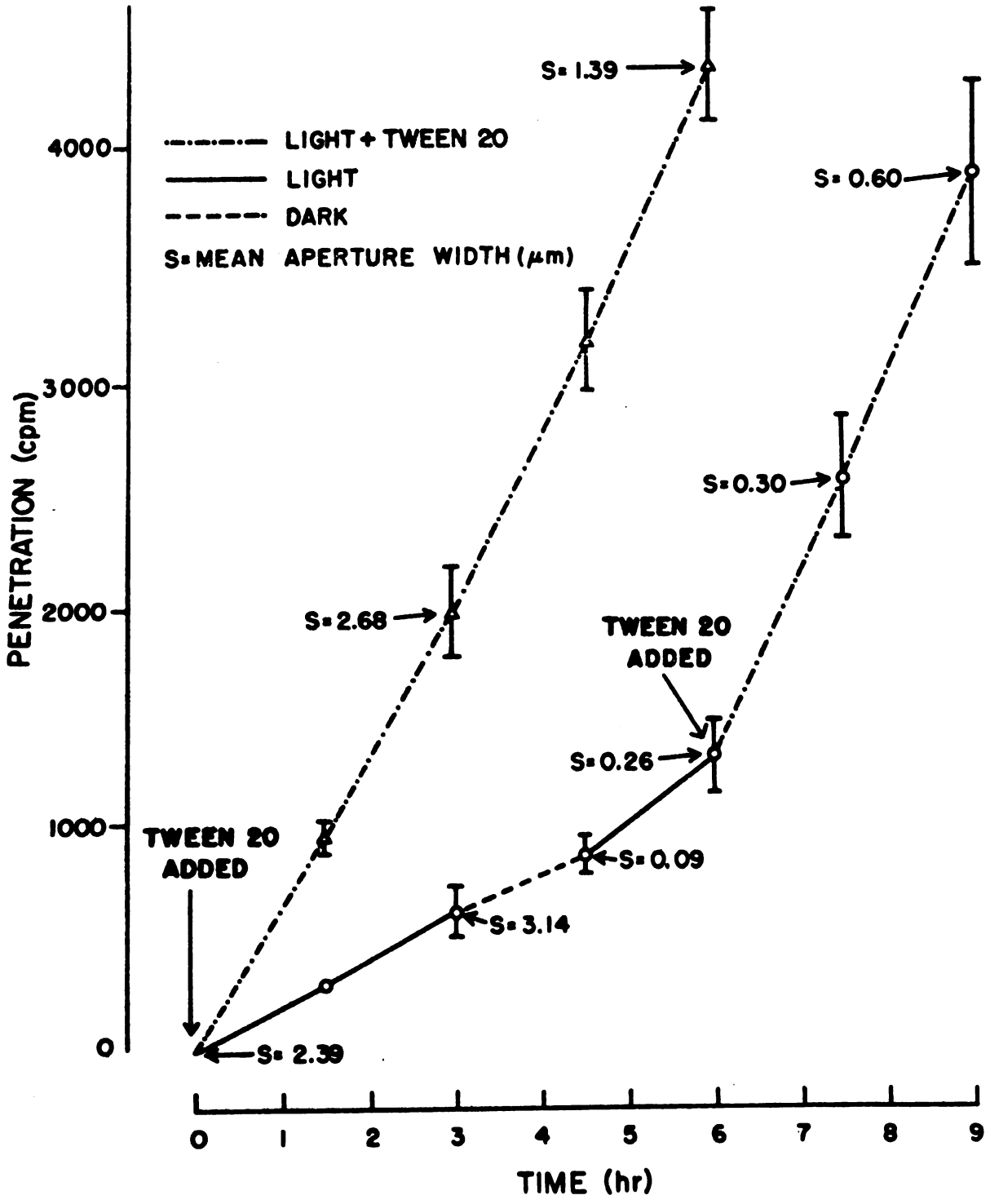
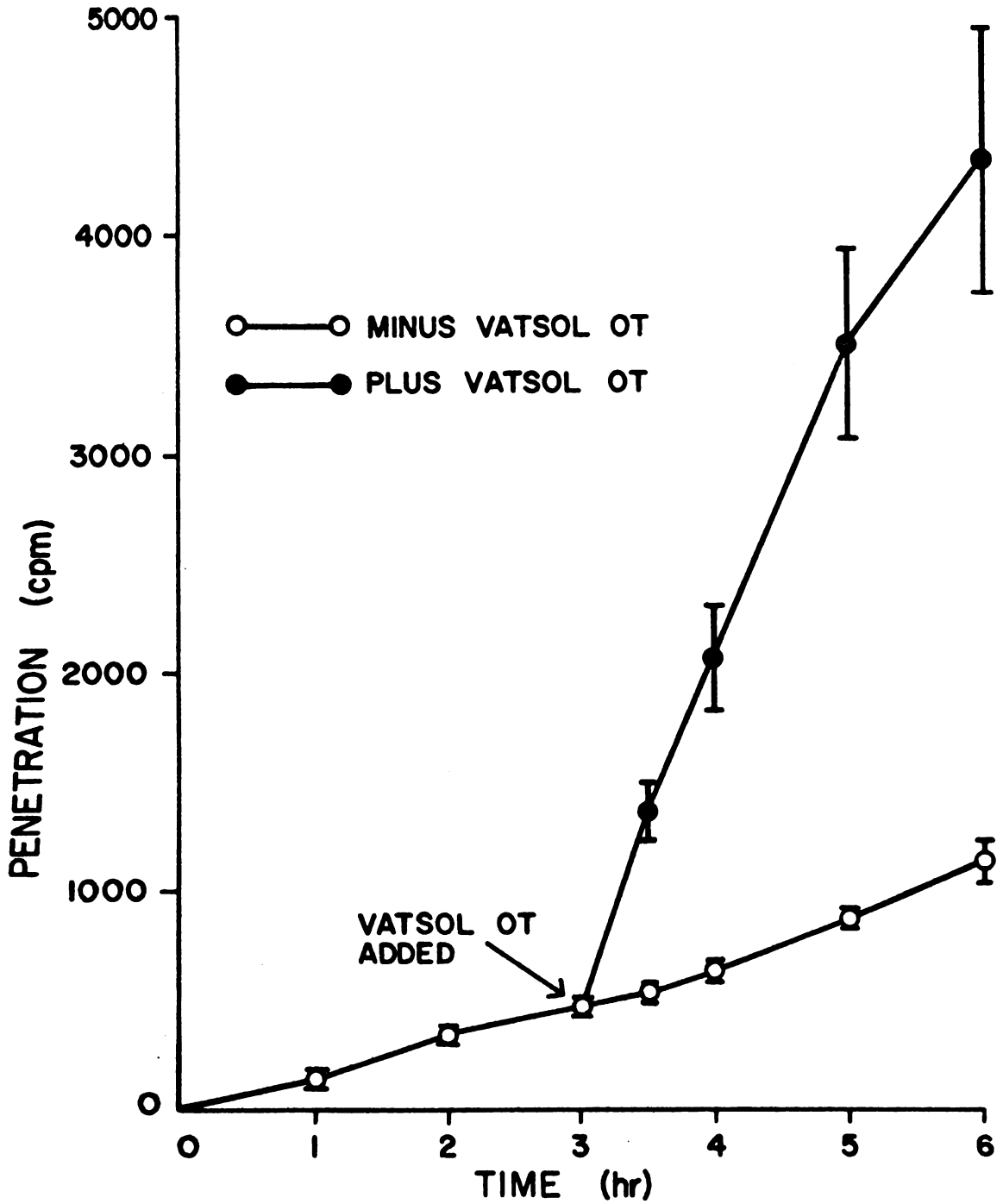

LIGHT + TWEEN 20
LIGHT
DARK
S = MEAN APERTURE WIDTH (μm)
PENETRATION (cpm)
4000
3000
2000
1000
0
S = 1.39
S = 0.60
S = 0.30
S = 2.68
TWEEN 20 ADDED
S = 0.26
TWEEN 20 ADDED
S = 0.09
S = 3.14
S = 2.39
0
1
2
3
4
5
6
7
8
9
TIME (hr)

Figure. 21. -- Effect of Vatsol OT on penetration of NAA into pear leaf disks through the lower surface after 3 hr exposure to the light.


5000
4000
3000
2000
1000
0
PENETRATION (cpm)
MINUS VATSOL OT
PLUS VATSOL OT
VATSOL OT
ADDED
0
1
2
3
4
5
6
TIME (hr)

areas varied in number from leaf disk to leaf disk but generally between 20 and 60 appeared. This would suggest that Vatsol OT may have decreased the surface tension to the point where stomatal entry occurred.

Effect of Surfactants on Penetration of pH 6.0 Treating Solution

To differentiate between the effect of surfactants on enhanced cuticular penetration and stomatal entry, penetration of NAA from solutions buffered at pH 6.0 was followed subsequent to the addition of surfactants (Figure 22). At pH 6.0 about 98% of the NAA is in the dissociated form and thus has a negative charge. Cuticular penetration of NAA buffered at pH 6.0 is very slow but stomatal entry should not be influenced. There was a rapid increase in penetration immediately following addition of all three surfactants. There was a significantly larger increase in penetration after 45 min with Vatsol OT or X-77 compared to Tween 20. Vatsol OT and X-77 were also found to reduce surface tension to the greatest extent (Figure 19). The Tukey w value shown on the graph is the difference required for significance at the 5% level after 45 min.

Effect of CO_2 Treatment on Penetration

High CO_2 concentrations will induce closure or prevent the opening of stomata. Results showing the influence of Tween 20, X-77, and Vatsol OT on penetration of NAA for 10 min into leaf disks maintained in atmospheres of high or low CO_2 levels are shown in Tables 16, 17, and 18, respectively. In addition, two different NAA concentrations but with the same radioactivity were used for each CO_2 treatment. Microscopic examination of leaf disks confirmed that CO_2 completely closed stomata, with all three surfactants. Penetration was significantly greater in leaf disks kept in the low CO_2 atmosphere and having open stomata. It may be further noted that there was

Figure 22. -- Effect of Vatsol OT, X-77, and Tween 20 on penetration of pH 6.0 NAA solution into pear leaf disks through the lower surface after 3 hr pretreatment in light.

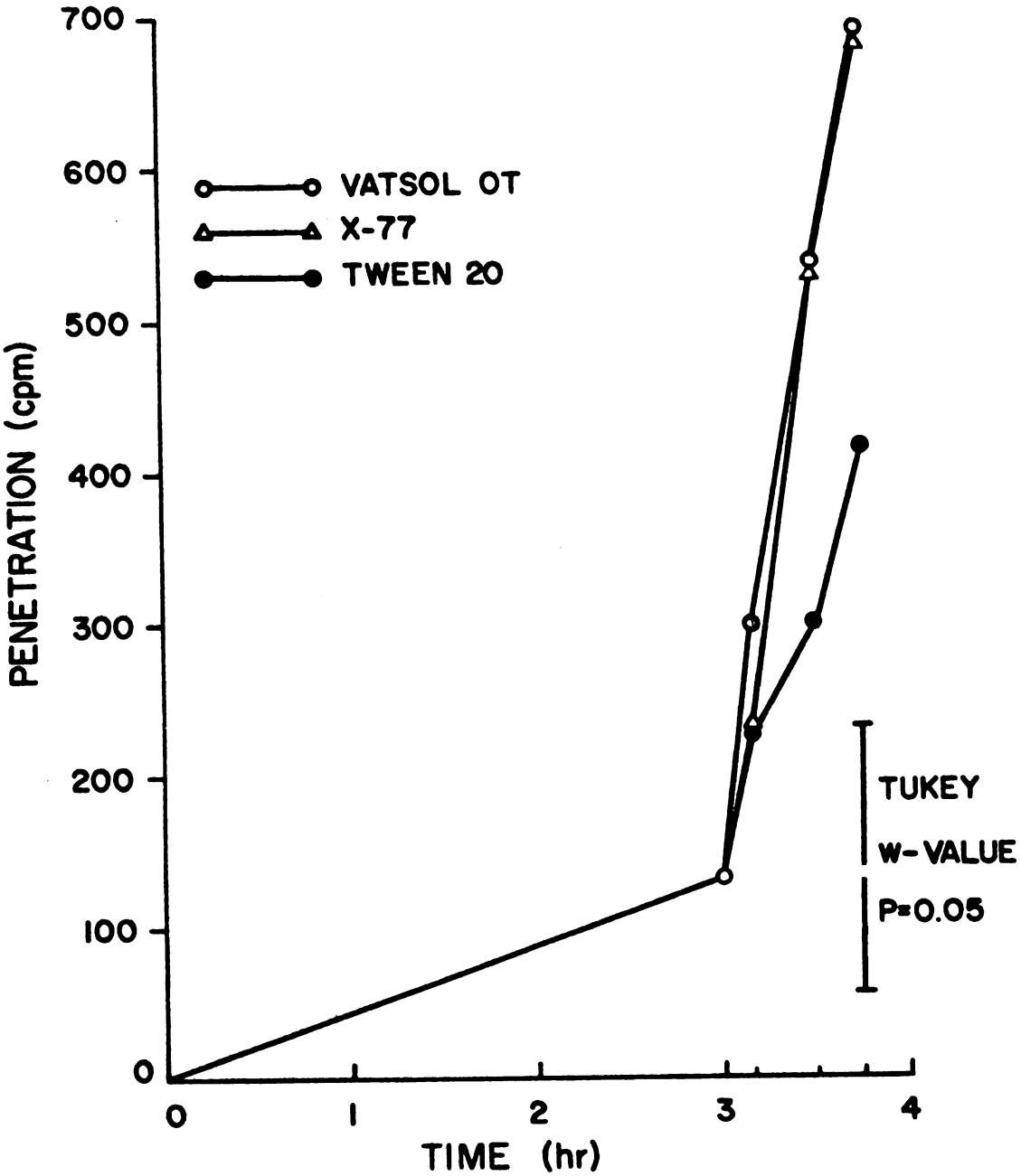

700
600
500
400
300
200
100
0
PENETRATION (cpm)
VATSOL OT
X-77
TWEEN 20
TUKEY
W-VALUE
P=0.05
0
1
2
3
4
TIME (hr)

Table 16. Effect of Tween 20 surfactant on penetration of NAA into pear leaf disks through the lower surface with stomata open ($-CO_2$) and closed ($+CO_2$).

Treatment	Penetration		Mean
	6×10^{-5}	1×10^{-3}	
	(cpm)		
$-CO_2$	116	92	104**
$+CO_2$	62	59	61
Mean	89	76	

**Significant at P = 0.01.

Table 17. Effect of X-77 surfactant on penetration of NAA into pear leaf disks through the lower surface with stomata open ($-CO_2$) and closed ($+CO_2$).

Treatment	Penetration		Mean
	6×10^{-5}	1×10^{-3}	
	(cpm)		
$-CO_2$	230	241	236**
$+CO_2$	98	62	80
Mean	164	152	

**Significant at P = 0.01.

Table 18. Effect of Vatsol OT surfactant on penetration of NAA into pear leaf disks through the lower surface with stomata open ($-CO_2$) and closed ($+CO_2$).

Treatment	Penetration		Mean
	6×10^{-5}	1×10^{-3}	
	(cpm)		
$-CO_2$	534	505	520**
$+CO_2$	112	83	98
Mean	323	294	

**Significant at P = 0.01.

no difference between penetration of NAA from the two different concentrations. The 10^{-3} M solution contained 16.7 times more NAA than 6 x 10^{-5} M solution yet the activity (1.0 μm/ml) was the same. Therefore, it was the volume of solution entering the leaf and not the NAA concentration that was the important factor in determining a difference in penetration with stomata open and closed. Although experiments were done at different times, it appears that the amount of penetration may be related to the extent to which the surfactant reduced the treating solution surface tension and permitted movement through the stomatal pore.

Penetration of Silver Nitrate

$AgNO_3$ (0.1 M) was utilized to document whether or not the treating solution entered the substomatal chamber. Treating solution containing 0.1% Vatsol OT was applied for 4 min to leaf disks pretreated for 3 hr in the light to open the stomata. Photomicrographs of the leaf cross sections are illustrated in Figure 23. Reduced silver, photographed as black spots, was present in the substomatal cavity to varying degrees (Figure 23 A-D). Reduced silver may also be observed in the stomatal pore (Figure 23 C). Cuticular penetration, even for this short period of time, was observed especially over veinal areas (Figure 23 E, F). Reduced silver was frequently found in the xylem (Figure 23 E).

Effect of Leaf Age on Penetration

As the leaf age increased, penetration through the upper surface tended to decrease but this difference was not significant (Table 19). However, penetration of NAA through the lower surface decreased significantly with increasing age. The influence of increasing leaf age on penetration was most pronounced in the younger leaves.

Figure 23. -- Transverse sections of leaf disks treated with silver nitrate for 4 min.

A.- D. Silver nitrate in substomatal cavity
E.- F. Silver nitrate in the cuticle over veins

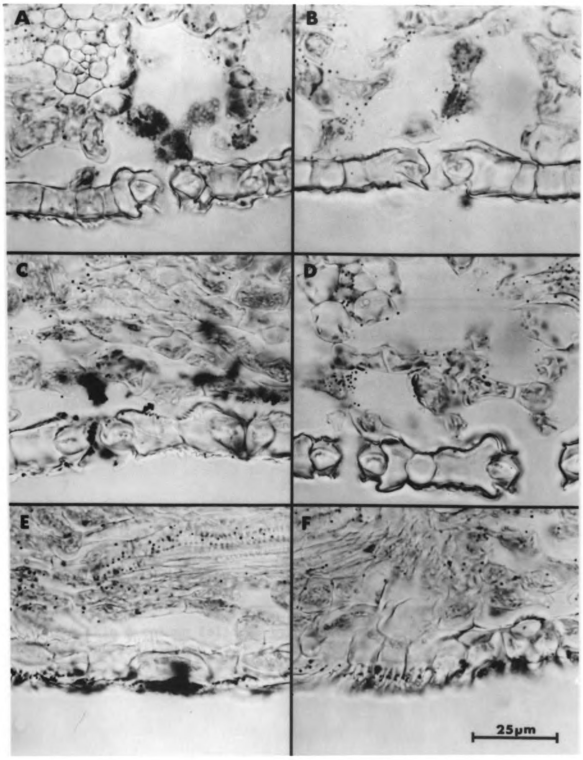

A
B
C
D
E
F
25µm

Table 19. Effect of leaf age on penetration of NAA into pear leaf disks through the upper and lower surfaces.

Leaf number	Penetration[1]	
	Upper	Lower
	(cpm)	
3 (youngest)	2106a	7959a
5	1561a	6166 b
7	956a	4875 c
9	1035a	3355 c
11	750a	2649 c
13 (oldest)	733a	3246 c

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

Penetration of NAAm through the upper and lower surfaces decreased as leaf age increased (Figure 24). There was not a significant difference between penetration through the upper and lower surfaces. However, the interaction was significant at P = 0.01. More NAAm entered the youngest leaves through the upper surface. As leaf age increased NAAm penetration was reduced to a greater extent through the upper surface so that more penetration occurred through the lower surface in the older leaves.

A direct comparison of NAA and NAAm penetration as influenced by leaf age is not possible since the trees used in the NAAm experiment were grown in the growth chamber and the trees used in the NAA experiment were grown under natural conditions in the greenhouse during March.

Effect of Droplet Drying on Penetration

The effect of droplet drying on subsequent penetration of NAA and NAAm by the upper and lower surfaces was manifest in all cases as an immediate increase in penetration. Following the initial increase after drying, penetration appears to continue to increase for the upper surface. This was much more apparent for NAAm. However, subsequent to the initial surge in penetration following droplet drying there appears to be little additional penetration for the duration of the experiment through the lower surface.

Microradioautographic Study of ^{3}H-NAA Penetration Through Isolated Cuticle

The results of a microradioautographic investigation of possible pathways of ^{3}H-NAA penetration through the enzymatically isolated upper cuticle of pear leaves are shown in Figure 26. The cuticle in cross

Figure 24. -- Effect of leaf age on penetration of NAAm into pear leaf disks through the upper and lower surfaces.

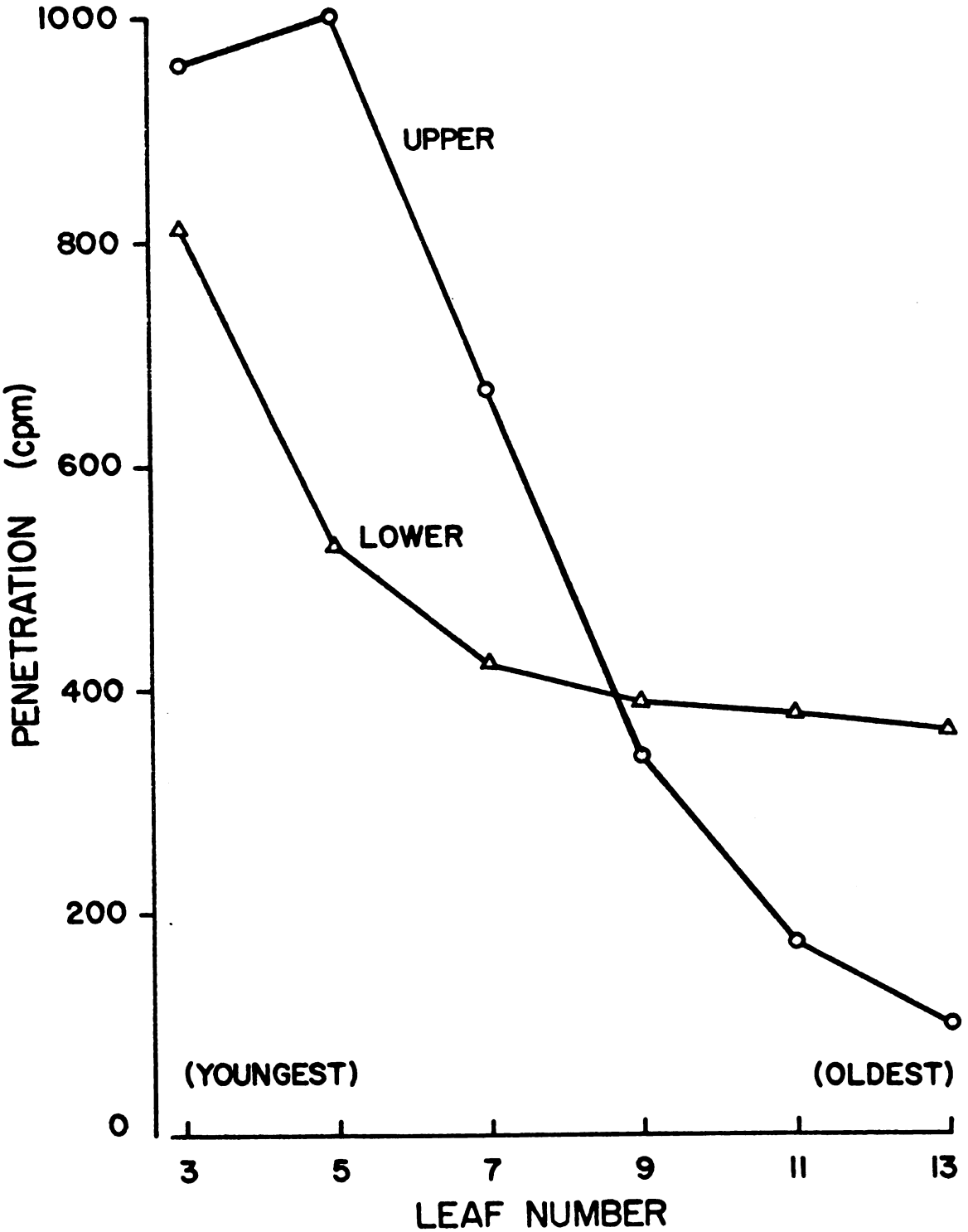

1000
800
600
400
200
0
PENETRATION (cpm)
UPPER
LOWER
(YOUNGEST)
(OLDEST)
3
5
7
9
11
13
LEAF NUMBER

Figure 25. — Time-course showing the effect of droplet drying on penetration of NAA and NAAm into pear leaf disks through the upper and lower surfaces.

A. NAA upper surface
B. NAAm upper surface
C. NAA lower surface
C. NAAm lower surface

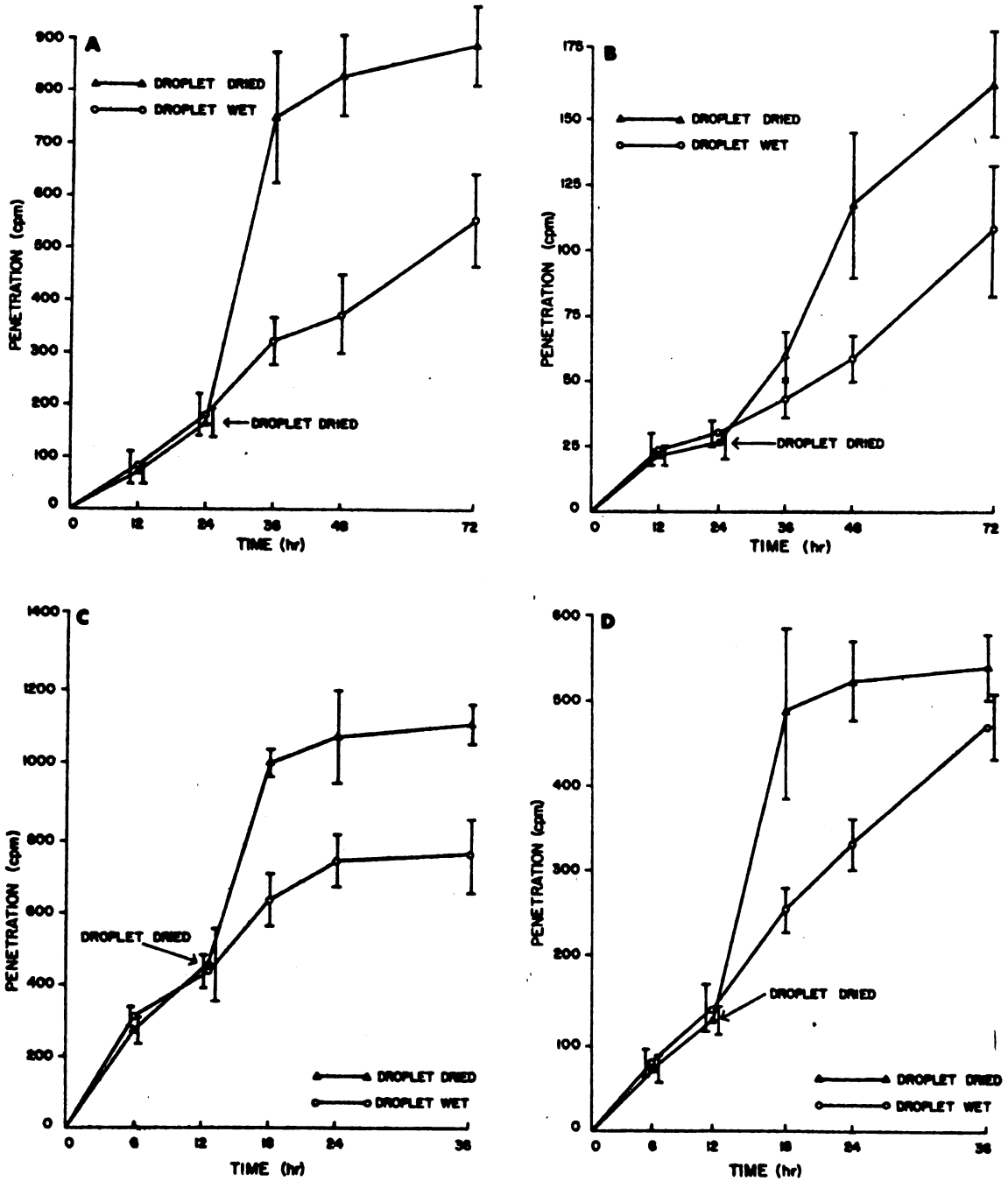
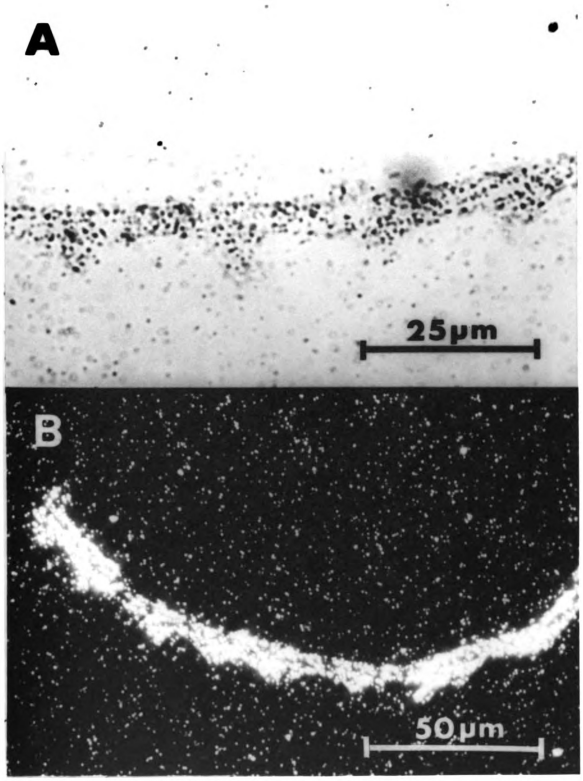

A
DROPLET DRIED
DROPLET WET
DROPLET DRIED
PENETRATION (cpm)
TIME (hr)
B
DROPLET DRIED
DROPLET WET
DROPLET DRIED
PENETRATION (cpm)
TIME (hr)
C
DROPLET DRIED
DROPLET DRIED
DROPLET WET
PENETRATION (cpm)
TIME (hr)
D
DROPLET DRIED
DROPLET DRIED
DROPLET WET
PENETRATION (cpm)
TIME (hr)

Figure 26. -- Microradioautograms illustrating the localization of ^{3}H-NAA in isolated upper pear leaf cuticle in cross section.

A. Light field photomicrograph
B. Dark field photomicrograph


A
25µm
B
50µm

section appears to be uniformly labeled with no preferential areas of penetration following a 12-hr penetration period.

DISCUSSION

The process by which a foliar applied chemical can pass through the cuticular barrier and enter the living continuum of the plant is complex and may be controlled or influenced by a number of factors. An improved method of studying foliar penetration is described that allows a critical evaluation of the relative importance of the factors and the conditions under which they are most important in affecting penetration.

Penetration of NAA and NAAm into pear leaf disks through the upper surface was found to be linear with time. This is in agreement with results of Bukovac and Norris (1966) for penetration of NAA through the isolated upper cuticle of the pear leaf. Extrapolation of the penetration curve back to zero time reveals that the curve does not pass through the origin but intersects the abscissa somewhat above this point. Rapid initial binding of NAA and NAAm to the upper surface of pear leaf disks has been shown to occur by Bukovac and Norris (1966). This may explain the initial deviation from linearity of NAA and NAAm penetration.

Rapid initial absorption for the first 24 to 48 hr followed by a gradual decrease in rate of penetration with time characterized the penetration of NAA and NAAm through the lower surface. Time-course curves similar to this have been reported by Bukovac (1965) for 3-CP penetration into peach leaves, Hughes and Freed (1961) for IAA penetration into bean, and Luckwill and Lloyd-Jones (1962) for NAA penetration into apple leaves. The end of rapid initial absorption through the lower surface occured after about 24 hr for NAA and 48 hr for NAAm.

The amount of NAA penetration at any given time was approximately twice that of NAAm. Therefore, the difference in the shape of the time-course curve may be attributed to different rates of penetration.

Penetration curves for NAA when applied via glass cylinders or in the form of microdroplets were similar. This point is particularly significant if information gained using glass cylinders is to be applicable to penetration from a spray droplet applied to a leaf. Rapid penetration occurred as the droplet dried. Although penetration was not determined immediately following droplet drying, most of this increased penetration was assumed to have occurred during the drying process. Norris and Bukovac (1969) have suggested that increased penetration, as the droplet dried, through isolated pear cuticle may be related to the increased concentration resulting from the loss of the aqueous phase. NAA and NAAm penetration into the lower surface appeared negligible from the droplet residue. Some penetration from the residue appeared to occur through the upper surface but the rate was much more pronounced for NAAm.

Penetration through the cuticle is thought to occur by diffusion (Currier and Dybing, 1959; Franke, 1967). The linear relationship of penetration with time and concentration observed in this study would tend to substantiate this for leaf disks. At high treating solution concentration (above 10^{-4} M) for NAA, or after a considerable amount of NAA or NAAm penetration (24 to 48 hr) into the lower surface, deviation from linearity occurred probably because of an approach to saturation. The rapid increase and decrease in penetration with the cycling of light and dark showed that the underlying cells may play an active

role and thus regulate diffusion through the lower cuticle by influencing the diffusion gradient. However, this is unlikely to be true for penetration into the upper surface of older leaves, where in some instances penetration was less than 1% of that observed for the lower surface.

Zenk (1962) has shown that NAA can influence its own uptake by pea epicotyl sections. There was a lag phase in NAA absorption which represented the time required for sufficient NAA to be taken up by the sections to induce an enzyme which conjugates NAA with L-aspartate. Following this induction phase there was an increase in NAA absorption. No attempt was made to identify an NAA-aspartate conjugate in pear leaf disks. However, if this or other NAA conjugates were formed in the pear leaf disks resulting in increased penetration, this should have been apparent as a deviation from linearity in the time-course study. None was observed. In addition if an inducible enzyme was formed then there should not be a linear increase in penetration with increasing concentration. Some threshold NAA value should be required to bring about induction of the enzyme. Although the cuticle on the lower surface may impede NAA penetration, it is not considered a great barrier preventing a sufficient amount of NAA from entering the leaf disk to bring about the induction of an enzyme. This conclusion is reached for several reasons. (a) Saturation of the leaf disks occurred after about 24 hr. (b) Leaf disks could also be saturated when NAA concentrations above 10^{-4} M were used. (c) Light-stimulated penetration could be shown up to 7 times greater than penetration in the dark.

The temperature coefficients (Q_{10}) of 2.0 or above have been used as criteria for an active uptake process (Franke, 1967; Rice, 1948;

Sargent and Blackman, 1962). A Q_{10} value of 2.0 is representative of many biological processes (Giese, 1962), forming the basis for the conclusion that foliar penetration of a compound is an active process. However, recently Norris and Bukovac (1969) observed Q_{10} values for penetration of NAA through isolated pear cuticle as high as 5.5-6.0 between 15 and 25 C. Therefore, this study of temperature influence on leaf disk penetration must be interpreted in light of at least two potential temperature dependent processes as determining the Q_{10} value, the cuticle and the underlying cells.

The leaves used in this investigation were fully mature. Photomicrographs of transverse sections stained with Sudan III and Sudan IV reveal a cuticle present of significant thickness. The cuticle is lipoidal in nature. Higher Q_{10} values may be expected for lipoid membranes (Suttcliffe, 1962). A diffusing molecule must acquire sufficient kinetic energy to overcome the large potential energy barrier when passing from a solution into the cuticle and also overcome a series of smaller energy barriers while passing through the cuticle. High Q_{10} values exist because at higher temperatures more molecules acquire sufficient energy to diffuse in a given time. Further, within the cuticle there is a continuous layer of oriented embedded waxes in the upper cuticle and a discontinuous layer in the lower (Norris and Bukovac, 1968a). It has been suggested (Van Overbeek, 1956) that at low temperatures permeability of this layer of oriented waxes may be low and hydrogen bonding would tend to be extensive.

Penetration at all temperatures was higher through the lower surfaces than the upper surface. This difference may be partially

attributed to the discontinuity of embedded waxes in the lower surface previously mentioned (Norris and Bukovac, 1969). Greater temperature dependence would be expected for penetration through a layer of oriented waxes. Greater penetration into guard cells and accessary cells, present only on the lower surface, may also contribute to the greater penetration through the lower surface. If penetration through the lower cuticle is greater than metabolically mediated uptake by the cells, at a given temperature, then the Q_{10} could be attributed to active uptake. At 25 C penetration through the lower surface of field grown pear leaves was sufficient so that light stimulation of penetration could be demonstrated. Therefore, the Q_{10} at this temperature is at least in part metabolically determined. This is not true for the upper surface.

Temperature coefficients for penetration of NAA through the upper surface of pear leaf disks would indicate that the high Q_{10} values for penetration are due to the embedded waxes and not the surface waxes. Surface wax accumulation usually ceases with leaf expansion but embedded waxes may still be deposited (Schieferstein, 1957). The Q_{10} value for penetration into young mature leaves, between 15 and 25 C, was 1.61. The Q_{10} value for leaves 6 weeks older, between 17 and 27 C was 7.7.

Penetration of NAA through the upper and lower surfaces increased as the pH of the treating solution decreased. The pK of NAA is 4.2. Below the pK more NAA is in the nondissociated form, has no charge and is lipophilic. As the treating solution pH was increased above 4.2 an increasing number of NAA molecules become dissociated, have a negative charge and are more hydrophilic. Since the cuticle is lipoidal in nature, greater penetration would be expected of the more lipophilic

nondissociated molecules. Increased penetration of NAA at pH values below the pK is in agreement with other investigators who have reported greater penetration of weak acids in the nondissociated form (Albert, 1951; Crafts, 1953; 1956; Swanson and Whitney, 1953).

It has been suggested that weak acids are taken up largely in the nondissociated form (Weintraub et al., 1954). Diffusion studies of 2,4-D into and out of Chlorella cells led Wedding and Erickson (1957) to conclude that the relative permeability of dissociated and nondissociated species was a constant ratio, independent of each other in the external solution. Penetration of NAA, from solutions buffered at a high pH, especially for the lower surface, leave no doubt that the dissociated NAA species can enter the leaf. Differences in the method used in this investigation do not allow direct comparison with results of Wedding and Erickson (1957). However, if the relative permeabilities of cells to dissociated and nondissociated species exists then it must be concluded (Table 7) that this does not occur under all conditions. Penetration in the light from a pH 5.2 solution was nearly twice as great as that from a pH 3.2 solution when compared with their respective dark controls. It is suggested that in the dark some NAA from the pH 3.2 solution can partition into the cell because 90% of the molecules are undissociated and thus lipophilic. NAA penetration from the pH 5.2 solution in the dark will be less because only 5% are in the nondissociated lipophilic form. Under conditions where light is present it would appear that energy may be provided to transport the dissociated NAA species across the plasmalemma and thus increase the diffusion gradient across the cuticle.

Penetration of NAAm into the upper and lower surfaces was independent of the treating solution pH. Since the pK of NAAm is about 14, the NAAm molecule was uncharged over the pH range studied (pH 3.0-7.0). Therefore, it is concluded that the influence of pH on the penetration is related to its effect on the penetrating molecule and not on the cuticular surface.

The influence of pH on penetration of NAA and NAAm is in agreement with the influence of pH on binding of NAA and NAAm by pear leaves (Bukovac and Norris, 1966). It also substantiates evidence from the above studies that electrostatic binding may not be critical for the uptake of growth substances.

The effect of light on increasing foliar penetration of plant growth substances is not well documented in the literature. Sargent Blackman (1962, 1965) were able to show light dependent uptake of 2,4-D by Phaseolus vulgaris leaf disks. This system was complex in that it was dependent upon pretreatment time in the light or dark, 2,4-D concentration and light intensity above 1000 ft-c. The absence of a light effect or a small light effect (Herrett and Linck, 1961; Kamimura and Goodman, 1964; Smith et al., 1959; Weintraub et al., 1954) may be due to several reasons: (a) the growth substance was not taken up, thus penetration would be unaffected by energy provided by light, (b) the method of application was such that spray droplets dried soon after application, thus limiting the amount entering the plant, (c) the cuticle limited penetration to such an extent that a light effect could not be detected as was demonstrated herein (Figures 11 and 12) for penetration into pear leaf disks through the upper surface.

Increasing light intensity was shown to bring about increased NAA and NAAm penetration through the lower surface until a light intensity of about 600 ft-c had been reached (Figures 11 and 12). Bohning and Burnside (1956) have shown that CO_2 fixation in shade leaves responded in almost an identical manner to NAA and NAAm penetration with increasing light intensity. The seemingly greater stimulation of NAAm penetration by increasing light intensities as compared with NAA is misleading. In subsequent experiments where NAA penetration was measured in the light and dark (Tables 6, 7 and Figures 12, 13, and 14) the increase over the dark controls was always at least 6-fold unless young leaves, with incomplete chlorophyll development were used (Table 6). Therefore, the light stimulated uptake of NAA and NAAm was quantitatively similar.

The fact that the light effect may be related to photosynthesis because of the similarity of the CO_2 fixation and penetration curves has already been mentioned. Further evidence supporting this is shown in Table 6. Penetration into young leaves, having incomplete chlorophyll development, was about 3 times less than penetration into mature leaves, with complete chlorophyll development, when compared with their respective dark controls. However, chlorophyll development was an observation and not an actual determination. Further characteristics of the light effect are: it was immediately lost or gained when leaf disks were transferred from light to dark or from dark to light (Figure 12). The effect can be "turned on" or "turned off" repeatedly with quantitatively related penetration in each case (Figure 13). The light-enhanced uptake of NAA was not related to stomatal opening (Figure 15).

It is therefore proposed that the energy for the light effect was provided by photosynthesis. Since the light effect is lost and gained

so rapidly, degradation of starch to provide energy is unlikely. The probable source of energy was one or a combination of the primary products of photosynthesis such as ATP, NADPH, or simple sugars.

The most definitive evidence that photosynthesis was involved in the light effect was provided by studies using photosynthetic inhibitors which specifically block the Hill reaction. Atrazine is readily absorbed by the Leaf (Klingman, 1961) and is a specific inhibitor of the Hill reaction (Moreland et al., 1959). No inhibition by Atrazine occurred in the dark, some occurred at low light intensity (Table 9). Two other Hill reaction inhibitors, Terbicil and Monuron (Crafts, 1961), significantly inhibited NAA penetration in the light, which is further evidence implicating photosynthesis in light-stimulated NAA penetration.

Difficulties were encountered when metabolic inhibitors were employed. Determination of the amount of each inhibitor diffusing through the cuticle to the underlying cells was not possible. Concentration and length of pretreatment were established so that there was no visible injury to the leaf at the end of the experiment. However, comparison of inhibitor effects on penetration is not valid because the actual amount of each inhibitor reaching the site of action in the cell remains unknown. Infiltration of the leaf disks proved unacceptable because cracks in the cuticle were created during the infiltration process. The most satisfactory method to inhibit metabolic processes in the leaf disks was to conduct penetration experiments in an atmosphere of nitrogen. Penetration of NAA into the lower surface was significantly reduced in an anerobic atmosphere (Table 8). This inhibition was probably due to both a lack of oxygen for metabolic processes and insufficient

CO_2 for photosynthesis. At present there is no explanation for increased penetration in two instances with NAA through the upper surface. A lack of inhibition is most likely due to the cuticle offering such a barrier to NAA penetration that an active component could not be detected.

Uncouplers of oxidative phosphorylation, m-Cl-CCP (Heytler, 1963), p-F-CCP (Hopfer et al., 1968) and DNP (White et al., 1964) all significantly reduced NAA penetration suggesting that catabolism of simple sugars provided some energy stimulating NAA penetration. Heytler (1963) has also shown that m-Cl-CCP can also inhibit photophosphorylation in the chloroplast. Phenylmercuric acetate, an inhibitor of stomatal opening, is postulated to act by combining with sulfhydryl groups in or near the membrane (Zelitch, 1963). Since stomatal aperture width has been shown not to be associated with the light effect (Figures 15 and 16), it may be that PMA reacts with the sulfhydryl group of an enzyme or enzymes responsible for transport across the plasmalemma. Stomatal entry in the control leaf disks is precluded because stomata do not open completely when treating solution is present during the period of opening. The lack of inhibition with sodium azide does not necessarily imply that NaN_3 does not inhibit NAA penetration. As explained earlier, it may not have entered the cell in sufficient quantities to bring about inhibition.

Surfactants reduce surface tension and thus ensure greater contact of the treating solution with the plant surface. This is especially true with irregular or rough leaf surfaces. The upper surface of the pear leaf was shown to be relatively smooth. Of the surfactants studied, none significantly increased penetration of NAA and NAAm through

the upper surface. However, penetration of both NAA and NAAm was increased through the lower surface, which was observed to be rougher and more irregular. There was a significant difference in penetration of NAA through the lower surface at surfactant concentrations between 0.001 and 0.01%. From Figure 18 it is seen that the surface tension of the treating solution must be reduced to between 45 and 42 dynes/cm^2 before a significant increase in penetration can be detected.

Westwood and Batjer (1960) reported that Tween 20 increased penetration of NAA by both surfaces of apple leaves. However, in this study no increased penetration of NAA through the upper surface of the pear leaf was observed. There are numerous reports in the literature of increased penetration through the lower surface by various surfactants.

In addition to increased penetration as a result of a reduction of surface tension Freed and Montgomery (1958), Jansen (1964), and Westwood and Batjer (1960) have emphasized that the molecular interaction between the surfactant and the growth substance may be of equal or greater importance and this would appear to be the case with surfactant influences on penetration of NAA and NAAm into the lower surface. Tween 20 at 0.1% increased NAA penetration by less than 2-fold but increased NAAm penetration by 9-fold. Tween 20 and Triton B-1956 were equally effective in increasing NAAm penetration into the lower surface. However, X-77 was significantly better than these which is in agreement with Westwood and Batjer (1960).

The highest surfactant concentration used in this study was 0.1%; a concentration that is at or slightly above the critical

micelle concentration. Therefore, the results reported here are not likely to be the result of solubilization of waxes or other water-insoluble material in the cuticle that is suggested to occur at surfactant concentrations above the critical micelle concentration (Osipow, 1964; Parr and Norman, 1965).

Several approaches were taken to determine the importance of stomatal penetration. Surfactants have been shown to differ in their ability to reduce surface tension (Figure 19). Vatsol OT was found to be the most effective in this respect. When Vatsol OT was added to the treating solution in a time-course study (Figure 21) there was a dramatic increase in penetration. This was considered an indication of, but not proof for, stomatal penetration since the treating solution was buffered at pH 3.0. When the surfactant was added, more NAA in the nondissociated form could come in contact with the surface thus increasing penetration. The effect of pH on penetration has also been shown to be dramatic (Figure 10). To differentiate between the effect of surfactants on enhanced cuticular penetration and stomatal entry an experiment was performed with NAA at pH 6.0 and using three surfactants: Vatsol OT, X-77, and Tween 20. Immediately following addition of surfactants there was a rapid increase in penetration. Stomatal penetration is indicated since essentially all of the NAA molecules were dissociated and cuticular penetration would be expected to be at a minimum. The surfactants which reduced the surface tension to the greatest extent were most effective in causing enhanced penetration.

Additional evidence for stomatal penetration is shown in Tables 16, 17 and 18, where surfactant-containing treating solutions were

added to leaf disks with stomata open, or closed with CO_2. In all cases penetration was significantly greater with stomata open, and the degree of penetration was related to the surface tension of the treating solution. There was no significant difference in penetration from 1 X 10^{-3} M and 6 X 10^{-5} M solutions containing 1.0 µc/ml even though the 1 X 10^{-3} M solution was diluted by a factor of 16.6 with non-labeled NAA. Therefore, differences in penetration must be attributed to stomatal entry since the volume of solution and not the concentration was shown to be the important factor.

Calculations were made to preclude the possibility that the increase in penetration was due to the filling of the stomatal pore and not actual passage of the solution into the substomatal cavity. The amount of NAA attributed to stomatal entry from a Vatsol OT solution was 422 cpm (Table 18). This was determined by subtracting the cpm entering leaf disks maintained in a high CO_2 atmosphere with stomata closed (cuticular penetration) from the control leaf disks having stomata open (cuticular plus stomatal penetration). Since the efficiency of the Low Beta II counter was 10% and a 1.0 µc/ml solution was used, 1.9 X 10^9 μm^3 of treating solution entered each leaf disk. It was determined that 12,500 stomata were exposed to the treating solution. Therefore, if the increased penetration was due to the filling of stomatal pores, the volume of each would have to be 160,000 μm^3. Approximate pear stomatal pore dimentions were determined: length 15 µm, width 5 µm, and depth 6 µm, giving a volume of 450 μm^3. Therefore, the volume of solution entering the leaf was approximately 350 times greater than the volume of the stomatal pores. Similar calculations for X-77 (Table 17) and Tween 20 (Table 16) indicated that the

volume of treating solution entering the leaf was 156 and 44 times as great, respectively, as can be accounted for by stomatal pore volume. It is concluded that NAA passed through the stomatal pore and entered the substomatal cavity.

The extent to which the treating solution filled the internal air space was also calculated. Leaf thickness was determined to be about 225 μm and the area of the leaf exposed to treating solution approximately 0.5 cm^2 giving a leaf volume of 1.1 X 10^{10} μm^3. If 20% of the leaf is assumed to be air space, the leaf volume available to accommodate treating solution entering through stomata would be 2.2 X 10^9 μm^3. From the previous calculations it was found that 1.9 X 10^9 μm^3 of Vatsol OT-containing solution entered the leaf. Therefore, approximately 86% of the available leaf air space was occupied with the treating solution after 10 minutes. Similar calculations for X-77 and Tween 20 show that 32% and 9% of the air space, respectively, was occupied by treating solution containing these surfactants.

Further evidence that solutions may enter the pear leaf through stomatal pores was demonstrated with localization of reduced silver in the substomatal chamber after a 4 minute treatment time (Figure 23 A-D). Although cuticular penetration was also observed in this short period of time, localization as shown is best explained by stomatal entry. If silver filled the substomatal chamber following passage through the stomatal pore then more reduced silver might be expected to be present. However, immediately following the 4 minute penetration period, the disks were infiltrated with Craf III killing and fixing solution which contained 0.3% chromic acid, a very powerful oxidizing

agent. This may have prevented further reduction of the silver already present in the substomatal chamber.

Stomata are probably not important as portals of entry of treating solution into the pear leaf because of the rather precise conditions required to open stomata fully. After leaf disks were illuminated with light for longer periods than 5 or 6 hr, stomatal aperture widths were observed to decrease. Further, stomata did not open fully when the treating solution was present during opening. Penetration of NAA from a treating solution containing 0.1% Tween 20 was similar irrespective of stomatal aperture width (Figure 20). In this experiment aperture widths were less than if the stomata were allowed to open on moistened filter paper in the Petri dish (Figure 18).

Evidence of stomatal penetration provided here is in agreement with the work of Currier, Pickering, and Foy (1964), Dybing (1958), Dybing and Currier (1961), Hull (1964), and Pickering (1965). However, the factors which bring about stomatal penetration are not well documented. Adam (1948) pointed out that the two most important factors influencing the penetration of a liquid through a pore were (a) low contact angles of the liquid and (b) the shape of the pore. The contact angle is influenced by the surface tension of the solution and the nature of the surface. The pore shape changes with degree of opening. In this study the extent of stomatal penetration was related to the reduction of surface tension, confirming observations made by Dybing (1958) and Dybing and Currier (1961). It was previously mentioned that stomatal penetration was believed to occur through only 20-60 stomata (less than 1.0%) out of the approximately 12,500 present

in a leaf disk. Therefore, if stomatal aperture width is a determining factor and less than 1.0% of the stomata are involved, then aperture width must be larger than 7.0 μm to allow a surfactant-containing treating solution through the pore (Figure 18). Currier, Dybing, and Foy (1964), and Dybing (1958) found stomatal penetration was correlated with the degree of stomatal opening. Foy (1962) found a correlation between 2,4-D penetration and larger stomatal apertures induced by high humidity. Data presented here and references cited in the literature would indicate that stomatal penetration occurs through stomata because of large aperture widths. However, alternative explanations may conform more to the mathematical considerations presented by Adam (1948). The high humidity conditions used in this study and those reported by Foy (1962) would partially hydrate the cuticle around the stomata. Cuticle hydration reduces the contact angle of the solution (Fogg, 1944) below that caused by the surfactant thus increasing stomatal penetration. Since the cuticle is lipoidal in nature and a surfactant has a lipophilic tail, it may be that the surfactant is sorbed from the treating solution into the cuticle around and in the stomatal pore. It this were the case then a cuticle-treating solution interface would not exist but there would be a continuum of cuticle, surfactant and surfactant treating solution. Such a situation would then result in a zero contact angle and passage through the pore would be very much facilitated. It is also probable that the degree of opening could influence the shape of the stomatal pore to make penetration feasible.

Recently, Idle (1969) has demonstrated that the silicone rubber-cellulose acetate method of measuring stomatal apertures may be

unreliable for plants having stomata with cuticular ledges. This cuticular ledge is apparent on pear stomata (Figure 23 A-D). However, it is also emphasized that relative differences in stomatal aperture widths are valid even though actual μm measurements may be in slight error.

Increased penetration of NAA and NAAm by young leaves is consistent with 3-CP penetration into peach leaves (Bukovac, 1965), NAA and NAAm binding by pear leaves (Bukovac and Norris, 1966), and 2,4-D penetration into bean leaves (Sargent and Blackman, 1962). Surface waxes are continually deposited on the surface until leaf expansion ceases but embedded waxes are continually deposited (Crafts and Foy, 1962). Increased cuticle thickness with age may contribute to reduced penetration (Richmond and Martin, 1959).

Penetration of NAA through the lower surface was always greater than through the upper surface. In general, differences became progressively greater with increasing leaf age. There are numerous reports of greater penetration through the lower surface (Fogg, 1958; Sargent and Blackman, 1962; 1965; Schieferstein, 1957). Penetration of NAAm is unique in that it was greatest through the upper surface of young leaves. This condition was reversed with the older leaves. The increased penetration through the upper surface is difficult to explain. However, the leaves developed under relatively low light (1800 ft-c) in the growth chamber and the epicuticular wax layer may have been insufficiently developed to provide a barrier. Penetration through the lower surface of the same leaves may have been less because this surface is rougher and there may have not been complete contact with the treating solution.

Microradioautographic studies with the isolated upper pear leaf cuticle indicated that with the resolution possible preferential absorption did not occur over anticlinal walls. This was similar to the uniform binding of 3-CP by peach leaf cuticle (Bukovac, 1965). Fogg (1948) and Dybing and Currier (1961) have indicated anticlinal walls as sites of preferential absorption. However, intact leaves were used and the underlying cells may have influenced the pattern of cuticular penetration.

Absorption of silver nitrate (Figure 23) showed cuticular penetration. At least 3/4 of the time when cuticular penetration was observed it was associated with veins. Although the cuticle is thicker over veins due to deeper indentations, embedded waxes were shown to be discontinuous (Norris and Bukovac, 1968a), thus perhaps allowing preferential penetration.

SUMMARY

An improved method of studying foliar penetration and the effect of various factors on foliar penetration of NAA and NAAm into leaves of the pear Pyrus communis L. is described.

1. Time Course

Penetration of NAA and NAAm into the upper surface was linear. Penetration into the lower surface is characterized by rapid initial NAA uptake for 24 hours and NAAm for 48 hours followed by a phase where uptake occurred at a reduced rate. Penetration of NAA when applied either via glass cylinders or as microdroplets was similar, provided that the droplet was not allowed to dry.

2. Droplet Drying

Droplet drying resulted in an increase in NAA and NAAm penetration through both the upper and lower surfaces. Subsequent to droplet drying NAA penetration into the upper surface proceeded at a slower rate and NAAm penetration was not reduced. Following the initial surge in penetration as the result of droplet drying, little additional NAA or NAAm entered the lower surface.

3. Concentration

Penetration of NAA and NAAm was linear with increasing concentration into the upper and lower surfaces with the exception of NAA penetration into the lower surface at the highest concentrations where uptake started to plateau.

4. Temperature

Penetration of both NAA and NAAm increased with increasing temperature. The greatest increase was between 25 and 35 C. Temperature generally had a greater influence on NAAm penetration.

5. pH

The penetration of NAA into the upper and lower surfaces was related to the degree of dissociation of the molecule. More NAA was taken up from treating solutions buffered at a low pH (3.0) than those at a high pH (6.0). NAAm penetration was not influenced by treating solution pH between 3.0 and 7.0.

6. Light

Penetration of NAA and NAAm through the lower surface increased with increasing light intensity up to about 600 ft-c. Beyond this light intensity there was little increase in penetration with increasing light intensity. Light stimulation of NAA and NAAm penetration could not be demonstrated in the upper surface of field grown leaves because the cuticle presented such a great barrier to penetration. Light-stimulated penetration of NAA into the upper surface could be shown with greenhouse grown leaves but stimulation was not as great as for the lower surface. The light stimulation of NAA penetration was not influenced by stomatal aperture width. The stimulation of penetration from light is immediately gained and when the light is turned off the stimulation is also immediately lost.

7. Inhibitors

Specific inhibitors of the Hill reaction, Atrazine, Monuron, and Terbicil, can partially reverse the light stimulated increase in penetration of NAA into the lower surface. It was concluded that

primary products of photosynthesis were responsible for increased penetration in the light. A 100% atmosphere of nitrogen, carbonylcyanide m-chlorophenylhydrazine, carbonylcyanide p-trifluoromethoxyphenylhydrazine, 2,4-dinitropheriol, and phenylmercuric acetate all inhibited NAA penetration through the lower surface in the light.

8. Surfactants

No surfactant studied increased penetration of NAA or NAAm into the upper surface. A 0.01% or higher Tween 20 or Tergitol 15-S-9 solution increased NAA penetration into the lower surface. Tween 20 and Triton B-1956 at 0.1% solutions increased NAAm penetration into the lower surface, but X-77 was significantly better than either of these. Surfactants increased NAAm penetration to a much greater extent than for NAA.

9. Stomatal Penetration

Stomatal penetration of applied treating solutions has been established. However, it is not considered to be a major pathway of entry of foliar applied compounds.

10. Leaf Age

Penetration was greater in younger than older leaves with the exception of NAA penetration into the upper surface. Uptake of NAA into the lower surface was always greater than into the upper. NAAm penetration was greater into the upper surface until leaf expansion was completed then the situation was reversed and the greatest absorption was into the lower surface.

11. Pathways

No preferential areas of NAA penetration through the isolated upper cuticle were observed. Silver nitrate was preferentially

absorbed through the cuticle above veins.

12. Active Uptake

It is concluded that NAA and probably NAAm are actively taken up by the leaf disks.

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APPENDICES

Table A1. Scintillation solvent mixture used for the selection of a detection method and for calibrating treating solutions.

Ingredient	Amount
Naphthalene	80 gm
BBOT	14.5 gm
Dioxane	385 ml
Xylene	385 ml
Ethanol	230 ml
Cab-O-Sil	37.5 gm

Table A2. Standard deviation for penetration of NAA and NAAm into pear leaf disks through the upper and lower surfaces from different concentration treating solutions.

	Standard Deviation (picomoles)			
	NAA		NAAm	
Concentration	Upper	Lower	Upper	Lower
10^{-6}	1	2	--	--
5×10^{-6}	1	7	--	--
10^{-5}	9	54	--	--
5×10^{-5}	19	175	9	8
10^{-4}	40	169	18	31
5×10^{-4}	389	614	202	183
10^{-3}	369	932	326	191

Table A3.

Trade name	Chemical name and class	Source
Tergitol 15-S-9	Polyethylene glycol ether of linear alcohol	Union Carbide Corporation New York, New York
Tween 20	Polyoxyethylene sorbitan monolaurate	Atlas Chemical Industries Wilmington, Del.
Triton B-1956	Sodium alkylaryl polyether sulfate	Rhom and Haas Company Philadelphia 5, Pa.
Triton X-100	Alkyl phenoxy polyethoxy ethanol	Rhom and Haas Company Philadelphia 5, Pa.
Vatsol OT	Diocytl ester of sodium sulfosuccinic acid	American Cyanamid Co. New York 20, New York
X-77	Alkylarylpolyoxyethylene glycols, free fatty acids and isopropanol	Chevron Chemical Company Ortho Division San Francisco, Calif.