ABSTRACT

MECHANISMS OF FOLIAR PENETRATION AND TRANSLOCATION OF MINERAL IONS WITH SPECIAL REFERENCE TO COFFEE (COFFEA ARABICA L.)

By Samu-Negus Haile-Mariam

Critical to an understanding of the mechanisms of foliar uptake is to consider the penetration of the solutes through the first barrier that sheaths the leaves of all plants - the cuticular membrane. The property of the cuticular membrane as a barrier, specifically to the penetration of solutes, was studied after enzymically separating it from leaf surfaces. New techniques were developed and fortified enzyme solutions were utilized after having failed to separate the leaf cuticles from some species of plants by existing procedures.

Penetration studies were made after affixing the cuticular membranes of Euonymus japonicus and coffee (Coffea arabica L.) leaves onto the open end of a 15mm diameter glass tube, containing the labelled ion solution (1.0mM for monovalent and 0.1 mM for divalent cations). This tube was then submerged into deionized distilled water in a 30mm diameter large test tube which in turn was submerged in a constant temperature water-bath. Each treatment was replicated thrice. After pre-determined times, 1 ml aliquots from the large test tube were sampled and radioassayed by a scintillation-well detector for $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, $^{137}Cs^{+}$, $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$; and by

an open window G-M detector for $^{45}Ca^{++}$, $^{89}Sr^{++}$, and $^{133}Ba^{++}$. Results were expressed as absolute values (mμmoles/time).

The order of permeability for the monovalent cations through Euonymus leaf cuticles was $^{137}Cs^{+} > {}^{86}Rb^{+} > {}^{42}K^{+} = {}^{24}Na^{+}$ and $^{133}Ba^{++} > {}^{89}Sr^{++} > {}^{45}Ca^{++}$ for the divalent cations. The rate of penetration was related to the Hofmeister or lyotropic ion series, being inversely correlated with the size of the hydrated ion. The rate of ion penetration through the stomatous cuticular membrane isolated from the dorsal leaf surface of Euonymus was 3-fold greater than through the astomatous membrane.

Iron, manganese, and zinc were combined with several carrier chemicals, hopefully to facilitate their passage through cuticular membranes. Two synthetic chelating agents - ethylenediamine tetraacetic acid (EDTA) and ethylenediamine di-o-hydroxyphenylacetic acid (EDDHA) combined with $^{59}Fe^{++}$, $^{54}Mn^{++}$, $^{65}Zn^{++}$ gave no enhancing effect on ion penetration through coffee leaf cuticular membranes. In fact, permeability of the ions was impeded when they were chelated. Dimethyl sulfoxide (DMSO) also had no enhancing effect on $^{59}Fe^{++}$ penetration through Euonymus leaf cuticles.

Application of $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ chelates onto the dorsal surface of the coffee leaf by the "sticking" method reduced absorption but increased translocation over that from the metal salt. Both EDTA and EDDHA, particularly EDDHA

increased by 2 and 3 fold the mobilization of $^{59}Fe^{++}$ and $^{65}Zn^{++}$ beyond the site of application.

Studies utilizing excised coffee leaves by the "immersion technique" and with cells enzymically isolated from green coffee leaf lamina confirmed that chelation with EDTA or EDDHA reduced ion uptake. Absorption of $^{59}Fe^{++}$ and $^{54}Mn^{++}$ by excised coffee leaves was generally greater at pH 3 than at higher pH levels.

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Samu-Negus Haile-Mariam

A THESIS

Submitted to

Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1965

DEDICATION

To my wife, Tsega Haile-Mariam

ACKNOWLEDGMENTS

I am deeply indebted to Dr. Sylvan H. Wittwer for his unending assistance and unerring guidance throughout my academic studies.

I am also indebted to members of my committee: Drs. R. L. Carolus, A. L. Kenworthy, C. J. Pollard, and H. M. Sell for their helpful suggestions in editing this manuscript.

The financial assistance granted by the Rockefeller Foundation and the Biological and Medical Division of the United States Atomic Energy Commission (Contract No. AT (11-1)-888) is gratefully acknowledged. Acknowledgment also goes to my wife, Tsega Haile-Mariam, to my friend and colleague, Mr. Seshadri Kannan, Dr. Abraham H. Halevy, and to Mrs. Geri Burkhardt for all their assistance.

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INTRODUCTION

Absorption by foliage might seem surprising since normally the root of the plant is primarily responsible for the uptake of solutes. Increased use of foliar applied sprays to effect weed, insect, and fungus control; to correct certain mineral deficiencies; and attain specific responses by growth regulators have directed some importance to studies of pathways and mechanisms for foliar absorption and subsequent translocation. The rapid fixation of some nutrients in the soil in unavailable forms to crop plants and their slow responses to soil application contributes to the interest in foliar feeding of fertilizers.

The most important consideration in foliar spraying has been the problem of solute penetration into leaves, and its transport to other parts of the plant. The first step in understanding the pathways and mechanisms of foliar absorption is to consider the penetration of solutes through the barrier that sheaths the leaves of all plants - the cuticular membrane. The intrinsic property of the cuticular membrane as a barrier to penetration of solutes can be alienated from leaf uptake only by separating it from the leaf surface.

Existing cuticular membrane separation procedures were improved in this study, and the following questions were considered to better understand the permeability properties of isolated leaf cuticular membranes:

1. Is there a differential rate of ion penetration, through cuticular membranes because of ion size?

2. Are there differences in ion penetration between the stomatous and astomatous leaf cuticular membranes?

3. Could dimethyl sulfoxide (DMSO), which has been described as capable of passing through some membranes and tissues "as a knife through butter," aid in ion penetration through cuticular membranes?

4. Do synthetic chelating agents such as ethylenediamine tetraacetic acid (EDTA), and ethylenediamine di-o-hydroxyphenylacetic acid (EDDHA) enhance penetration of ions through leaf cuticles? The importance and success of synthetic chelating agents as soil applications to correct plant micronutrient deficiencies is now a legion, but knowledge of the action of synthetic chelating agents on foliar sprays of micronutrients is scant.

This thesis is also devoted, in part, to an inquiry of the mechanisms of absorption and translocation of chelated iron, manganese and zinc applied as foliar sprays to the coffee plant. Coffee plants were utilized in many of these studies because coffee is of great importance in Ethiopia, and plants frequently exhibit micronutrient deficiency disorders.

LITERATURE REVIEW

Historical

Studies of foliar absorption of inorganic and organic substances have been phenomenal since Gris in 1844 (63) first used iron sprays to correct chlorosis on grapes. Other early studies on foliar feeding were those of Mayer 1874 (115), Bohm 1877 (10), Sachs 1888 (144), Metzger 1890 (116), Molisch 1892 (122), and Johnson 1916 (80).

It has been reported by Tukey 1952 (158), that a 12 year old apple tree provides in its foliage, both upper and lower surfaces, an area of 1/10 of an acre or approximately ten times the spread of the tree. Therefore, leaves provide by far the most extensive surfaces for the absorption of nutrients from sprays.

A 100-bushel yield of corn requires approximately 160 lbs. of nitrogen, 30 lbs. of phosphorus, and 125 lbs. of potassium, but less than one pound of Cu, Mn, Zn is required to develop all parts of the corn plant (42). The amount of Cu, Mn, Zn, Fe required by plants is not great, therefore, it is easily possible to meet the plant needs for these elements by leaf feeding. It is not practicable to supply all the major nutrient requirements through the leaves and foliar feeding of these nutrients should be as a supplement to soil applications.

Hamilton, Palmiter and Anderson (70) sprayed 5 lbs. of urea/100 gallons of water on apple trees and observed an increase in leaf chlorophyll and total nitrogen. Others had also similar results with urea on apple trees (51, 52, 53). The commercial use of urea sprays on other crops has been tried. These include coffee, cacoa, and banana (29, 110, 126); sugar cane (96); citrus (81, 97, 100, 163); corn (136, 173); and tomato (114). In general, the experience of investigators with urea sprayed on stone fruits and grapes has been disappointing or inconclusive (55, 124, 166).

Partial success from P, K foliar application has been reported: tomato (3, 149); apple (27, 28, 45, 54, 93); cotton (12); sugar beets (153); coffee (110, 127, 128); chrysanthemum (11, 119).

Iron chlorosis is one of the most widely known nutritional disorders in plants, especially when plants are grown in poorly drained and alkaline soils. Application of iron to these soils has not been productive or the response was not rapid enough to influence quality and yield of tree fruits. Therefore, the first attempt with foliar sprays was to correct iron chlorosis (63). The earliest successful large scale commercial use of iron sulfate sprays was on chlorotic pineapple fields in the Hawaiian Islands (80). Under Hawaiian conditions, pineapple chlorosis resulted from unavailability of

iron due to extremely high Mn in the soil (91). Chlorosis was widely linked with carbonate soils in which the soil iron was not sufficiently available for plants (60, 118). However, from analysis of plant tissues, it has been established that plants afflicted with chlorosis often contain as much or even more iron than healthy plants (72, 120). Further investigations have established that the reasons for chlorosis is a deficiency in soluble iron in the plant because of its conversion into bound form (142). Binding of iron in plant tissues is also facilitated by increase in pH of the cell sap when the plants are grown on carbonate soil (107, 142), and a rapid uptake of phosphorus (130), particularly when the copper content is large (21).

Wallace (162) hypothesized that binding sites for micronutrient cations are natural chelating agents. He said that Ca^{++} and soil organic matter could enter into chelate competition with iron. Therefore, he attributed the problem of iron chlorosis in plants grown in overlimed and manured soils to the increased competition of Ca^{++} and iron with the chelate.

Chandler (31) discovered that mottle-leaf and little-leaf conditions of citrus fruit trees could be cured by trunk injection of zinc nails. Since then, positive responses have been obtained in many crops using foliar spray of zinc sulfate

(71, 93, 110, 131, 164). Sweet cherry and walnut do not respond satisfactorily to zinc sprays (31).

Manganese deficiency may be easily controlled by foliar spray of $MnSO_4$ (17, 30, 99, 172), and copper deficiencies by Bordeaux misture (16, 103).

Factors Affecting Absorption and Transport of Nutrients Sprayed on Leaves

Nature and Chemical Composition of Nutrient Sprays.

Carrier Ions:

Nitrogen - Urea is the most frequently used form of nitrogen for foliar sprays. It is soluble in water, high in nitrogen, readily available and easily absorbed by leaves (29, 51, 52, 53, 70, 81).

Posphorus - Silberstein and Wittwer (149) testing the absorption of a number of solutions of inorganic and organic phosphorus compounds by greenhouse grown plants found that the ortho-phosphoric acid form was the best, and Fisher and Walker (54) likewise preferred ortho-phosphoric acid for apples. Bridger and his associates (18) suggested metal-ammonium phosphate as good a formulation to aid metal penetration.

Potassium - Neptune, et al. (127) observed that sulfate of potash was the best form absorbed by coffee plant, especially at high rates.

Magnesium and Calcium - A 2% spray of Epsom salts ($MgSO_4 \cdot 7H_2O$) was an effective remedy for magnesium deficiency on apples (15, 154), although Fisher, et al. (54) preferred magnesium nitrate ($Mg(NO_3)_2 \cdot 6H_2O$). Calcium nitrate or chloride sprays controlled Blackheart disease of celery (108).

Trace elements - Fe, Mn, Zn in sulfate forms gave the best responses as foliar sprays (17, 64, 100, 134, 172).

Chelating Agents:

The success of chelated metals in correcting deficiencies in the soil has been phenomenal, but as agents for foliar sprays they have thus far been relatively ineffective (54, 94, 99, 161). On the other hand, Wallace (161) amongst others (43, 65, 90) has reported that chelated metals are more mobile in plants than non-chelated ones. Millikan and Hanger from Australia (121) have recently suggested that calcium, considered immobile once it is deposited on leaves (25), could be mobilized by the addition of small amounts of EDTA or HCl or both, or by citric acid. In spite of a few successes, chelates, by virtue of their aid in the mobility of ions, could be important components

of foliar sprays if they facilitate the subsequent mobility of the absorbed nutrients.

Urea:

Many citrus fruits in Arizona and California (162) are successfully sprayed with a combination of urea sulfate or iron chelate. Fisher and Walker (54), however, observed no effect of urea on phosphorus and magnesium uptake by apple trees.

Growth Substances and Other Metabolites:

Growth substances that modify polarity in plants such as triiodobenzoic acid (TIBA) and trichlorobenzoic (TCBA) have given increased basipetal transport of otherwise immobile calcium (25, 92). Addition of gibberellic acid (GA_3) with urea sprays enhanced the utilization of urea in corn leaves (136), but the presence of Naphthalene acetic acid (NAA) was of no significance in the absorption of P and Mg (54). Nicotinic acid (12) and glycine (54) increased markedly the foliar uptake of phosphorus, but not that of magnesium.

Dimethyl Sulfoxide (DMSO):

DMSO is the widely hailed "wonder drug" liquid extracted

from paper-pulp wastes and commonly used as a solvent. Among the many interesting properties claimed, the most characteristic is its ability to penetrate skin easily (77). Jacob et al. (78) have claimed that DMSO enhanced penetration of antibiotics and fungicides into plants, and also stimulated the active transport of desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) across frog cell membrane. Mussell, et al. (125) have observed an occasional enhancement of 2,4-D activity when applied with DMSO, but their observations on plant membranes failed to show either alteration of the membrane behavior or increased rate of water movement from DMSO. The ability of DMSO to form complexes with many metal ions (78) gives it an interesting property to study if DMSO could act as a carrier aiding in foliar penetration and translocation.

pH:

Rapid phosphate uptake by leaves was facilitated by pH 2 to 3 as compared to pH 8 (54, 149, 155, 156). Swanson and Whitney (155) ascribed the increased P uptake and translocation at the lower pH level, to the suppression of the dissociation of phosphoric acid, and to a possible direct effect on the permeability of the adjacent cells. On the other hand, Boroughs, et al. (14), using P^{32} -labelled sodium, potassium, and ammonium

phosphates on cacao leaf found maximum absorption at pH 2 for potassium, 10 with ammonium, and 5 with sodium. Teubner, et al. (156) found K and Rb uptake was least at pH 2 and 4 and greatest at pH 8. Unlike phosphate, pH did not affect the uptake of sulfate (7).

Surface-active Agents:

The value of surfactants in commercial sprays to ensure wetting of leaf surfaces and to increase penetration of sprays into plants has long been recognized (39, 44, 79). Surfactants facilitate both stomatal and cuticular penetration presumably by reducing suface tension of solutions (44). Cook and Boynton (36) and Guest and Chapman (64) obtained a 100% increase in urea absorption by apple leaves by using 0.1% Tween 80 (a sorbitan mono-oleate polyoxyalkylene derivative) or 0.01% Tween 20 (a sorbitan mono-laurate polyoxyalkylene derivative). The effects of surfactants on foliar uptake, however, have not always been positive (4, 95, 155, 156).

Morphological Characteristics of Leaf Surfaces

Pathways of entry of sprayed substances into leaves have not been clearly delineated. Suggested preferential routes include hairs (47), guard-cell walls (182), tears or punctures

(132), stomata (36, 66), and through cuticular membranes (143, 165).

Cuticular Membrane:

By virtue of its position, the cuticular membrane, which sheaths the aerial parts of higher plants, is the first barrier to foliar penetration. Brongniart (19, 20) first described this thin membrane as non-cellular in structure and named it "cuticle." Later, Lee and Priestley (102) suggested that the cuticle was formed from epidermal lipoid substances which migrated to the surface and oxidized and polymerized on exposure to the atmosphere to form a varnish-like covering.

Frey-Wyssling (59) summarized the chemical nature of cutinized plant cell walls as follows: they are made up of four distinct substances, all of which may vary in distribution within the wall. These substances are: 1. cutin, 2. cutin wax, 3. pectin and 4. cellulose. Anderson (1) showed that adjacent to the cell lumen the cellulose-pectin become of decreasing order with cutin and cutin waxes becoming of increasing order as it extended to the outermost zone.

The chemical components of cuticles have been separated by many workers. Fremy (58) saponified cutin from agave and obtained two acids, stereocutic and oleocutic acids. Legg

and Wheeler (104) further resolved the cutin of agave into cutic acid ($C_{26}H_{50}O_6$) and cutinic acid ($C_{13}H_{22}O_3$). Matic (112), Roberts, et al. (140) and Yamada (174) examined wax and cellulose free cuticles of several species of plants. Matic found upon saponification of agave a mixture of acids, 58% of which was ether-soluble and 17% was water-soluble. The ether-soluble fraction consisted largely of a mixture of hydroxylated octa- and hexadecanoic acids. The cutin was thus a polyester with polar properties. This gives it an affinity for water and cations because of its pronounced negative charge. Higher development of cutin was observed on the upper surface than the lower surface of leaves (111). Martin (111) described waxes as consisting of paraffins, alchols, ketones, acids, triterpenes and hydroxy acids. Cellulose is composed of very long chain molecules that are relatively stable imparting tensile strength and elastic cell walls. Pectins are made of long chain polygalacturonic acid molecules having side carboxyl groups. These are capable of forming salts, most notably Ca-pectate. Polygalacturonic acid is readily soluble in water but its Ca salt is insoluble. Pectins are soluble in pectic acid and hydrogen peroxide and readily break down upon hydrolysis and by treatment with pectric enzymes.

Utilizing this knowledge, Roelofsen (141) isolated the

cuticles of Clivia nobilis leaves by extracting the underlying pectic layer with 5% hydrogen peroxide or by 2% ammonium oxalate solution. Skoss (150) employed a culture of Clostridium roseum to enzymically decompose various plant tissues and as a consequence caused the release of cuticles. Wood, et al. (171) were able to release cuticles of various plants by using filtrates from culture solutions of Bacterium aroideae. Orgell in 1955 (132), as a partial fulfillment for his Ph.D. degree, successfully and conveniently isolated leaf cuticles by use of pectinase.

Probably the most characteristic feature of plant cuticles is their extreme variability among plants and plants of different environment. This involves not only chemical variability as already discussed, but variability in distribution over the plant surfaces (89, 132), and in thickness on various parts of a single leaf (89, 155). Succulent xerophytes such as cacti and agave have thick cuticles; whereas, the hydrophytic water lily have undetectably thin cuticles. Herbs that characteristically grow in the shade have thin cuticles and a low wax content (74, 150, 174). Many workers have observed differences in thickness between upper and lower leaf surface cuticles (41, 61, 66, 89).

Although the cuticular layer is acceptably viewed as continuous over the surface of leaves, a controversy still rages whether or not the cuticle sheathing the stomata is perforated.

Electron micrograph by Franke (56) failed to show any holes on the cuticle where the stomata existed. Other observers have given substantial proof to the absence of holes through the stomatal pores (82, 105, 148, 177, 178).

Although cuticular membranes have long been described and their chemical properties analyzed, it was not until recently that the permeability properties were studied thoroughly. Hurst (75) working with insect cuticles first reported that the rates of movements of organic substances and even water may not be the same when they are escaping (efflux) as when they are entering (influx). Schieferstein (146) while studying ivy leaf cuticular membranes confirmed that there was 1.44 times greater permeability to water in the inward than the outward direction. Moreover, Yamada, et al. (177) conclusively demonstrated by using tomato fruit cuticle (astomatous) and onion leaf cuticle (stomatous) that influx was much greater than efflux for organic substances such as urea, maleic hydrazide and N, N-dimethylamino succinamic acid and inorganic ions such as SO_4^{--} and Ca^{++}. The order of permeability through the two cuticular membranes was organic substances > cations > anions. The difference in the rate of urea influx and efflux through onion leaf cuticle was not significant.

The same authors (176) conducted interesting experiments on the ion binding capacity of the outer and inner cuticular

surfaces. They found the anion and cation binding capacities of the inner surfaces were 2 and 13 times greater, respectively, than that of the outer surfaces. Yamada, Rasmussen, Bukovac and Wittwer (178) also localized ion binding sites on cuticular surfaces by using microautoradiography. These vividly showed that the inner surface had more ion binding capacity, especially for cations. This is concordant with the fact that cuticles are negatively charged on the inside surface because cutin, the major constituent of cuticles, is made of polymerized dicarboxylic and hydroxylcarboxylic acid with negatively charged end groups.

Any association of penetration of organic and inorganic substances with cuticle thickness is difficult because of chemical and structual differences. It has been observed that with the cuticular membrance of Hedera helix L., that permeability to water increased with leaf age; whereas it decreased greatly to the sodium salt of 2,4-D (147). Repp (137) attempted to relate herbicidal sensitivity to cuticular structure, and found that the thickness of the outer epidermal cell wall was not as important as cuticular structure.

A possible pathway for sprayed substances across cuticular membrane was first proposed by Roberts, et al. (139). Crafts and Foy (38) later suggested two preferential routes: "Some apparently penetrate rapidly via the lipid phase of the

cuticle, but others of a highly polar nature, for instance, maleic hydrazide (MH), PO_4^{-3}, and K^{+1} apparently enter the leaf via an aqueous route."

Stomata, Guard Cells, Ectodesmata:

Teubner, et al. (156), and others (5, 165, 168), showed there was little or no entry of sprayed substances through stomata. On the other hand, Dybing and Currier (44) along with others (37, 159, 160) stated that entry of aqueous solutions through the stomata is possible by the addition of proper surfactant to the solution. Cook and Boynton (36) found with proper wetting agents that penetration was always greater through the lower surface of an apple leaf (stomatous) than through the upper surface (astomatous). Contradictory results were obtained on bean plants by Teubner, et al. (156), wherein absorption of P^{32} was almost double on the upper as compared with the lower leaf surface. Frequency of stomata, however, was over seven times greater on the under surface of bean leaves.

According to the more refined observations of Sargent and Blackman (145) absorption, at least of 2,4-D, cannot take place through stomatal pores but must occur through the outer walls of the guard-cells and around them in adjacent accessory cells. It is a striking fact that in guard-cells and around adjacent accesory cells there is found as a rule a concentration of

ectodesmata (57). Franke (57), after observing ectodesmata by electron microscopy described them as thread- or ribbon-like forms which extended across the outer walls of epidermal cells from the lumen to the cuticle. To give the proof lacking for Sargent and Blackman's conclusion, that guard-cells and not the stomatal pores are the sites of absorption, Franke demonstrated with droplets of radioactive solutions applied on the epidermis and by subsequent microautoradiography that the guard-cells of spinach and Viola were the favored absorption sites. Autoradiograms of ectodesmata distribution, demonstrated that areas of favored absorption corresponded to areas of greatest ectodesmatal concentration. No black spots from the autoradiograms were observed in the stomatal pores.

Ion Uptake by Isolated Leaf Cells:

It is of interest to know if the mode of solute penetration and absorption through the whole leaf and isolated leaf cells is similar. Smith and Epstein (151) working on corn leaf discs found that penetration through the surface layer of the leaf does not contribute to absorption, rather, the uptake of Rb on a fresh weight basis varied inversely with the diameter of the disc. This indicated that only those cells near the cut edges absorb ions from the solution.

The rate-limiting barriers, cuticular membranes and epidermis, may be eliminated and the mesophyll cells directly exposed to the solution by enzymic isolation of single cells (32, 43, 86, 87, 88, 179, 180); or by leaf tissue slices (49, 50, 151, 152). Smith and Epstein (151) found maximum uptake of ions when the leaf tissue slices were cut 300u wide and 12 mm long.

Unpublished data of De, et al. (43) indicated that $^{59}Fe^{++}$ in $FeSO_4$, and FeEDDHA had a similar order of absorption in both intact bean leaves and isolated leaf cells, even though the quantitative uptake per weight was higher for single cells.

Environmental Factors

Temperature:

Absorption and translocation increase with temperature (61, 66, 84, 133, 145, 155, 156, 181). On the basis of the relatively high temperature coefficients obtained (84, 85, 155, 156), and supported by other tests (84, 85) foliar penetration could be considered as metabolically governed.

Light:

The effect of light on foliar uptake is not conclusive.

Numerous workers (33, 34, 66, 67, 156) found apparent stimulation of foliar uptake by the presence of light. Weintraub, et al. (167), on the other hand, could find no effect of light on the absorption of 2,4-D by bean leaves. Gustafson (66) observed decreased transport of Co^{60} through the phloem if the plant was kept in darkness depleting the leaves' sugar, and the addition of sucrose to the dark-grown plant considerably stimulated the rate of transport. Rice in 1948 (138) stated that penetration into the leaf is not influenced by light, but that subsequent translocation is light-dependent.

Relative Humidity:

Experiments of Clor (33) and Clor, et al. (34) showed that absorption and translocation of chemicals by cotton leaves were greatly increased under conditions of high humidity.

Techniques Employed in Foliar Absorption Studies by Use of Radioisotopes

Techniques of Applying Solutions:

Jyung (83) has given a comprehensive review of the techniques used for applying mineral solutions to leaf surfaces for study of the mechanisms and pathways of foliar absorption. The following are some of those commonly used. Spraying the

entire above ground parts (68, 73, 149); or spraying of single leaves or selected leaves (6, 36, 46, 48, 95, 156). The leaf surface may be painted (4, 157) or dipped (23, 113, 156, 170). Solutions have also been applied to above-ground parts as single drops (4, 23, 24, 44, 83, 95, 156); or multiple drops held in place from running-off by a lanolin ring (66), and the "sticking" method of Okuda (129, 174). The recently developed "leaf-immersion technique," of Jyung and Wittwer has given reproducible experimental results (84, 85). Undoubtedly the lack of one single standard experimental method has greatly contributed to the present capricious experimental results and theories for the mechanisms and pathways of foliar absorption of mineral nutrients.

Methods of Removal of Non-Absorbed Residues:

Foliar absorption can only be expressed accurately after the non-absorbed or adsorbed residues are completely removed from the leaf surface. The most common procedure for removal of residues has been a washing technique. Washing solutions have consisted of distilled water (24, 29, 44, 83, 97, 129, 149, 174); acidified water (68); and water with detergent (4, 36). Jyung (84) tested the effectiveness of different washing solutions for removing the non-absorbed residue remaining from one drop of P^{32} solution of H_3PO_4 applied on the leaf of

bean plant. He found no statistical significance in the effectiveness of removing the residues among solutions of distilled water, H_3PO_4, at 10^{-1}, 10^{-2}, or 10^{-3} M, and $CaCl_2$, KH_2PO_4, or NaOH at 10^{-2} M.

Methods of Measuring Cuticular Penetration:

Cuticular penetration studies utilizing labelled organic and inorganic substances have been performed by several types of apparatus: Diffusion through and into an agar block (40, 89); a diffusion cell apparatus (8, 9); and Yamada's water-bath method which will be dealt with in detail later (174, 175, 177).

Sample Preparation and Expression of Results:

With the advent of radioisotopes the study of mechanisms of uptake of mineral nutrients has become simplified and precise. The relative amounts of absorbed or translocated labelled nutrients may be determined by counting of the dried plant samples. Direct counting may also be made after ashing the dried tissue (23, 24, 44, 83, 155, 156). Oven-dried samples may be alternatively ground and only an aliquot radioassayed (12, 13, 101). Depending upon the characteristics and amount of radioactivity present, fluid samples may be assayed directly from planchets or vials, or the liquid evaporated on a hot plate or

by an infrared heat and counted.

It is often necessary to prepare solid counting samples from suspensions, for instance, isolated cell suspensions. In this instance, collection of precipitates or cells may be performed on filter paper mounted on a precipitation apparatus (86).

Data from radioisotope studies have been reported in various ways. Radioactivity may be expressed in counts per minutes or seconds (4, 22, 44, 89, 95, 129, 149, 156, 174); percentage of dose applied (4, 23, 25, 29, 83, 95, 156, 177); or in absolute quantity (μg, μM, mg) per specified material per unit time (84, 87, 88, 98, 156, 175, 176, 177).

EXPERIMENTAL METHODS

Chemicals for Leaf Cuticle Isolation and Procedures:

The most frequently used method for isolating cuticular membranes from leaf surfaces is Orgell's (132) enzymic method. After numerous trials with Orgell's enzyme solution on leaves of some woody plants improvements were introduced to facilitate easy separation of cuticles from some plant species. These modifications of Orgell's enzymic method are summarized in Table I, and the isolation procedures are outlined briefly in Table II.

Cuticular membranes were separated from the leaves of the following species using these procedures: *Agave americana* L., *Malus pumila* (apple), *Prunus persica* (peach), *Prunus armeniaca* (apricot), *Prunus cerasus* (sour cherry), *Pyrus communis* (pear), *Syringa vulgaris* (lilac), *Coffea arabica* (coffee), *Lycopersicon esculentum* (tomato), and *Allium cepa* (onion). Plant materials were obtained from the University campus and nearby Horticultural farms. The mature coffee leaves were sampled from a 10 year old tree growing in a greenhouse.

Studies on Penetration Through Cuticles:

Monovalent cations, Na^{+}, K^{+}, Rb^{+}, Cs^{+}, and divalent cations, Ca^{++}, Sr^{++}, Ba^{++}, differing in ion size were selected from respective groups of the Periodic Table and labelled with

Table I. Chemicals and Enzymes for Separation of Cuticular Membranes:

1.	Acetate Buffer (Walpole Buffer) pH 3.5 - 4.5	2.0M
2.	Pectinase (Nutritional Biochem. Corp.)	2.0%
3.	Cellulase (Nutritional Biochem. Corp.)	0.2%
4.	Hemicellulase (Nutritional Biochem. Corp.)	0.2%
5.	Ethylenediamine Tetraacetic Acid (EDTA)	0.2M
6.	Merthiolate (Disinfectant)	100 ppm

Table II. Procedures for Isolation of Cuticular Membranes:

1. Cut-out midribs and trim edges of leaves. Place leaves in buffered enzyme solution.
2. Aspirate. Weight leaves down.
3. Incubate at 20 - 35°C.
4. Separate one edge. Slowly peel-off.
5. Wash cell fragments from surface with wet tissue paper and rinse.
6. Store in distilled water or dry. Identify origin.
7. Check for imperfections.

radioisotopes. All ionic solutions were prepared as the chloride salt. Permeability studies with the monovalent cations were conducted with Euonymus japonicus cuticles derived from both leaf surfaces (stomatous on the dorsal and astomatous on the ventral). Studies with divalent cations were restricted to the upper leaf surface membranes.

DMSO (10^{-4}M) was added, after preliminary experiments to determine desirable concentrations, to $^{59}FeSO_4$ and to ^{59}FeEDDHA solutions, and the effects noted on the penetration of $^{59}Fe^{++}$ through the cuticle derived from the upper surface of Euonymus leaves. The effects of ethylenediamine tetraacetic acid (EDTA), and ethylenediamine di-o-hydroxyphenylacetic acid (EDDHA) on the penetration of $^{59}Fe^{++}$ through cuticles (astomatous) isolated from the upper surface of coffee leaves was also studied.

The monovalent ion treating solutions containing $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, $^{137}Cs^{+}$ were all 1.0 mM and their specific activity about 0.3 µc/µmole. Solutions containing the divalent cations were 0.1 mM, and had a specific activity of 3µc/µmole for $^{45}Ca^{++}$, $^{89}Sr^{++}$, $^{133}Ba^{++}$, and 5µc/µmole for $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$. The synthetic chelates were prepared in equimolar concentration as the ions. A pH of 6 was maintained for all treating solutions.

The Yamada, et al. (175) apparatus was used to study the

penetration of ions through isolated cuticular membranes. A large test tube (35mm diam.) with 20ml deionized water was suspended in a constant temperature (20°C) water bath. A smaller tube (15mm diam.) was in turn suspended inside the large test tube after the cuticular membrane to be studied was affixed onto it with rubber cement and wrapped by narrow stripes of Parafilm[1]. Fixation of the cuticular membrane was carefully done to resemble the normal surface orientation on leaves. 3 ml of the labelled solution was added to the small tube and it was carefully lowered into the deionized water in the large test tube to the level of the meniscus in the small tube.

The rate of ionic penetration through the membranes was determined by radioassays of 1 ml aliquots taken periodically from the outer solution in the large test tube. A gamma scintillation-well detector was employed for $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, $^{137}Cs^{+}$, $^{59}Fe^{++}$, $^{54}Mn^{++}$, $^{65}Zn^{++}$ and an open-end G-M detector for $^{45}Ca^{++}$, $^{89}Sr^{++}$, and $^{133}Ba^{++}$.

Permeability Studies with Dormant Euonymus japonicus Leaves:

Euonymus leaves were harvested in February from plants over-wintering outdoors and used for a comparative study of the passage of ions through cuticles intact on the leaf surface

[1]A product of the Marathon, Division of the American Can Company, Neenah, Wisconsin.

with those enzymically isolated from the leaf. Metabolic activity of the leaf cells in midwinter was assumed to be negligible.

These freshly harvested leaves were placed in 40 ml of an isotopic solution of $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, or $^{137}Cs^{+}$ in Petri dishes, with either the upper or lower, or both leaf surfaces, in contact with the solutions. The isotopic solutions were of the same concentration and the specific activity as those used in the isolated Euonymus leaf cuticle study. After 5 hours the leaves were washed in running tap water for one minute, blotted and a 15mm diam. (area = 1.6cm^2) leaf disc removed from the center of the leaf by #8-size cork borer. The radioactivity was determined by a scintillation-well gamma detector.

Foliar Uptake and Translocation by Coffee (Coffea arabica L.)

One pound of fresh coffee seeds Coffea arabica, L. Puerto Rican strain of "Borbon" was obtained through the courtesy of Dr. G. J. Rigau, at the Agricultural Experiment Station of the University of Puerto Rico. Seeds were sown in flats containing vermiculite and placed in a greenhouse. Seedlings were transplanted 5 months into a sand-muck mixture and spaced 2 by 2 inches. When the seedlings were about 9 months old or about 6 inches in size, they were transferred to aerated one-half gallon

glass jars filled with one-fourth strength Hoagland solution. There were 4 seedlings in each jar. After 3 days the middle of the underside of the second leaf from the apex was treated with the particular labelled solution according to the "sticking" method described by Okuda and Yamada (129). Two layers of cheese cloth about 1.2 cm diam. were used to retain 0.4 ml of solution.

The treating solutions contained $^{59}FeSO_4$, $^{54}MnSO_4$, $^{65}ZnSO_4$, with or without synthetic chelates (EDTA or EDDHA). The ligands and the metals were equimolar at 0.1 mM. Specific activities for $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ was 20, 15, 15 μc/μmole, respectively. All solutions were adjusted to pH by μ = 0.02M (μ = ionic strength) acetate buffer. Tween-20 (0.01%)was added as a wetting agent.

The cheese cloth used in the "sticking" technique was wetted 3 times at 3 hour intervals with distilled water. Seven days later the experiments were terminated by excising the treated leaf area with a cork borer. The samples were dried at 60°C for 24 hours, and counted for total radioactivity in gamma scintillation detector. Before the leaf discs were dried each was washed with 10 ml distilled water to remove the non-absorbed residues.

Absorption values were summed from the total counts of the leaf disc and the remainder of the plant and expressed in

absolute values as mμmoles/cm^2 area; whereas, translocation was expressed as a percentage of the total absorbed that was found in plant parts other than the treated leaf disc.

All treatments were randomized with 3 replicates. Significant differences among the treatment means were determined by analysis of variance, and expressed as the least differences necessary for significance (LSD).

Ion Uptake by Excised Coffee Leaves:

The excised leaves were also obtained from 9-month old seedlings grown as already indicated. The leaves second from the apex were harvested, rinsed in distilled water, and the ends of the petioles sealed by dipping in rubber cement. The leaves were treated according to the "leaf immersion" technique of Jyung and Wittwer (85). A Petri dish, containing a leaf held in position, was filled with 40 ml of the labelled nutrient solution. Except for pH levels, 3, 4, 5, 6, obtained by use of 0.06M citrate buffer, all experimental solutions were identical to those used in the foliar uptake and transport studies of intact coffee seedlings. Specific activity of the solutions was about 1 μc/μmole.

The Petri dishes were placed under 600 ft-c. fluorescent lamps and evaporation from the surfaces were reduced by mylar covers. All experiments were performed in an air conditioned

laboratory maintained at 22°C, and solutions were likewise maintained at that temperature.

After predetermined times the leaves were removed, thoroughly rinsed for one minute by running tap-water, and blotted. Later, instead of measuring the leaf surface area by means of a planimeter as Jyung and Wittwer (85) specified, 2 cm^2 area leaf discs were removed from the middle of the leaves by means of a size #10 cork borer. The discs were dried for 24 hours in an oven, weighed, and counted in a scintillation gamma detector.

Uptake was expressed in µmoles/mg/time instead of as "specific absorption" which Jyung and Wittwer defined as µmole/cm^2 leaf/24 hours. Three randomly placed replicates were used and the least differences necessary for significance were determined.

Ion Uptake by Isolated Single Cells of Coffee Leaves:

Young leaves from 9-month old coffee seedlings were selected for the cell separation studies. The leaves were rinsed 3 times in distilled water and then shredded into 2 mm or smaller size sections by sharp razor blades. The shredded leaves were then put in the Murashige and Skoog (123) nutrient culture solution to which was added 2% pectinase and 0.1% cellulase and

hemicellulase enzymes. The pH was adjusted to 6. Isolation of coffee leaf cells was difficult without light maceration by a homogenizer for several minutes. The macerated leaf tissue was then transferred into 125 ml Erlenmeyer flasks. These were shaken for 4 hours under light at 150 excursions per minute. Cell suspensions consisting of mixtures of cell walls, mesophyll, palisade cells and organelles were thus obtained.

Procedures which followed were essentially the same as those outlined by Zaitlin (179) and Jyung, et al. (86, 87). The cell suspensions were filtered through 4 layers of cheese cloth, centrifuged at 600 x g for 10 minutes and washed with ice-cold 0.35M sucrose successively 3 times. Cells were then suspended in cold 0.35 sucrose and held at 4°C until used.

The cell incubation procedures outlined by Jyung, et al. (86, 87) were adopted with few modifications. Sodium-acetate buffer was used to give pH 4 and 5 instead of tris-maleate because a lower pH was necessary for a comparison of ion uptake by the isolated leaf cell and the excised leaf.

The composition of the incubation solution in μmoles per 15 ml was as follows: Sucrose 1755, Na_3-citrate 30, Na_2-succinate 30 $NaHCO_3$, and $MgCl_2$ 1.5. The sodium acetate buffer (pH 4 or 5) was $\mu = 0.02M$. The concentration of mineral ions were the same as in those studies just described for excised coffee

leaves. Specific activity was 0.1μc/μmole for $^{59}Fe^{++}$, $^{54}Mn^{++}$, $^{65}Zn^{++}$.

Approximately 80 mg dry weight of living cells was suspended in 15 ml of incubation solution in 50 ml Erlenmeyer flasks and placed in a water bath shaker. It was adjusted to 50 excursions per minute and maintained at 25°C under 600 ft-c illumination from cool-white fluorescent bulbs.

At predetermined times, 2 ml of the incubation mixtures were collected under mild suction on filter paper mounted on a precipitation apparatus, and washed with 1.5 ml deionized distilled water. The samples were then dried, weighed, and radioassayed.

Treatments were replicated twice and significant differences determined by LSD.

Estimates of Variability:

Fluctuations in radioactive decay often result in error in the observed counting rates. To establish the magnitude of the statistical errors one must search for a standard. For practical purposes, Comar (35) gave a simple formula; "The standard deviation will equal the square root of the number of counts." Therefore, in samples that have total counts of 100, 1,000, or 10,000, the standard deviation becomes 10, 32, or 100 counts, respectively. If statistical errors are to be minimized to 1% or 5% then a total count of about 10,000

or 400, respectively, are required. Thus, if a sample has a predetermined count of 100 CPM (minus background), then 5% and 1% errors are obtained if counted for 4 or 100 minutes, respectively. In all experiments reported herein, no more than a 5% counting error was tolerated.

All data were expressed in absolute values (mμmoles per time). Treatments were randomized with 2, usually 3 replications. Significant differences among treatment means were determined by analysis of variance and obtaining values for least differences necessary for significance (LSD).

RESULTS

Isolation of Plant Leaf Cuticular Membranes:

Cuticular membranes were successfully separated from both the upper and lower surfaces of leaves of 10 species. With coffee, only the upper leaf surface was readily obtained. With the exception of agave and onion all species yielded cuticular membranes derived from leaves which were astomatous on the ventral and stomatous on the dorsal leaf surfaces. Microscopic observation of cuticles derived from the stomatous surface showed no evidence of punctures or holes associated with stomatal apertures.

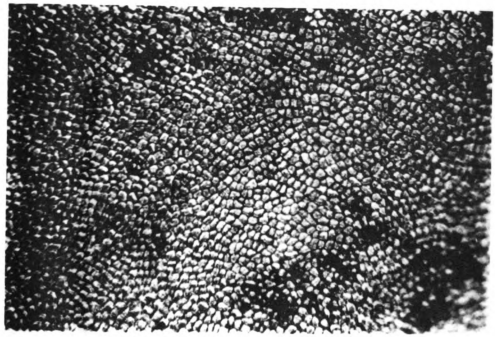
The general appearance and outer surface features of enzymically isolated cuticular membranes from leaves of Euonymus japonicus are illustrated in Figure 1.

Ion Size as Related to the Rate of Penetration Through Stomatous and Astomatous Euonymus Leaf Cuticles:

Comparative rates of penetration of $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, and $^{137}Cs^{+}$ through cuticular membranes derived from both surfaces of Euonymus leaf are illustrated in Figures 2 and 3 and summarized in Table III.

Ion penetration rates were approximately 3-fold greater in the stomatous than the astomatous cuticles. Penetration rates for the 4 monovalent cations through the lower stomatous

Figure 1. Surface view of Euonymus japonicus cuticle enzymically isolated from the ventral (top picture) and dorsal (bottom picture) leaf surfaces. The dark spots are cell fragments, and the granulated appearance is the result of epidermal cell indentations or the cuticular surface. Note the stomata guard-cell impressions observed only on the dorsal leaf surface (bottom picture).



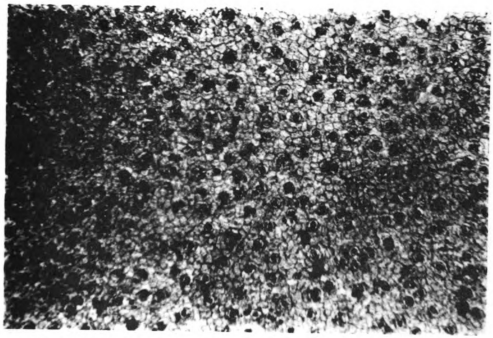
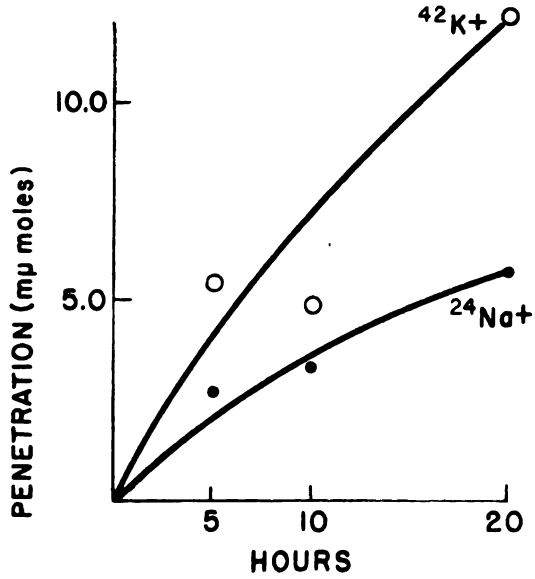
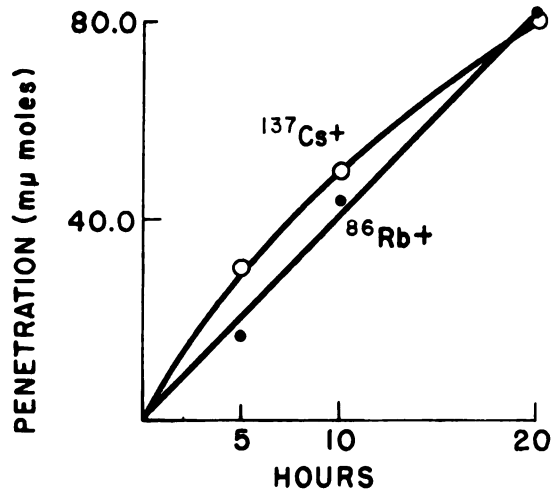


Figure 2. Top -- Penetration of $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, and $^{137}Cs^{+}$ (1.0mM conc.) through Euonymus japonicus cuticle separated from the ventral leaf surface (astomatous). Each value a mean of 3 replicates.

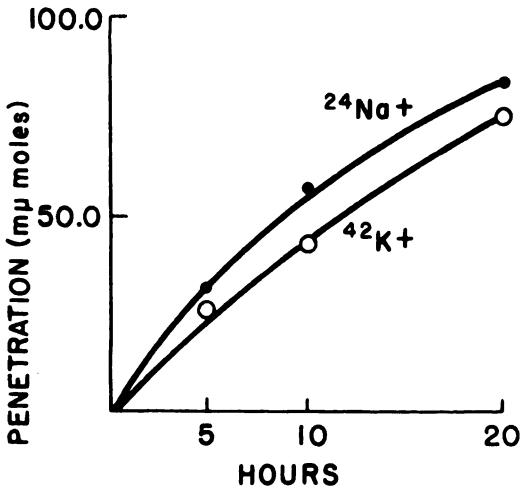
Figure 3. Bottom -- Penetration of $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, and $^{137}Cs^{+}$ (1.0mM conc.) through Euonymus japonicus cuticle separated from the dorsal leaf surface (stomatous). Each value a mean of 3 replicates.

EUONYMUS CUTICLE-UPPER LEAF SURFACE (ASTOMATOUS)





EUONYMUS CUTICLE-LOWER LEAF SURFACE (STOMATOUS)



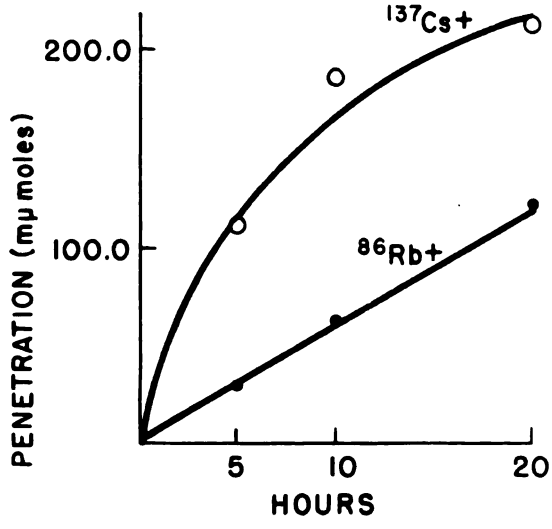


Table III. Monovalent ion penetration through Euonymus leaf cuticles (mμmoles/20 hrs).

Origin of cuticle	Ions				Average effect for cuticle
	$^{24}Na^{+}$	$^{42}K^{+}$	$^{86}Rb^{+}$	$^{137}Cs^{+}$	
Upper (astomatous)	5	12	83	83	46
Lower (stomatous)	85	76	122	213	124
Average effect for ions	45	44	102	148	

LSD (5%)

Ions	39
Cuticles	27
Ions x Cuticles	55

Euonymus leaf cuticular membranes was Cs > Rb > K = Na and Cs = Rb > K > Na for the upper astomatous surface. The ratio of Cs to Na that penetrated through the upper astomatous Euonymus leaf cuticles was 17 to 1, but only 2.5 to 1 for the lower stomatous cuticle.

Penetration rates for three divalent cations, $^{45}Ca^{++}$, $^{89}Sr^{++}$, and $^{133}Ba^{++}$ through cuticular membranes from the upper leaf surface of Euonymus japonicus are illustrated in Figure 4. The order of permeability was $^{133}Ba^{++} > ^{89}Sr^{++} > ^{45}Ca^{++}$.

Effects of DMSO on Ion Penetration Through the Astomatous Cuticular Membrane of Euonymus Leaves.

The additon of 10^{-4}M DMSO did not result in a significant difference in either $^{59}FeSO_4$ or ^{59}FeEDDHA permeability through the upper Euonymus leaf cuticle (Figure 5).

Penetration of $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ as the Sulfate and When Chelated with EDTA or EDDHA Through Upper Coffee Leaf Cuticular Membrane (Astomatous).

All data are summarized in Figure 6. The amounts of $^{59}Fe^{++}$ that penetrated after 36 hours as the sulfate was 2 and 4 times greater than when $^{59}Fe^{++}$ was complexed with

either EDTA and EDDHA, respectively. $^{54}Mn^{++}$ and $^{65}Zn^{++}$ as sulfate salts were likewise more permeable than when chelated with EDTA or EDDHA. The total that penetrated was several fold greater for $^{59}Fe^{++}$ than for $^{54}Mn^{++}$ and $^{65}Zn^{++}$. Penetration rates varied among replicates.

Comparative Uptake of $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, $^{137}Cs^{+}$ by Different Surfaces of Excised Dormant Euonymus Leaves.

Data in Table IV show that the uptake of the four ions, $^{24}Na^{+}$, $^{42}K^{+}$, $^{86}Rb^{+}$, and $^{137}Cs^{+}$ by excised dormant Euonymus japonicus leaf surfaces was not significantly different. On the other hand, there was approximately a 5-fold greater uptake of all ions from the lower stomatous leaf surface than from the upper astomatous surface. Ion uptake by the submerged leaf was 2-fold greater than through the upper leaf surface alone.

Absorption and Translocation of Ions from Leaves of Coffee Seedlings:

Table V shows the absorption of $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ as the sulfate salts and in combination with EDTA and EDDHA applied by the "sticking" method on the lower leaf surfaces of 9-month old coffee seedlings.

Figure 4. Penetration of $^{45}Ca^{++}$, $^{89}Sr^{++}$, and $^{133}Ba^{++}$ (0.1mM conc.) through Euonymus japonicus cuticle separated from the ventral leaf surface (astomatous). Each value a mean of 3 replicates.

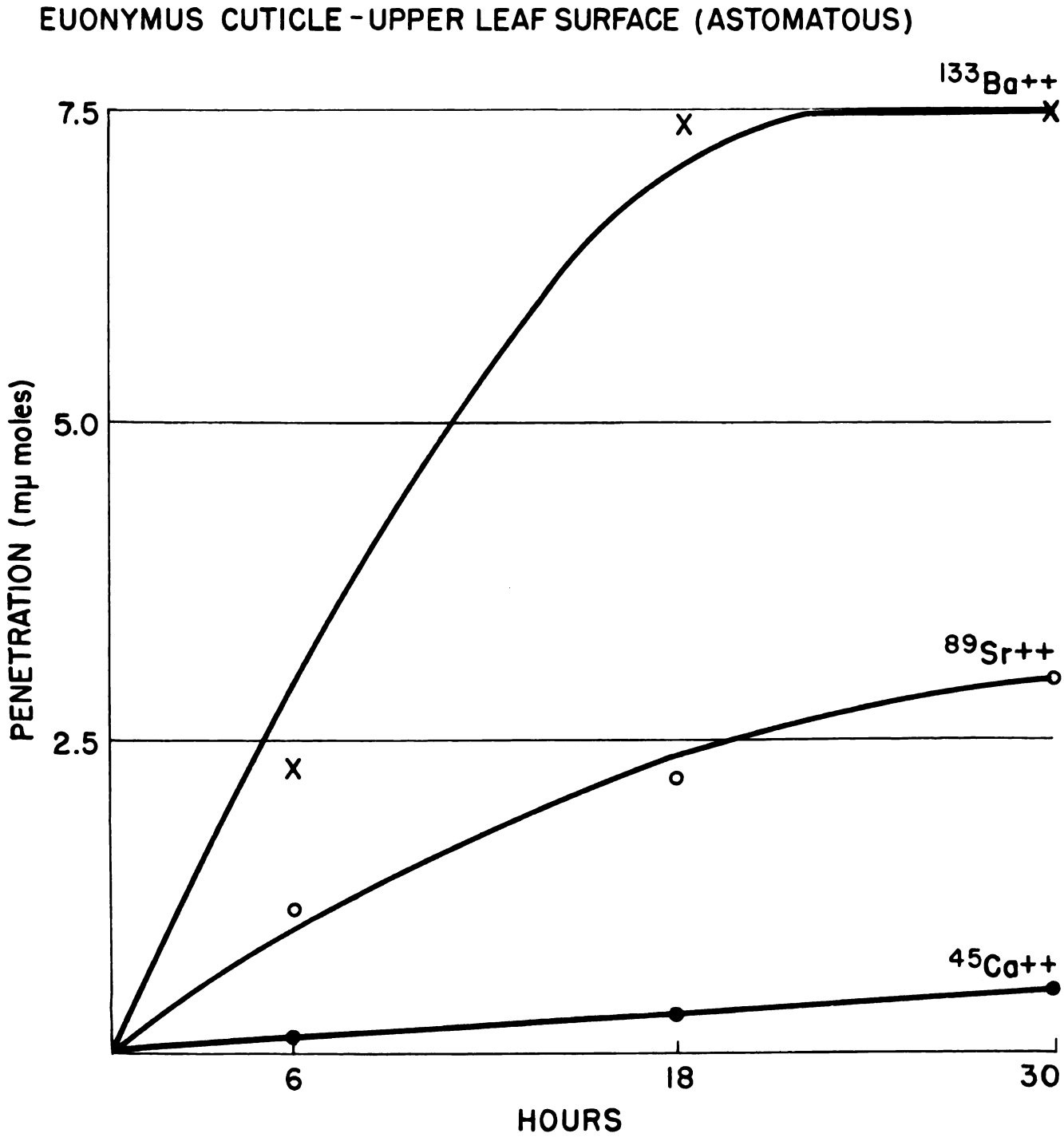

EUONYMUS CUTICLE - UPPER LEAF SURFACE (ASTOMATOUS)
$^{133}Ba^{++}$
$^{89}Sr^{++}$
$^{45}Ca^{++}$
7.5
5.0
2.5
PENETRATION (mμ moles)
6
18
30
HOURS

Figure 5. Effect of DMSO (10^{-4}M) on the penetration of $^{59}Fe^{++}$ as the sulfate or chelated with EDDHA (0.1mM) through Euonymus japonicus cuticular membranes isolated from the ventral (upper) leaf surface (astomatous). Each value a mean of 2 replicates.

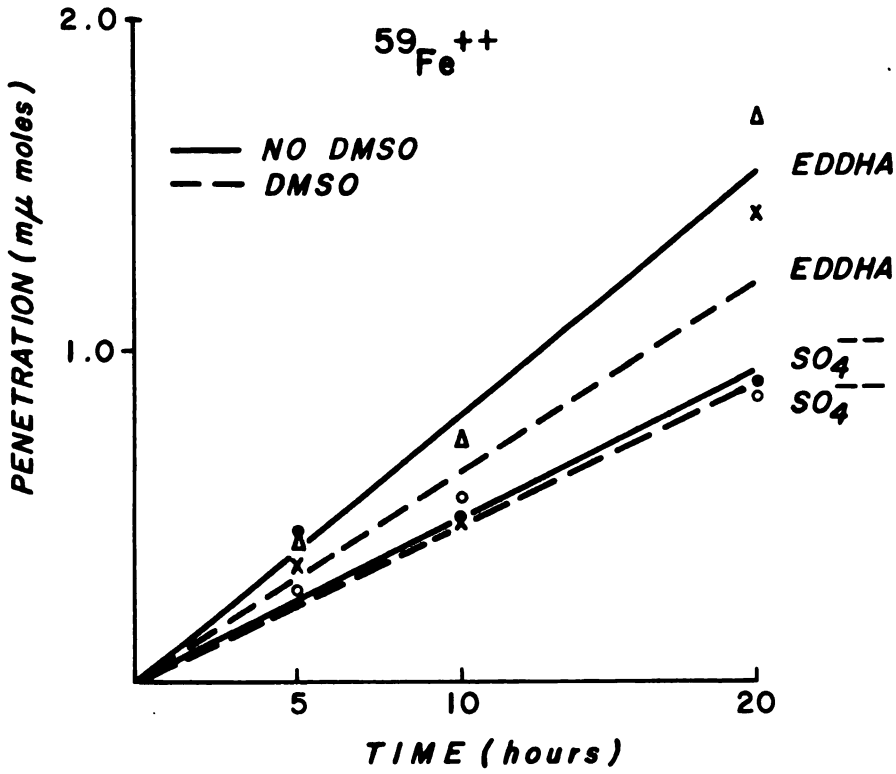

2.0
1.0
$^{59}Fe^{++}$
NO DMSO
DMSO
EDDHA
EDDHA
SO_4^{--}
SO_4^{--}
PENETRATION (mμ moles)
5
10
20
TIME (hours)

Figure 6. Effect of synthetic chelating agents and the sulfate on the penetration of $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ through coffee cuticular membrane isolated from the ventral (upper) leaf surface (astomatous). Concentration of both ligands and ions at 0.1mM. Each value a mean of 3 replicates.

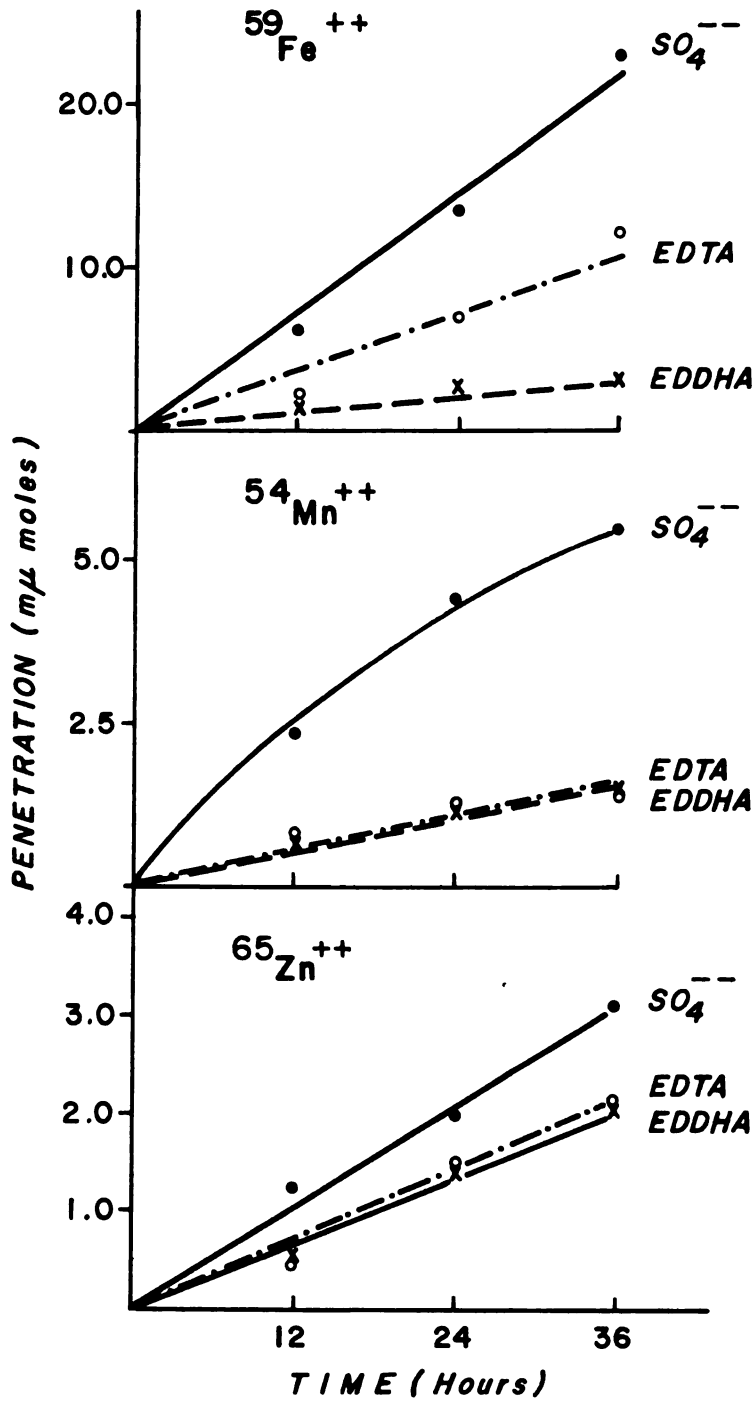

59Fe++
SO4--
EDTA
EDDHA
54Mn++
SO4--
EDTA
EDDHA
65Zn++
SO4--
EDTA
EDDHA
20.0
10.0
5.0
2.5
4.0
3.0
2.0
1.0
12
24
36
PENETRATION (mμ moles)
TIME (Hours)

Table IV. Comparative absorption (mμmoles/1.6 cm^2/5 hrs.) by different surfaces of dormant Euonymus japonicus leaves.

Surfaces	$^{24}Na^+$	$^{42}K^+$	$^{86}Rb^+$	$^{137}Cs^+$	Average effect for surfaces
Upper (astomatous)	30	32	28	34	31
Lower (stomatous)	142	184	129	156	152
Both (submerged)	71	81	60	61	68
Average effect for ions	81	99	72	84	

LSD (5%): Surface = 21; Ions = N.S.; Surface x Ions = N.S.

Table V. Absorption of ions (mμmoles/cm^2) by leaves of coffee seedlings. (Mean of 3 replicates).

	SO_4^{--}	EDTA	EDDHA	LSD (5%)
$^{59}Fe^{++}$	9.3	7.4	6.9	1.9
$^{54}Mn^{++}$	5.3	3.9	7.0	N.S.
$^{65}Zn^{++}$	9.0	6.1	8.2	N.S.

EDTA and EDDHA added to $^{59}FeSO_4$ decreased the absorption of $^{59}Fe^{++}$ by about 20% as compared with $^{59}Fe^{++}$ applied as the sulfate alone. $^{65}Zn^{++}$ absorption by the coffee leaf was likewise reduced by 32% and 10% when applied with EDTA and EDDHA, respectively, while $^{54}Mn^{++}$ absorption was greatest when complexed with EDDHA. Statistically the effects of chelation were significant only for iron.

Translocation subsequent to foliar absorption of $^{59}Fe^{++}$ and $^{65}Zn^{++}$ by coffee leaves was greatly altered by the form in which the labelled metals were applied (Table VI). Mobility of $^{59}Fe^{++}$ in combination with EDDHA was about 3 times greater than when applied as the sulfate, and about twice as great with EDTA. $^{65}Zn^{++}$ translocated was 2.5 times greater as the ^{65}ZnEDTA or ^{65}ZnEDDHA complex than as a sulfate. $^{54}Mn^{++}$ mobility, however, was not significantly affected by chelation. The percentages of the metals translocated from the sulfate salts as compared to the total amounts absorbed were 0.6 for $^{59}Fe^{++}$, 1.4 for $^{65}Zn^{++}$, and 7.6 for $^{54}Mn^{++}$.

Uptake of Ions by Excised Coffee Leaves as Modified by Chelation and pH.

Data are portrayed in Figure 7, and statistically

Table VI. Mobility of ions absorbed by coffee leaves as affected by chelation (Mean of 3 replicates).

	Percent translocated			
	SO_4^{--}	EDTA	EDDHA	LSD (5%)
$^{59}Fe^{++}$	.6	.7	1.5	.4
$^{54}Mn^{++}$	7.6	8.9	5.7	N.S.
$^{65}Zn^{++}$	1.4	3.4	3.4	1.3

Figure 7. Effects of synthetic chelating agents and pH on $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ absorption by excised coffee leaves. Leaves were immersed in 40 ml of 0.1mM solutions for 4 hours at 22°C and 600 ft-c. Each value a mean of 3 replicates.

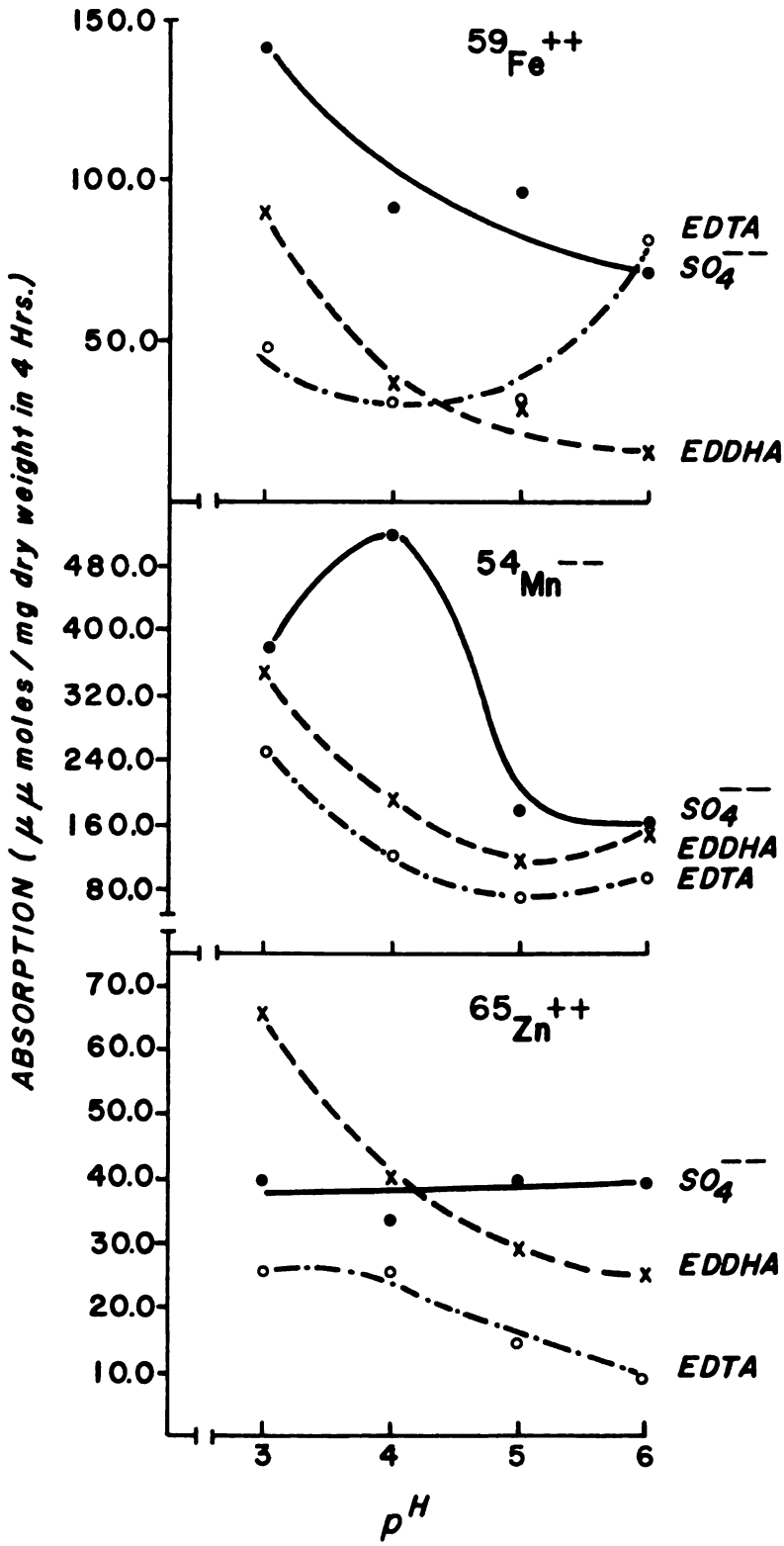

150.0
100.0
50.0
59_{Fe}^{++}
EDTA
SO_4^{--}
EDDHA
480.0
400.0
320.0
240.0
160.0
80.0
54_{Mn}^{--}
SO_4^{--}
EDDHA
EDTA
70.0
60.0
50.0
40.0
30.0
20.0
10.0
65_{Zn}^{++}
SO_4^{--}
EDDHA
EDTA
3
4
5
6
pH
ABSORPTION (μμ moles / mg dry weight in 4 Hrs.)

Table VII. Effects of pH and chelations on the uptake (mμmoles/mg/4 hrs) of $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ by excised coffee leaves.

pH	SO_4^{--}	EDTA	EDDHA	Average effect for pH
		$^{59}Fe^{++}$		
3	142	49	90	94
4	92	32	35	53
5	99	34	30	54
6	71	81	14	55
Average effect for chelates	101	49	42	
LSD (5%) Chelates = 24; pH = 28; Chelates x pH = N.S.				
		$^{54}Mn^{++}$		
3	386	254	354	331
4	520	121	189	277
5	172	73	114	120
6	160	99	147	135
Average effect for chelates	309	137	201	
LSD (5%) Chelates = 61; pH = 70; Chelates x pH = 122				
		$^{65}Zn^{++}$		
3	40	26	67	44
4	33	26	41	33
5	40	14	29	28
6	38	8	25	24
Average effect for chelates	38	18	45	
LSD (5%) Chelates = 12; pH = N.S.; Chelates x pH = N.S.				

summarized in Table VII. In general the greatest uptake of $^{59}Fe^{++}$ and $^{54}Mn^{++}$ occurred at pH 3. Exceptions were $^{54}MnSO_4$ and $^{59}FeEDTA$ where maximum absorption was at pH 4 and 6, respectively. $^{65}ZnSO_4$ as the sulfate or complexed with chelates gave no statistically significant response to pH variation even though absorption was higher at pH 3 than at pH 6 for the chelated metal. At a given pH, non-chelated forms of all three ions were generally most readily absorbed. Least uptake of $^{54}Mn^{++}$ and $^{65}Zn^{++}$ occurred with EDTA.

Uptake of Ions by Isolated Coffee Leaf Cells as Modified by Chelation and pH.

Absorption rates of ions at pH 4 and 5 by cells isolated from the lamina of green leaves are summarized in Table VIII and Figure 8. The effects of pH were generally insignificant except for $^{54}Mn^{++}$ where absorption was generally greater at pH of 5.

As with excised excised coffee leave, the non-chelated ions were absorbed more readily than the chelated forms. Metals complexed with EDTA were absorbed less rapidly than when chelated with EDDHA.

Figure 8. Effect of synthetic chelating agents on $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ absorption by cells isolated from coffee leaves. The two pH levels, 4 and 5, were achieved by μ = 0.02M acetate buffer. Cells were incubated in 15 ml 0.1mM conc. of the respective ion. Cells were exposed to 600 ft-c light and 25°C for 2 hours. Each value is the mean of 2 replicates.

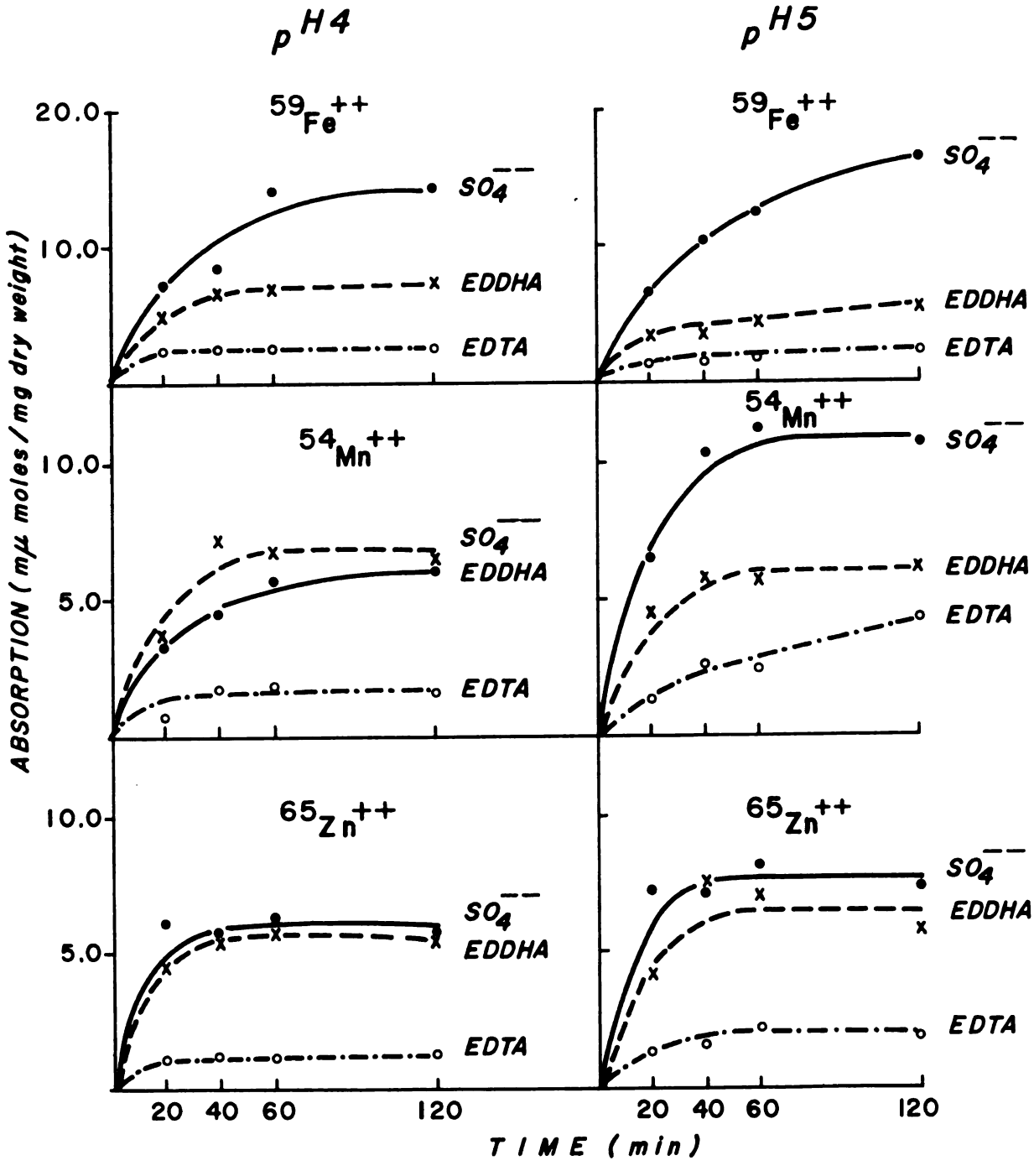

pH4
pH5
59Fe++
54Mn++
65Zn++
SO4--
EDDHA
EDTA
20.0
10.0
5.0
20
40
60
120
ABSORPTION (mμ moles/mg dry weight)
TIME (min)

Table VIII. Uptake of ions (mμmoles/mg/ 2 hours) by cells isolated from coffee leaves as modified by chelation and pH.

pH	SO_4^{--}	EDTA	EDDHA	Average effect for pH
		$^{59}Fe^{++}$		
4	14.1	2.7	7.4	8.1
5	16.3	2.4	5.7	8.1
Average effect for chelates	15.2	2.5	6.5	
LSD (5%) Chelate = 2.4				
		$^{54}Mn^{++}$		
4	6.0	1.6	6.3	4.6
5	10.9	4.3	7.2	7.5
Average effect for chelates	8.4	2.9	6.7	
LSD (5%) Chelate = 3.1; pH = 2.5				
		$^{65}Zn^{++}$		
4	5.8	1.3	5.4	4.2
5	7.4	1.9	5.9	5.1
Average effect for chelates	6.6	1.6	5.6	
LSD (5%) Chelate = 1.0				

DISCUSSION

Permeability of Cuticular Membranes:

Improved techniques for cuticular membrane seperation from the surfaces of the leaves of some of the plant species employed was necessary. Orgell's (132) method of enzymic isolation and later modified by Yamada, et al. (175) was further improved. Leaves of 10 species of plants were successfully skinned of their cuticle by following newly developed techniques with leaves incubated in additionally fortified enzyme solutions.

Numerous reports have conclusively vindicated that the cuticular membrane is the most important barrier to entry of sprayed solutes into plants (75, 149, 174, 175). These workers have also shown that there is greater unidirectional penetration of solutes from outside to inside than inside to outside. Yamada, et al. (175) in studying the comparative ion binding capacity of outer and inner surfaces of isolated cuticular membranes found that the inner surface had a many fold greater ion binding capacity than the outer surface. This was related to the presence of a greater electrical charge balance found on the inside surface of the cuticle. The physical nature of the cuticle surfaces was also considered in explaining this phenomenon. Enzymically isolated

show a smooth and glossy surface on the outside, while the inside surface, being the side previously in contact with the epidermal cells, showed many cell fragments even after thorough washing. This and the absence of wax should naturally increase the sorptive power of the inside surface. Yamada, et al. (175) have also reported that cation penetration was much greater than anions through cuticular membranes. These and other interesting properties of cuticular membranes have raised the question, if the penetration through cuticular membranes are affected by particle or ion size.

Accordingly, penetration of the 3 divalent cations, Ca^{++}, Sr^{++}, and Ba^{++} and the 4 monovalent cations, Na^{+}, K^{+}, Rb^{+}, and Cs^{+} through astomatous and stomatous Euonymus leaf cuticles was followed to partially answer this question, and also to ascertain the extent to which permeability was related to the presence of stomates on leaf surfaces from which cuticles were derived (Figure 2, 3 and 4, Table III). The order of the rate of penetration of the ions through an astomatous cuticle was Cs = Rb > K > Na and Ba > Sr > Ca. This was correlated closely with the Hofmeister line of ion series (lyotropic series). There was an indirect correlation of the rate of penetration with the size of the hydrated ion. The values for ion and hydrated ion size are

as follows:

	Hofmeister line of ions:			
	Na	K	Rb	Cs
Size of ions (Å)	0.98	1.33	1.46	1.66
Size of hydrated ion (Å) (dilution 1:100)	2.81	1.88	1.81	1.80
No. of H_2O mol. per ion	4.3	0.9	0.5	0.2

The order of cation permeability through semipermeable membrane, for example, through a copper ferrocyanide membrane is (117):

$$K = Na > Li > Ba > Sr > Ca = Mg$$

This variable rate of penetration was attributed to the size of the hydrated ions. Hofmeister's series of ions was based on the fact that smaller ions attract the largest number of H_2O molecules and hence have the thickest water hull.

The penetration results reveal that the size of the hydrated ion was a determining factor in the rate of ion penetration through isolated leaf cuticles. A similar study on penetration rates, but conducted on intact cuticular membranes on the surface of dormant Euonymus leaves revealed that ion size was insignificant as a factor controlling penetration (Table IV). The leaves employed in the study were previously exposed to a long cold Michigan winter and it was

assumed that the uptake detected was mainly from diffusion through the intact cuticle.

Uptake of the four ions (Na, K, Rb, Cs) through lower leaf surface of Euonymus was 5-fold greater than through the upper surface, and 2-fold over both surfaces when submerged (Table IV). A 3-fold increase in diffusion of the ions was obtained through the separated stomatous cuticular membrane over that of the astomatous cuticle (Table III). The increased rate of passage of ions through the stomatous surface of an excised Euonymus leaf over that through the isolated stomatous cuticular membrane, may be because the stomatal cavities lined with cuticles exist in their normal condition in the leaf, and thus provide a more permeable environment than the enzymically isolated cuticles.

The studies of Kamimura and Goodman (89) have confirmed that the dorsal leaf surface of apple absorbs 3-5 times as much as the ventral surface. This is attributed to the fact that the cuticle on the dorsal surface is thinner, and stomates are present. Another important factor may be chemical differences between the cuticles from the 2 surfaces. Cuticles from the dorsal surface contain less cutin, and a high cutin content is often associated with a decrease in permeability (111, 140).

The reduced uptake of all 4 ions (Na, K, Rb, Cs) by

submerged Euonymus leaves, as compared to that by the lower leaf surface, may be related to oxygen deficiency in the submerged leaves.

Panacean claims have been made about DMSO (dimethyl sulfoxide), a chemical extracted from paper-pulp wastes. Among the many attributed qualities discussed by Jacob, et al. (77, 78), the most characteristic properties of DMSO are their ability to penetrate skin easily and enhance the penetration of antibiotics and fungicides into plants. They have also reported that DMSO could form complexes with many metal ions. The question thus arises as to whether or not DMSO could act as metal ion carrier aiding in foliar penetration through cuticular membranes. In the studies herein reported, the penetration of $^{59}FeSO_4$ and ^{59}FeEDDHA in the presence or absence of DMSO was studied with leaf cuticles separated from the upper surfaces of the Euonymus leaf. No enhancement in $^{59}Fe^{++}$ penetration was found from DMSO (Figure 5).

The penetration study conducted on cuticular membrane isolated from the upper coffee leaf surface revealed that the inorganic sulfate of iron as well as manganese or zinc diffused much more than the chelated ions. This is in accord with the former observation on isolated Euonymus leaf cuticles that variation in permeability rates

through cuticular membranes may be because of the molecular size of the penetrant.

Foliar Uptake and Transport of Fe, Mn and Zn in Coffee.

As indicated earlier, the penetration rate for Fe, Mn, and Zn through coffee cuticular membrane was generally greater for the non-chelated metals. This result on coffee leaf cuticular membrane was also found to be in agreement with the results obtained for excised leaves and isolated leaf cells of coffee.

In excised leaves and isolated leaf cells of coffee, the non-chelated ions of Fe, Mn, and Zn were preferably absorbed and the addition of synthetic chelates - EDTA and EDDHA was in all cases inhibitory to absorption (Figures 7, 8, and Tables VII, VIII). The depressing effects of synthetic chelates on foliar uptake have been confirmed in many field trials (54, 94, 99, 161).

Uptake of Fe, Mn, and Zn applied as sulfate salts and as chelates with EDTA and EDDHA was favored by a pH of 3, as compared to 4, 5, 6, except for Fe-EDTA and $ZnSO_4$. $ZnSO_4$ was absorbed equally well at all pH levels (Figure 7, Table VII).

The increase in absorption of $^{59}FeSO_4$ and $^{54}MnSO_4$ at

pH 3 may possibly be explained by the dissociation theory as proposed by Swanson and Whitney (155). But a similar increase at pH 3 in the case of chelated ions, which are normally undissociated, leads to the alternate suggestion that the nature of cuticle itself could have been altered by pH to possibly facilitate greater penetration.

In contrast to the effects of pH on absorption by the intact leaf, the uptake of Fe, Mn and Zn by enzymically isolated leaf cells is slightly favored by the higher pH. With leaf cells a pH 5 and 6 are optimum for cell activity and the increase in ion uptake at pH 5 is in line with the pH within the cell.

Although Fe, Mn, and Zn supplied on coffee leaves as sulfates were the most readily absorbed forms, little translocation from the sites of application were observed, especially for Fe and Zn. Chelation of these ions with EDTA and EDDHA, however, greatly increased their mobility and translocation beyond the treated leaves (Table VI). The recent works of Hale and Wallace (69), De, et al. (43), Kannan and Wittwer (90), and Millikan and Hanger (121) attest to the fact that small amounts of synthetic chelating agents greatly increase the mobility of otherwise relatively immobile nutrients. The present study revealed that only about 0.6% of $^{59}Fe^{++}$, and 1.4% of $^{65}Zn^{++}$ that was foliar absorbed was translocated while about 7.6% of $^{54}Mn^{++}$ was

mobilized when applied without cheltate. With EDDHA chelate there was a 3- and 2-fold increase in $^{59}Fe^{++}$ and $^{65}Zn^{++}$, respectively, that was translocated as compared to that foliar absorbed. The mobility of $^{54}Mn^{++}$ was not affected by complexing with either EDTA or EDDHA. The order of mobility of the three micronutrients within the coffee plant was $^{54}Mn^{++} > {}^{65}Zn^{++} > {}^{59}Fe^{++}$. The results reported herein also suggest that the most important effect of chelation, as with EDTA or EDDHA, is an aid to transport for the relatively immobile elements - $^{59}Fe^{++} > {}^{65}Zn^{++} > {}^{54}Mn^{++}$. This order of mobility compares favorable with that reported earlier by Bukovac and Wittwer (25) for bean plants.

SUMMARY

A study of the mechanisms of ion uptake from foliar sprays was approach by investigating the permeabilities of isolated cuticular membranes. Some species of plants have leaves from which it is extremely difficult to separate intact cuticular membranes by existing isolation procedures. Such cuticular membranes were readily separated by new mechanical procedures and fortified enzyme solutions.

Leaf cuticular membranes were studied to determine if permeability was a function of the size of ions applied. Four monovalent cations - Na^+, K^+, Rb^+, Cs^+, and 3 divalent cations - Ca^{++}, Sr^{++}, Ba^{++}, as chloride salts were labelled and diffusion studies made with isolated Euonymus japonicus leaf cuticles. Euonymus species provided strong but thin cuticular membranes from both upper and lower leaf surfaces, being astomatous and stomatous, respectively, and thus are ideally suited for permeability studies. Ion permeability studies with both types of membranes revealed that the rate of penetration was of the order Cs > Rb > K = Na and Ba > Sr > Ca. Penetration was thus inversely correlated with the hydrated ion size. Penetration of the 4 monovalent cations through the enzymically isolated stomatous cuticular membrane from the lower leaf surface was 3 times greater than that through the astomatous cuticle from the upper leaf surface. Penetration of the same ions through intact cuticles

on dormant leaves revealed a 5-fold increase for the lower as compared with the upper leaf surface. With intact leaves there was an overall increase in penetration for all ions and the size of the hydrated ion was insignificant.

The greater ion permeability properties of the cuticle on the lower stomatous leaf surface may be the function of a thinner cuticle, the presence of stomatal openings, or related to the relatively lower cutin content found in the cuticle covering the under surface of a leaf.

DMSO did not enhance the penetration of labelled $^{59}FeSO_4$ or ^{59}FeEDDHA through the cuticular membranes of the Euonymus leaf.

Mechanisms of foliar absorption of $^{59}Fe^{++}$, $^{54}Mn^{++}$, and $^{65}Zn^{++}$ from sulfate salts and complexed with the chelating agents - EDTA and EDDHA - were studied with enzymically isolated coffee leaf cuticular membranes. EDTA and EDDHA reduced the penetration of all three microelements through the cuticular membrane and as well suppressed the absorption by excised leaves and enzymically isolated leaf cells. Translocation of the foliar absorbed $^{59}Fe^{++}$, and $^{65}Zn^{++}$, however, was significantly increased when they were applied as chelates. The effect of EDDHA on enhancing mobility was greater than EDTA. The order of mobility observed in 9-month old

coffee seedlings was $^{54}Mn^{++} > {}^{65}Zn^{++} > {}^{59}Fe^{++}$ applied as the sulfate and $^{59}Fe^{++} > {}^{65}Zn^{++} > {}^{54}Mn^{++}$ applied as the EDDHA chelate. A pH of 3 induced maximum uptake of the three microelements by excised coffee leaves.

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