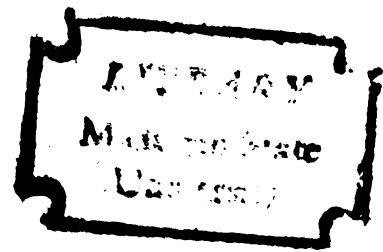


MECHANISMS OF FOLIAR ABSORPTION
IN HIGHER PLANTS WITH
SPECIAL REFERENCE TO IRON

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This is to certify that the

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ABSTRACT

MECHANISMS OF FOLIAR ABSORPTION IN HIGHER PLANTS WITH SPECIAL REFERENCE TO IRON

By Seshadri Kannan

Iron was probably the first micronutrient used successfully as a foliar spray for the correction of iron chlorosis. Today, more than 100 years later, its successful commercial use as a nutrient spray has spread only to a few species. The value of iron as well as other nutrients applied as sprays to aerial plant parts is dependent first upon the permeation of a cuticular membrane, which constitutes the first barrier to absorption, secondly, the absorption by living cells, and thirdly, the extent of translocation beyond the absorption site. Penetration of ^{59}Fe from ^{59}Fe labeled FeSO_4 and FeEDDHA , with and without urea, through enzymically isolated cuticular membranes, and of absorption by enzymically separated leaf cells, were studied in detail with the objective of resolving the contributing mechanisms.

Cuticular membranes were separated from dorsal and ventral leaf surfaces of Euonymus japonicus, and from ripe tomato fruits, by treating them with an incubating mixture containing 2% pectinase. These were affixed to one end of a glass tube (15mm. diam.) to resemble the normal surface orientation on leaves and fruits, and the edges hermetically sealed with rubber cement. This tube, containing 3 ml. of the labeled solutes,

was suspended into 30 ml. of deionized distilled water inside a large test tube (30 mm. diam). Suitable aliquots from the large test tube were periodically radioassayed by scintillation well and gas-flow detectors. The rates of penetration of ^{59}Fe from 0.1mM solutions of ^{59}Fe labeled FeSO_4 and FeEDDHA , in the absence and presence of 10mM urea, were expressed as millimicromoles per unit time.

The penetration rate of ^{59}Fe was higher for FeSO_4 than from other iron sources, through the 3 types of cuticles. Permeability was generally higher through cuticles from dorsal (stomatous) leaf surfaces of Euonymus, than through others. Permeability coefficients were derived from the results of penetration of ^{14}C -labeled non-polar solutes, namely, ethylene glycol, glycerol, D-ribose, D-glucose, sucrose, EDTA and EDDHA. A significant inverse relationship between molecular weights of these solutes and their penetration rates was obtained, particularly for cuticular membranes from tomato fruits and those from ventral leaf surfaces of Euonymus.

Leaf cells consisting of a mixture of pallisade and mesophyll cells were enzymically isolated from tobacco, bean and soybean leaves for studying mechanisms of ^{59}Fe absorption.

Light, Na_2 -succinate, and high temperature favored absorption. Metabolic inhibitors; viz, NaN_3 and DNP reduced it. K_3 -citrate in the incubation mixture also markedly reduced uptake, and NaHCO_3 was inhibitory,

especially in the dark. These results suggested that active metabolic processes were involved in ^{59}Fe absorption by leaf cells.

The presence of Ca at concentrations in excess of $5 \times 10^{-4}\text{M}$ in the absorption system was inhibitory to uptake of ^{59}Fe and ^{54}Mn by tobacco leaf cells. Fe and Mn mutually antagonized the absorption of each other, but the inhibition of Mn absorption by Fe was higher than that of Fe by Mn. A significant inverse correlation between the age of the leaf and a direct correlation between the chlorophyll content, and the rate of ^{59}Fe absorption by the tobacco leaf cells was demonstrated. Fe absorption was significantly lower for cells derived from (Fe deficient) chlorotic leaves than for those from healthy leaves of the PI 54619-5-1 variety.

Results of studies of ^{59}Fe absorption by leaf cells of tobacco and bean, using 0.1mM solutions of ^{59}Fe labeled FeSO_4 , FeEDTA , and FeEDDHA , revealed a reduction by chelation, and an increase in ^{59}Fe uptake from FeSO_4 in the presence of 10mM urea. Urea had no effect on ^{59}Fe absorption from FeEDTA and FeEDDHA . The data on Fe absorption from FeEDTA and FeEDDHA by tobacco leaf cells also revealed that Fe was absorbed by the cells after its separation from the chelates, and that the ligands were not absorbed by the cells.

For intact bean plants, foliar absorption of ^{59}Fe was measured by the "leaf immersion technique." Uptake of FeEDTA and FeEDDHA ,

as compared with FeSO_4 was greatly reduced but rate of translocation expressed as percentage of absorption was greatly enhanced. Intact leaves absorbed more ^{59}Fe from FeSO_4 than from chelated sources, both in the absence and presence of urea, but this iron was translocated the least.

The competitive nature of EDDHA on Fe absorption by tobacco leaf cells was revealed from enzyme-kinetic analysis. The synthetic chelate probably competes with an unknown natural chelate present in cells, for Fe. The affinity constant for Fe by the Fe-carrier was low.

Adsorption by cuticular surfaces, diffusion through the cuticles, and subsequent metabolic uptake of Fe by leaf cells were analyzed with data on absorption isotherms for excised leaves of Eucnymphus japonicus. The leaves were floated on ^{59}Fe labeled FeSO_4 solution (0.1mM) under 500 ft. c. of cool fluorescent white light, at $20^\circ \pm 2^\circ\text{C}$, or, in dark at $0^\circ \pm 2^\circ\text{C}$, and uptake was measured at predetermined times. The adsorption of ^{59}Fe by the cuticular surfaces was very rapid and instantaneous. Diffusion through both the dorsal and ventral cuticular surfaces was activated by light and temperature, perhaps prompted by the rapid removal of the diffused ions by the leaf cells.

It is concluded from these observations that foliar absorption is a multiphase process - initial adsorption on the cuticular surfaces, diffusion through cuticles and absorption, which is energy dependent.

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WITH SPECIAL REFERENCE TO IRON

By

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in memory of late Shri. G. Seshadri Aniyangar
and late Shri. M. S. Krishnamachari.

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INTRODUCTION

Iron was probably the first micronutrient used successfully as a foliar spray for the correction of a nutritional disorder (44). Today, more than 100 years later, its successful commercial use as a nutrient spray has spread, however, only to a few species. These include pineapple (154), grain sorghum (81, 162), and gardenia (147). With these exceptions, remedial measures for supplying iron as a spray to deficient foliage have not been entirely satisfactory. The effectiveness of foliar sprays of iron for most plants is limited, first by slow absorption (163) and secondly by a lack of mobility after absorption (16).

Much has been done, by contrast, to increase the availability of iron applied to root media. Reports of the experimental and commercial success of the synthetic chelates, ethylenediamine tetraacetic acid (EDTA) and ethylenediamine (di o-hydroxyphenylacetic acid) (EDDHA) in facilitating the availability of iron for plants grown in soilless culture and applied as a band in the soil as a component of a complete fertilizer are now legion. Synthetic chelating agents have proven most valuable as a means of increasing the availability of iron absorbed by roots. Comparably successful results have not apparently been obtained with iron chelates over iron sulfate applied as foliar sprays although a few reports (8, 9, 162) show chelated iron to be equally effective.

The value of iron, as well as other nutrients, applied as sprays to aerial plant parts is dependent first upon the permeation of a cuticular membrane which constitutes the first barrier to foliar absorption; secondly, the absorption by living cells; and thirdly, the extent of translocation beyond the absorption site.

Experimental data are lacking on the first two and are not extensive on the third.

This dissertation will describe for iron and the synthetic chelates of iron (EDTA and EDDHA) some of the mechanisms of penetration through enzymically isolated cuticular membranes, absorption by enzymically isolated leaf cells, and translocation in intact plants.

LITERATURE REVIEW

General Considerations

Iron as an Important Nutrient Element

Iron is well known as an important nutrient element that is required by and often deficient in agricultural crops. Symptoms of iron deficiency develop on crops grown in soils either lacking iron or having an insufficient reserve of iron in the plant. Chlorosis, a general term denoting the lack of chlorophyll, is also a symptom caused by inadequate iron. Iron chlorosis specifically refers to a chlorosis which could be alleviated by providing the plant with suitable iron compounds. Iron chlorotic plants are found on both acid as well as alkaline or calcareous soils.

Functions of Iron in Plants

Inorganic iron salts are known to possess properties of an oxidase and an electron transporter depending upon the pH of the medium, although their capacity to function as a catalase or peroxidase is very negligible (42). The role of iron enzymes in respiration has been well established. The cytochromes found in plant and animal cells (40) consist of a number of iron porphyrin enzymes. An adequate supply of iron is essential for the formation of chlorophyll in the plant. Recently, two different actions of iron on plant growth have been recognized (18, 19). An iron addition to roots in a iron free nutrient solution results in a reduced rate of elongation in light and an enhancement in darkness and this is recognized

as the high iron effect. Addition of EDTA strongly inhibited cell multiplication in darkness by chelating iron, and this is the low iron effect, produced by amounts of iron lower than those carried to the plant from the seed.

Iron Supply

In the natural environment, the inherent supply of iron is usually adequate for plants and an actual deficiency of iron in the nutrient medium is probably found only in sand or water cultures. Since iron is not deficient in most soils, variations in the capacity of plants to absorb and accumulate this element probably relate to factors other than the level of iron in the soil (12). Thus, there appears to be a regulatory mechanism for the absorption and translocation of iron in plants, similar to the mechanism in animals. Most iron compounds occurring naturally in soils are quite insoluble. Ferrous ions are unstable in aerated soils with a pH value of 6 or higher. Alkalinity and aeration favor oxidation of iron and waterlogging favors reduction (85). The extent of oxidation or reduction is also influenced by other factors, such as activity of microorganisms, level of soil organic matter, and the presence of other cations or anions. Bivalent iron is the form of iron readily absorbed by plants (12).

It has been recognized that some plants grow well where others will not grow without developing iron chlorosis and this has been attributed to the differences among plant species. Brown (11) tested the susceptibility to iron chlorosis of a number of plant species grown on calcareous and organic soils and found that

they responded variously. In iron absorption studies, with a few selected plant species, only the soybean (variety PI 54619-5-1) failed to absorb sufficient iron from a calcareous soil to maintain growth (13).

Iron Requirement and Distribution in Plants

Iron occurs in rather large amounts in most soils, but it is present only in small amounts in plants. Glenister (39) found that the lower (older) leaves of sunflower contained 10 times as much iron as the upper (younger) leaves. A continuous supply of iron was necessary to keep the younger leaves normal. Similar requirement has been indicated for soybean (13). On the basis of leaf analysis, 60 to 90 ppm. of Fe is considered as optimum (12). Mildly chlorotic citrus leaves contained about 50 ppm. (89), and nearly normal leaves contained 80 ppm. (155). It is reported (12) that 82% of the total iron in spinach leaves was found in chloroplasts, and five-sixths of the firmly bound iron was associated with phosphorus containing proteins. The cell nucleus contained only traces while the cell wall had no iron. Chloroplasts represented about one-third of the dry weight in sugar beet leaves and contained 61% of the total iron (161). Bennett (6) found that the active iron content of pear leaves built up rapidly early in growth, and then diminished, while residual iron was low at first and then accumulated as the growth progressed. He concluded that "active" iron can be transformed into residual iron, but that iron once transformed into the residual state cannot be utilized for chlorophyll formation.

Brown and Holmes (13) observed that iron was mobile in green leaves of soybeans with one source of iron, but was immobile to chlorotic leaves which developed on the same plant, if the source of iron was changed. In iron deficient bacterial cells of Aerobacter indologenes, there appeared to be a selective distribution of iron, and the iron requirement of cytochrome system was met first (157). With regard to the amount of iron required, the chlorosis susceptible soybean variety needed a higher iron concentration to produce green plants, than the non-susceptible variety.

Nutrient Factors Related to Iron Chlorosis

Iron chlorosis has been observed in both acid and alkaline soils (89). The causes of lime induced chlorosis have been discussed by Brown (3, 12, 15) and Wallace and Lunt (148). Phosphates might precipitate iron either in the soil or in plant tissues (7). High manganese in soils or plants might oxidise iron to an inactive state (128). Excessive amounts of potassium in leaves might displace iron and disrupt metabolic processes (12). Bicarbonate is known to induce iron chlorosis (53), which decreased the availability of iron at the root surface (29, 92, 115, 145). Increasing concentrations of KHCO_3 progressively depressed protein synthesis and oxidase activity with a decrease in respiration and metabolism of potato discs (12). Brown (12) suggested that NaHCO_3 might increase the concentration of HCO_3^- , $\text{CO}_3^{=}$, and OH^- ions, which might compete with

phosphate adsorbed on the surface of soil particles. The excess phosphate in soil solution would combine with iron to form insoluble iron phosphate, thus, indirectly inducing iron chlorosis.

The macronutrient elements associated with iron chlorosis are usually calcium, phosphorus, potassium and nitrogen. Consistent differences were obtained between chlorotic and green leaves in the Ca/K ratio (53, 101). Phosphorus could influence iron metabolism and plant species and varieties differ in their susceptibility to this effect upon iron (14, 159). Excess phosphate showed no relationship to iron chlorosis in Utah soils (138).

Of the micronutrients elements, the effectiveness for inducing iron chlorosis was of the order: $Ni > Cu > Co > Cr > Zn > Mo > Mn$ (55, 66). Pineapples developed iron chlorosis when grown on mangiferous soils (63). Accumulation of copper in soil caused iron chlorosis in Florida (12). Nickel, cobalt, zinc and copper induced iron deficiency in a number of annual crops (55, 56, 104, 105, 108, 109).

High concentrations of calcium in soil may be a major factor in iron chlorosis (12, 150), but the effect of this ion in soils is closely associated with its effect on soil pH. It was suggested (104) that an interfering ion like Mn, Cu, Zn and Ca might take the place of iron in the malic or malonic acid complexes in the plant. These ions could interfere with iron translocation, since most of the iron was translocated to the top in the form of iron malate or malonate.

Physiological Effects of Iron Chlorosis

Dekock and Morrison (30) showed that the organic acid content of chlorotic plants was often similar whether it was produced by genetic effects, lime, or even virus. The organic acids in chlorotic plants were high (158). Cytochrome oxidase activity was also inhibited (101), the succinic oxidase system was sensitive (4), and there was an effect on the oxidation and reduction of cytochrome c in the mitochondria of Ricinus. Peroxidase activity was not markedly different in normal and iron chlorotic leaves, whereas catalase activity was several times greater in the green leaves (12, 29). Changes in the malonic acid content in the leaves of Phaseolus vulgaris were associated with lime induced iron chlorosis (114), while an increase in malonic acid marked the onset of iron deficiency (171). In Neurospora crassa, iron deficiency reduced catalase, peroxidase, cytochrome c reductase, oxidase, and DNase activities (2, 110).

Correction of Iron Chlorosis

Effectiveness of Foliar Sprays of Iron Salts and Iron Chelates

The earliest successful commercial use of iron sulfate was on pineapples where a 2.8% spray of ferrous sulfate gave temporary recovery from chlorosis (72). Similar results with iron sulfate sprays have been obtained in citrus (47), cacao (44) and with certain vegetables and ornamental plants (160). Guest and

Chapman (45) tested 30 iron compounds and found that only with ferrous sulfate sprays were satisfactory results obtained.

Soil applications of chelated iron, though effective, are very expensive (132, 133). Iron chlorosis may be controlled in currants, plums, and pears by spraying 0.1% ferric EDTA, and in peaches by spraying ferric DTPA (Diethylene triamine pentaacetic acid) (8), although ferric DTPA was not translocated from the leaves and soil applications were more effective (58). On the other hand, Wallihan (155) and Wallihan et al. (156) found no benefit from soil application of EDTA or any other chelating agent for citrus. Synthetic chelates have been used effectively to keep iron soluble and available for plant growth when applied to the root medium (21, 54, 87, 88, 129, 130, 131). Tiffin et al. (142) found that chlorosis could not be corrected by an application of 20 ppm chelating agent, while foliar applied radio-iron caused greening only at the sites of application.

Use of Other Chemicals for Iron Spray

A combination of citric acid with iron sulfate applied as a spray has been advocated in Arizona (147). Since iron is transported in the form of iron malate (140), it is suggested that iron malate might be effective for foliar application of iron. The usefulness of an iron-fructose chelate in biological systems has also been suggested (147). The sprays of iron chelated with 2,4-pentanedione, which results in a non-ionic form of iron were not effective (148).

Since urea penetrates leaves readily, it may influence the permeability of leaves to other nutrients (75). Some citrus growers in Arizona and California claim that the combination of iron chelate and urea in foliar sprays corrected iron chlorosis (147). Iron sulfate combined with glucylglycine and applied as a foliage spray was exceptionally promising on macadamia (148). Triiodobenzoic acid may favor translocation and utilization of subsequently applied iron (80).

Mechanism of Absorption of Iron and Chelates by Roots

A chelating agent is considered as an inert metal carrier (124) which delivers the metals to absorbing surfaces, but is not itself absorbed (68). Several reports indicated that the entire chelating molecule may be absorbed by the roots (57, 58, 146, 153). Some (61, 82, 88, 149, 154) have suggested the uptake of the chelating agent or a decomposition product by the plant roots. Tiffin et al. (141), however, demonstrated a differential absorption of metal chelate components by roots. Tiffin and Brown (139) further showed a selective absorption of Fe from FeEDTA, FeDTPA, and FeEDDHA by Hawkeye soybeans. However, the increased concentration of iron in plant materials (147) in addition to the presence of ^{14}C suggested the probability that both chelate components (metal and ligand) were absorbed together. It was further found (162) that equivalent uptake of iron and ^{15}N chelating agent was obtained in citrus. In later work (147) the ratio between iron and

chelating agent was greater than unity, which suggested that the uptake of these components might not be equivalent. The same lack of equivalence was shown by Krauss and Specht (82) in studies with green algae. Tiffin et al. (136) concluded from work with soybeans, zinnia, and sunflower that these plants selectively absorbed the iron, and the EDDHA remaining, for the most part, in the nutrient medium. The studies by Hale and Wallace (50) showed that less chelating agent than iron found its way into the plants via the root system.

The effects of pH on the availability of iron from chelates are significant (146, 147). FeEDDHA supplies iron to plants reasonably well on any soil, but FeEDTA is effective only on acid and neutral soils. The differential behaviour of the two is related to the stability of these against hydrolysis in the soil. At pH values above 7, FeEDTA is easily hydrolyzed and the iron is precipitated as hydroxide, which is poorly available to plants. At pH 7 and higher, many times as much FeEDDHA remains in solution as FeEDTA.

Mechanism of Foliar Absorption

General

The foliar absorption of mineral nutrients is an active metabolic process (74, 164). Many have obtained the typical two phase time course curves in

foliar uptake of nutrients (16, 17, 73, 137), which correspond to those for root absorption (84). Typical of an active uptake process, foliar absorption is temperature dependent. Temperature coefficient values in excess of 2 were obtained for ^{32}P , ^{86}Rb and ^{42}K in bean leaves (136) over a range of 10 to 21.1°C, for Mn absorption by soybean leaves (102) over a range of 36° to 64° F, for ^{60}Co absorption by bean leaves between a low temperature of 70° to 76° F and a high temperature of 87° to 100° F (46), and for Br uptake by *Nitella* cells between 10° to 20° C (60).

Light is closely associated with the metabolic activity of all living plant protoplasm (90), and the positive effect of light on ion uptake by isolated green leaves has been thoroughly documented (10, 83).

Metabolic inhibitors provide a means to relate foliar absorption to corresponding processes of metabolism. Among commonly used inhibitors are 2,4-dinitrophenol (DNP), an uncoupling agent in oxidative phosphorylation, and arsenate and iodoacetate, uncouplers of the substrate level phosphorylation. Cyanide and azide inhibit respiration in uncoupled as well as coupled systems (152).

Other factors used as criteria for active uptake include accumulation against a concentration gradient, irreversibility, oxygen dependence, specificity, and ion competition (83, 164), and pH dependence (26, 28, 135, 136, 144, 167).

Penetration of Ions Through the Cuticles of Leaves

The cuticular membrane which sheaths the aerial parts of higher plants is the first barrier to foliar absorption (164). The chemical nature of cutinized outer epidermal cell walls has been summarized by Frey-Wyssling (37). The chemical components of cuticles have been separated by many workers (36, 86, 99, 100, 118, 167). The outer coating of the cuticle is made up of a wax film and plant waxes which consist of free alcohols, hydrocarbons, unsaturated ketones, long chain (C_{28}) aldehydes and glycerol compounds, the main component being neutral or hard wax (64, 65, 117, 118, 123). Differences in the nature of upper and lower leaf cuticles have been recorded for many species. There is generally greater development of cutin in the upper (ventral) than in the lower (dorsal) surface (99).

Cuticular membranes have long been described and their chemical properties analyzed, but only recently have their permeability properties been studied. Hurst (67) working with insect cuticles first reported that the rate of movement of organic substances and even water might not be the same when they were escaping (efflux), as when they were entering (influx). Evidence for permeability of the plant cuticle for water is found in the phenomena of cuticular transpiration to polar substances in the salt residues on the leaves of plants in saline habitats, and to the uptake of

non-polar substances after foliar application. Gases have been known to diffuse through leaf cuticles more readily than liquids. Schieferstein (125) found in the case of ivy leaf cuticular membranes, the influx of water was 1.44 times of efflux.

It has been explained that permeability of cuticles for liquids is based on pores in the cuticle. The lipoid nature of the cuticle enables the penetration of non-polar substances (the "lipoidal pathway"), and an "aqueous pathway" through which polar substances may penetrate is the result of imbibition by the cutin layers, as well as through the hydrophilic pectic layers (27, 32, 95). The identification of pores in the cuticle which served as excretory ducts, and are concerned with the formation of wax protuberances on the cuticular surface, and also of ducts extending up to the surface of the cuticle have also been recorded (51, 95, 106). Whether these pores are actually concerned with permeability of substance is, however, not known.

Recent studies of permeation of cations and anions through enzymically cuticular membranes have greatly contributed to the understanding of foliar absorption. Penetration of ions through cuticles has been assumed as a physical diffusion process which has also been recently confirmed (167, 169). It was demonstrated with ripe tomato fruit cuticles (astomatous), and green onion leaf cuticles (stomatous), that ion penetration followed a typical diffusion equation. Permeability of cuticular membranes to isotopically labeled

cations (^{45}Ca , ^{59}Fe), was greater than that for anions (^{32}P , ^{35}S).

Inorganic ions penetrated more rapidly from the "outer" side to the "inner" side (influx), than from the "inner" to the "outer" side (efflux) (169). The greater ion binding capacity of the "inner" surface was proposed as a possible explanation (166). This was found to be however not applicable to urea (170). Using chemically excised apple leaf cuticles, the reverse results for penetration of urea, benzoic acid, glucose, maleic hydrazide, and simazine have been reported (41).

Time course studies have revealed that urea penetrates cuticular membranes at a remarkable rate, and this increases with time (75). Furthermore, urea increased the rate of foliar uptake of other nutrients simultaneously applied. Facilitation of absorption by urea occurred at the cuticular level for anions and at both cuticular and cellular levels for cations (75).

Many investigators believe that isolated cuticular membranes still maintained their integrity and natural permeability properties. However, it has been found that the permeation rates of substances through isolated cuticular membranes was much less than when they were intact on the leaf surface (41). Jyung and Wittwer (75) have emphasized the importance of molecular size, charge, partition, volatility, solubility and adsorbability, in solute penetration through cuticular membranes.

Mechanism of Ion Uptake by Leaf Cells

Once the cuticular entry of nutrients into the leaf is accomplished, they may be either absorbed directly by leaf cells or diffuse within a free space volume. Ion uptake studies by isolated leaf cells of various types (spongy, mesophyll, palisade, epidermal, guard cells) may be one of the most useful approaches for the future in resolving mechanisms of foliar absorption (75, 76). Living cells removed from leaf tissues and suspended in solution constitute a useful system for studying the mechanisms of ion uptake. The structural and functional heterogeneity typical of intact leaves is minimized, and all concern for differences associated with stomatal and cuticular penetration and movement through cracks on leaf surfaces is eliminated (75).

Although extensive work has been reported on the presumed absorption of solutes by plant cells (71), only excised or intact organs, and slices of storage tissues have been used for such studies. Ion uptake studies exclusively by isolated cells is of very recent origin, especially those involving enzymically isolated leaf cells (76).

Various procedures have been described for isolating single cells from leaves (22, 76, 77, 172, 173). The procedure herein is based on enzymatic degradation of the intercellular pectic substances by pectinase (126), a technique applied previously to root meristems (22). Intact living cells have been isolated by this method from the leaves of Nicotiana glutinosa,

Datura stramonium, potato, Chenopodium amaranticolor, Quercus borealis, Crotalaria spectabilis, peach, cherry, cucumber, and Magnolia sp. (173).

Mechanisms of Absorption of Ions by Cells and Cell Organelles

Yeast cells have been used to study the mechanism of uptake of manganese (70, 71). It has been postulated that both phosphate and manganese are transferred into the yeast cell in an essentially irreversible manner by two systems which are coupled in an unknown way to glycolysis at the 3-phosphoglyceraldehyde dehydrogenase step. Yeast cells which ordinarily absorbed phosphate only after a lag period of thirty minutes, absorbed without delay, if pretreated with glucose and potassium. It was suggested therefore that a phosphate carrier was synthesized during glycolysis.

Absorption of Rb and phosphate was studied by Jyung et al. (76) using enzymically isolated cells from green tobacco leaves. It was found that absorption of Rb was temperature dependent. Energy sources such as light, succinate and bicarbonate as sources of CO₂ for photosynthesis promoted uptake. Absorption rates in the light for green leaf cells exceeded enormously those isolated from the pith. NaN₃ and DNP reduced the Rb and phosphorus uptake. The inhibitory effect of DNP on Rb uptake was negated by the addition of ATP (76).

Absorption of iron by isolated chloroplasts was studied by Hagen et al. (48) and the amount absorbed was governed by at least three factors, (a) the

mass of chloroplasts, (b) the concentration of iron in the system and (c) iron-binding capacity of the suspension medium. Mitochondria introduced into a wheat chloroplast suspension containing acetate and ^{55}Fe - ^{59}Fe , diminished iron absorption. This suggested a possible competition for iron between different particulates, which might affect the availability of iron in immature leaves (48). Waring and Werkman (157) studied the uptake of iron by six bacterial cell species and found a variance according to the cytochrome system contained.

Foliar Absorption and Transport

Foliar absorption and transport of mineral nutrients have been discussed extensively by Wittwer (163) and by Jyung and Wittwer (75). The absorption of potassium, calcium, magnesium, manganese, and zinc by leaf surfaces were found to be very rapid, but were not equal to that of the nitrogen from urea. Marked differences occurred in the extent of translocation from the foliage to other parts. Potassium, along with sodium, rubidium, and cesium, were the most mobile of all cations. Zinc, copper, and manganese were moderately mobile, with iron, molybdenum, and boron only slightly mobile, and calcium, and magnesium along with strontium and barium were essentially immobile. Iron and molybdenum in addition to being relatively immobile, were absorbed at slow rates.

EXPERIMENTAL METHODS

Procedure for Isolating Cuticles Enzymically

Penetration of iron and other substances through cuticles was studied with Euonymus japonicus cuticles obtained from both the dorsal and ventral leaf surfaces and from ripe tomato fruits. The method of enzymic separation was essentially that of Orgell (112) as modified by Okuda and Yamada (113). The enzyme solution used for separating cuticles consisted of the following:

Acetate buffer (pH 3.5 - 4.5)	2.0M
Pectinase	2.0%
Cellulase	0.2%
Hemicellulase	0.2%

The isolation procedure was as follows:

1. Ripe tomato fruits were sliced and the pulp scooped out.
2. The slices were transferred to the enzyme mixture and incubated for a week at 20-25^o C.
3. The slices were removed, washed with deionized water to eliminate the cell debris and further treated with the enzyme solution for 2-3 days if cell remnants still adhered.
4. Cuticles were washed with deionized water, and cleaned carefully with soft tissue paper until free of cells, air-dried, and stored.

The following procedure was adopted for the isolation of the cuticles from Euonymus leaves:

1. Edges of the leaves trimmed and transfer to enzyme mixture.
2. Leaves in solution aspirated repeatedly to permit diffusion of the enzyme solution through the cut edges.
3. Incubated at room temperature (20-25⁰C) for 2 weeks, or until the cuticle separated easily.
4. The delicate cuticles were peeled carefully from both surfaces and washed free of cell fragments with deionized water.
5. Cuticles resuspended in deionized water and stirred slowly with a magnetic stirrer for 1-2 hours. Resultant cuticular membranes were very clean and free from cellular fragments.
6. Cuticles carefully washed and stored dry. The origin of the cuticle was identified with microscope before use. Cuticles from dorsal surfaces were stomatous.

While it was rather easy to separate the cuticles from ripe tomato fruits, the separation was more difficult with Euonymus leaves. The tomato fruits were obtained from plants grown in the greenhouse, and the Euonymus leaves were collected during mid-summer from plants grown on the campus.

Studies on Penetration Through Enzymically Isolated Cuticles

The rates of penetration of iron, with and without EDDHA and urea, were studied using 0.1 mM solutions of FeSO₄ labeled with ⁵⁹Fe. Equimolar

solutions Fe and EDDHA were used for chelation. The urea concentration was 10.0 mM, and the pH was 6. ^{14}C -labeled organic compounds of different molecular weights in addition to ^{14}C -labeled EDTA and EDDHA were used to ascertain if molecular weight influenced the rate of penetration. Concentrations of all compounds were 0.1 mM.

The apparatus used and methods employed were those of Yamada et al. (1969). Slight modifications such as a metal jacket to hold additional tubes in the water bath, and the use of rubber cement, self sealing latex bandage (The Sealtex Company, Chicago, Illinois), and Parafilm (Marathon Parafilms) for sealing the cuticular membranes over the open end of the glass tubes, were made.

A large test tube (35 mm. diam.) with 30 ml. of deionized water was suspended in the constant temperature (20°C) water bath. The cuticular membrane was carefully affixed on one end of a smaller tube (15 mm. diam.) to correspond with surface orientation on the leaf. This smaller tube was held in position inside the large test tube by means of a rubber stopper. The labeled solution (3 ml) was added to the small tube, while it was carefully lowered into the deionized water in the large test tube, to the level of the meniscus in the small tube. The specific activity of ^{59}Fe was 3 uc/umole Fe, and ^{14}C was 10 uc/umole. Rates of penetration through the cuticular membranes was determined periodically by radioassay of 1 ml. or 2 ml. aliquots of the outer solution in the large tube. A gamma scintillation-well

detector was employed for the ^{59}Fe solutions. For ^{14}C an automatic gas flow counter was used after the liquid samples were dried in planchets under a Fisher Infra-Radiator.

Studies on Fe Absorption by Enzymically Isolated Leaf Cells

The uptake of Fe was studied using enzymically isolated leaf cells of tobacco and bean. The procedure for cell separation was a modification of methods by Zaitlin (172), Letham (91), and Jyung et al. (76). Fully expanded young leaves of tobacco, and primary leaves of bean were used. The modified procedure is outlined in Table I. The separation mixture consisted of the nutrient solution of Murashige and Skoog (107) to which was added pectinase and the pH adjusted to 6 (Table II).

Isolated leaf cells were incubated by suspending approximately 50 mg. dry weight equivalent of living cells in 10 ml. of incubation mixture in 50 ml. Erlenmeyer flasks. These were held in a water bath shaker at $20 \pm 2^\circ\text{C}$ and exposed to ca. 500 ft. c. of cool white fluorescent light. The incubation mixture contained 1170 umoles of sucrose and 0.5 umoles ^{59}Fe labeled FeSO_4 , FeEDTA , or FeEDDHA . 100 umoles of trismaleate, and 20 umoles each of Na_2 -succinate, K_3 -citrate, and NaHCO_3 were included only in a few experiments where interference or competition with Fe absorption by cells was not suspected.

The specific activity of all radioisotopes used in the cell uptake studies ($^{59}\text{Fe}^{++}$, $^{54}\text{Mn}^{++}$, $^{65}\text{Zn}^{++}$, or $^{86}\text{Rb}^+$) was 0.1 uc/umole. The salts

TABLE I

Procedure for Enzymic Separation of Leaf Cells.

1. Slice the leaves into $2\text{-}3\text{ mm}^2$ sections after removing the midrib.
2. Treat with the medium for cell separation (see Table II), in the ratio of 20 ml. per 1-2 gms. of leaf sections.
3. Shake vigorously in a reciprocating shaker for 3-4 hours at ca. 500 ft. c. (light intensity), and $20 \pm 2^\circ\text{C}$. (temperature).
4. Centrifuge at 600 x g. for 15 minutes; wash sediment with 0.25 M sucrose (ice cold) and again centrifuge. Repeat the process.
5. Re-suspend the cells in sucrose and refrigerate until used.

Table II
MEDIUM FOR CELL SEPARATION¹
A. Mineral Salts

Major elements		Trace elements	
Salts	mg/l.	Salts	mg/l.
NH ₄ NO ₃	1650	H ₃ BO ₃	6.2
KNO ₃	1900	MnSO ₄ ·H ₂ O	22.3
Ca Cl ₂ ·2H ₂ O	440	ZnSO ₄ ·4H ₂ O	8.6
MgSO ₄ ·7H ₂ O	370	KI	0.83
KH ₂ PO ₄	170	Na ₂ MoO ₄ ·2H ₂ O	0.25
Na ₂ EDTA	37.3	CuSO ₄ ·5H ₂ O	0.025
FeSO ₄ ·7H ₂ O	27.8	CoCl ₂ ·6H ₂ O	0.025

B. Organic Constituents

Sucrose	30 g/l.	Myo Inositol	100 mg/l.
Glycine	2.0 mg/l.	Nicotinic acid	0.5 mg/l.
Indoleacetic acid	1-30 mg/l.	Pyridoxin . HCl	0.5 mg/l.
Kinetin	0.04 - 10 mg/l.	Thiamin . HCl	0.1 mg/l.

¹After Murashige and Skoog (107).

Pectinase added to the final solution 10 gm/l. and pH adjusted to 6.

were FeSO_4 , MnSO_4 , ZnSO_4 , RbCl , and CaSO_4 .

At the end of an experimental period, the contents in the flask were centrifuged and/or a known aliquot was collected under mild suction, on a weighed filter paper mounted on a precipitation apparatus. It was washed twice with 1.5 ml. ice cold 0.1 M sucrose solution, and finally with deionized water. The filter paper containing the cells was then air-dried, weighed and radioassayed.

Measurement of Specific Absorption and Translocation of ^{59}Fe in Intact Bean Plants (Leaf Immersion Technique) (74).

Bean seeds were germinated in sand and 15 day old seedlings were used for specific absorption and translocation studies. One of the primary leaves were immersed in the ^{59}Fe labeled solution kept in a Petri-dish. All experiments were conducted under an illumination of 500 ft. c. and temperature of $20^{\circ} \pm 2^{\circ}\text{C}$. The dry weights of the leaves and other plant parts were determined and the radioactivity measured. Specific absorption was expressed as mumoles of ^{59}Fe per mg. dry weight, per unit time, and translocation on the basis of that recovered in plant parts other than the treated leaf.

Expression of Results and Estimates of Variability

The results of studies on cuticular penetration of substances are expressed as the absolute quantity penetrated during different time intervals.

All treatments were replicated three times and the results statistically analyzed (31, 127).

Treatments with enzymically isolated cells were also replicated three times and each experiment was repeated until reproducibility was certain. All data were statistically analyzed. Points in line graphs are averages of replicates.

Radioassay was based on methods described by Aronoff (1). Each sample was counted twice with sufficient time for at least 3000 counts.

RESULTS

Penetration of Solutes Through Cuticular Membranes

Penetration of $^{59}\text{FeSO}_4$ and $^{59}\text{FeEDDHA}$, With and Without Urea Through Cuticular Membranes

The comparative rates of penetration of $^{59}\text{FeSO}_4$ and $^{59}\text{FeEDDHA}$ in the absence and presence of urea, through cuticular membranes isolated from tomato fruits, and those from dorsal and ventral surfaces of Euonymus leaves were recorded at different time intervals. The results are summarized in Figure 1.

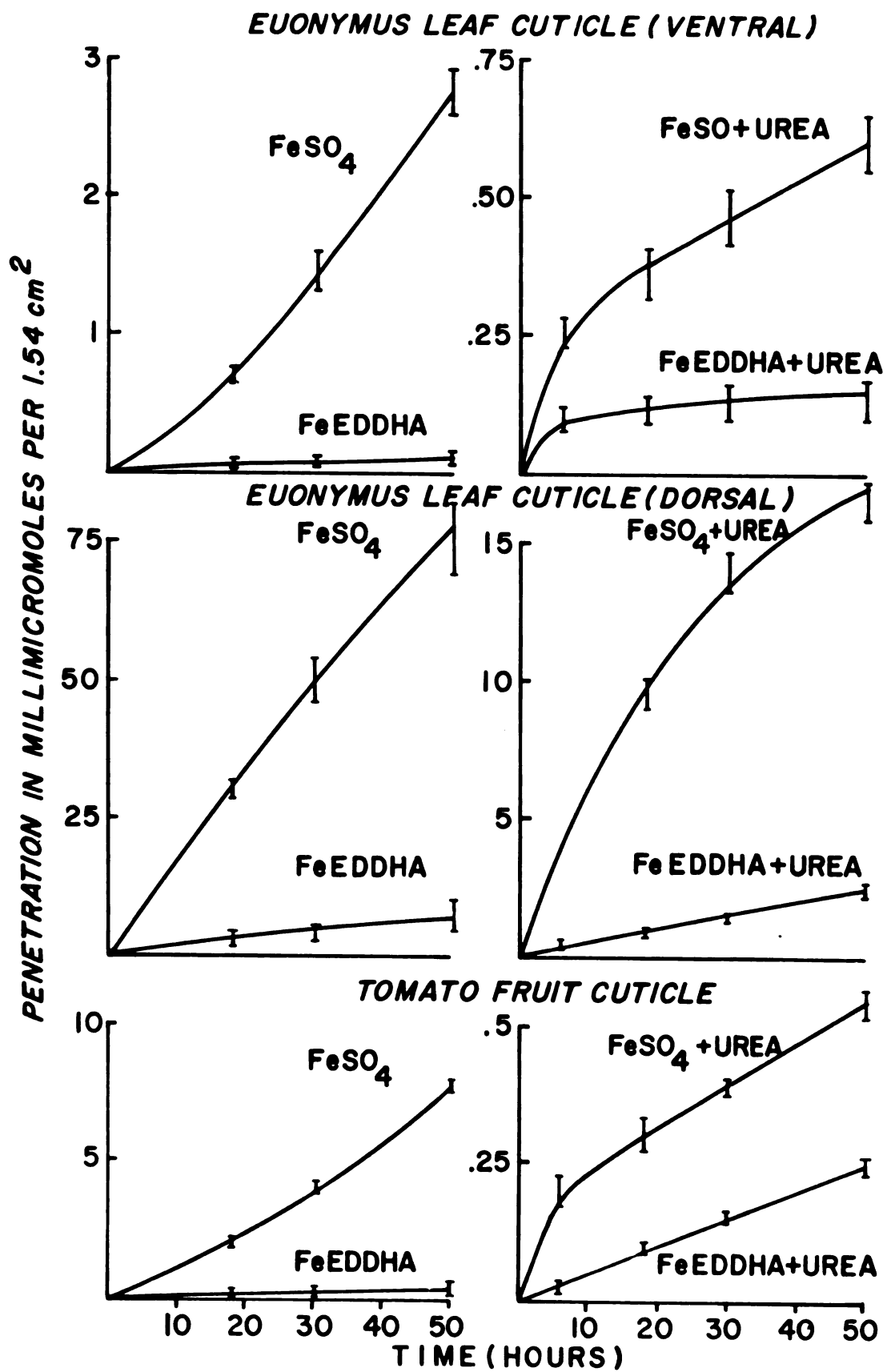
The penetration of Fe from FeSO_4 , far exceeded that from FeEDDHA , FeSO_4 +urea, or FeEDDHA +urea. Both the chelate and the presence of urea decreased the penetration of iron in the three types of cuticles. The permeability of ^{59}Fe through the cuticle removed from the stomatous (Euonymus-dorsal) surface was several times greater than that through the cuticles from the tomato fruit surface and astomatous-ventral surface of the Euonymous leaf.

Ion Binding by Tomato Fruit Cuticles

Ion binding on cuticular surfaces has been related to ion permeability properties (168). This was determined for FeSO_4 on disks of tomato fruit cuticles (3.1 cm^2) in the presence and absence of 0.1mM EDDHA. The disks were first floated in the ^{59}Fe labeled solutions for 5 seconds and the exposed (inner) surface then rinsed quickly in running deionized water for 15

Figure 1

Rates of penetration of ^{59}Fe from ^{59}Fe labeled FeSO_4 and FeDDHA in the absence and presence of urea through cuticular membranes, enzymically isolated from the ventral and dorsal leaf surfaces of Euonymus japonicus, and from ripe tomato fruits. Concentration of urea 10 mM, and others 0.1mM. The vertical intercepts are the standard deviations of the means.



seconds. After blotting the cuticular disks with tissue paper, the bound ^{59}Fe was estimated by radioassay. It is evident from Figure 2 that the binding of ^{59}Fe from $^{59}\text{FeSO}_4$ was considerably reduced by EDDHA, while it increased with increasing concentration in the absence of EDDHA. Cuticular penetration and binding of ^{59}Fe from $^{59}\text{FeSO}_4$ by inner cuticular surface were both reduced by EDDHA.

Effect of Molecular Weight of Solutes on the Rate of Penetration Through Cuticles

Since EDDHA greatly reduced cuticular penetration of Fe, the effect of molecular weight of certain organic compounds was next determined. This was suggested earlier (75) but not studied.

Accordingly, ^{14}C -labeled ethylene glycol, glycerol, D-ribose, D-glucose, sucrose, EDTA, and EDDHA were utilized for comparative penetration studies through the 3 types of cuticles. The results (Figures 3, 4, 5) show that glycerol penetrates very rapidly through all the cuticular membranes and this is also true for ethylene glycol except for the cuticle removed from the dorsal surface of the Euonymus leaf. This suggests that substances of small molecular weight diffuse more rapidly than large molecules such as sucrose and EDDHA. Correlations between molecular weights and rates of penetration are illustrated by the regression analyses (Figure 6). Higher correlations were obtained for astomatous than stomatous cuticles.

Figure 2

Differences in ^{59}Fe binding by the inner surface of tomato fruit cuticles as influenced by ^{59}Fe derived from solutions of FeSO_4 in the absence and presence of EDDHA (0.1mM).

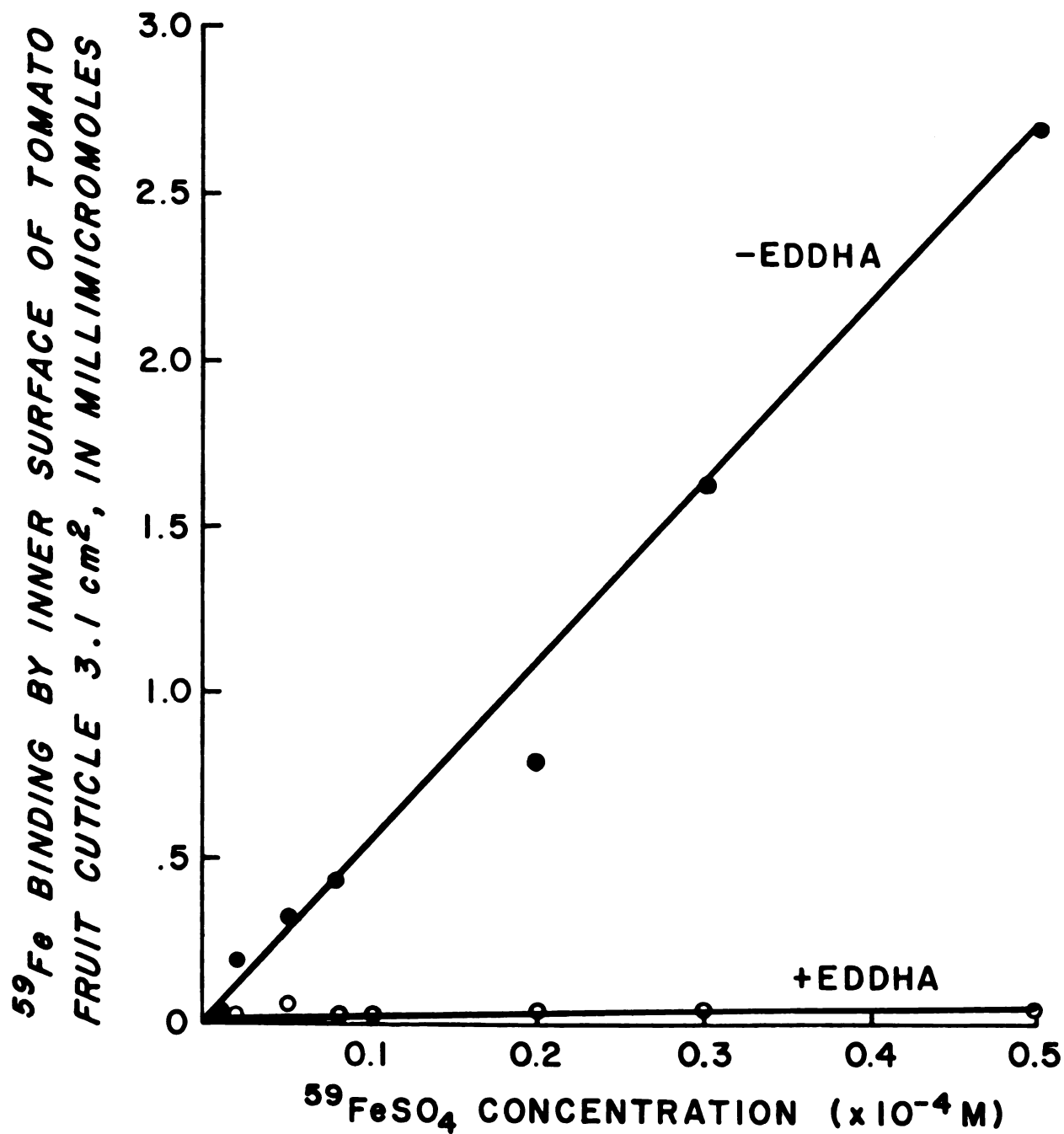


Figure 3

Rates of penetration of ^{14}C -labeled organic substances through cuticular membranes enzymically isolated from the ventral leaf surfaces of Euonymus japonicus. The concentration of each was 0.1mM (Figures in parentheses are molecular weights).

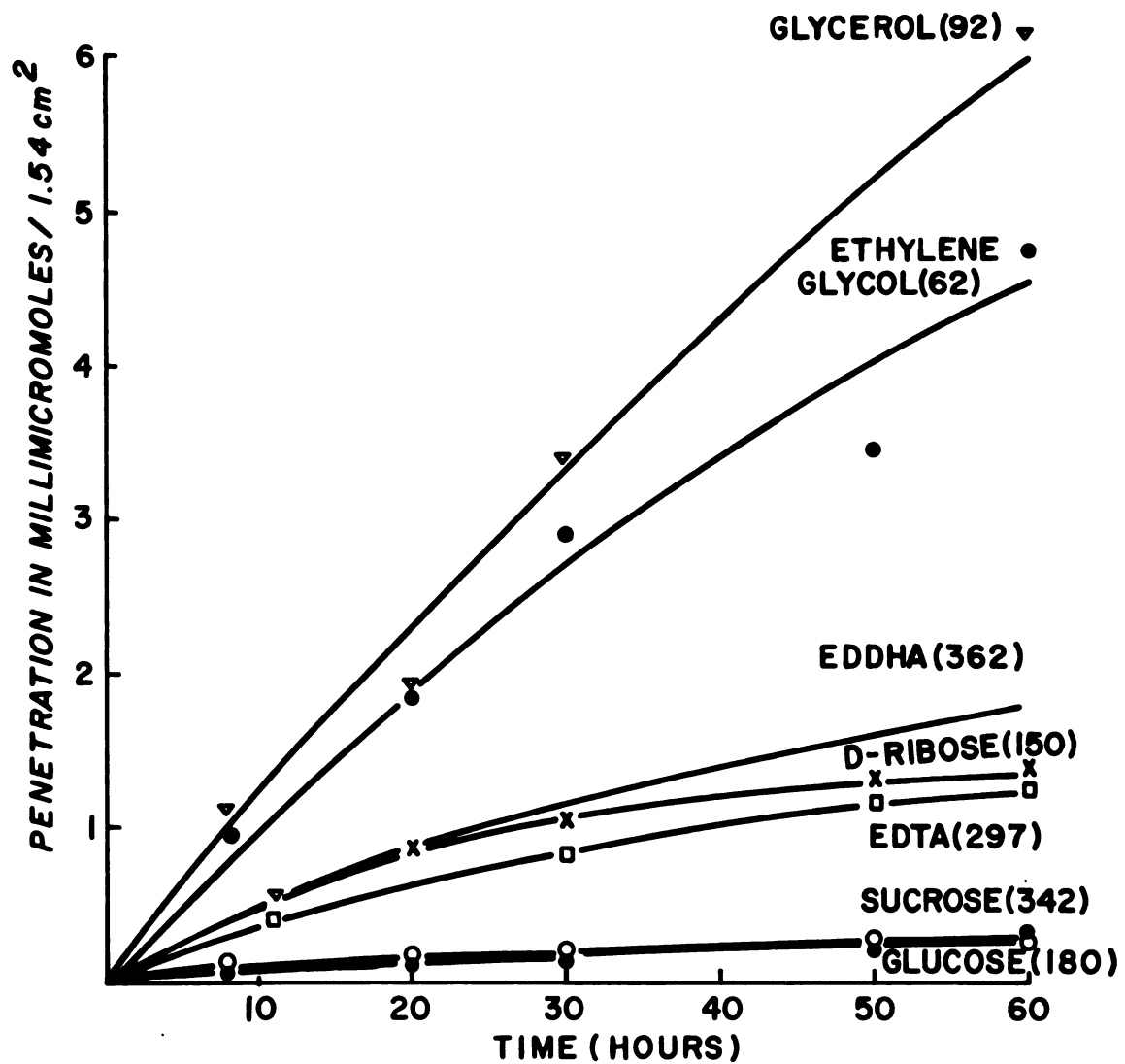
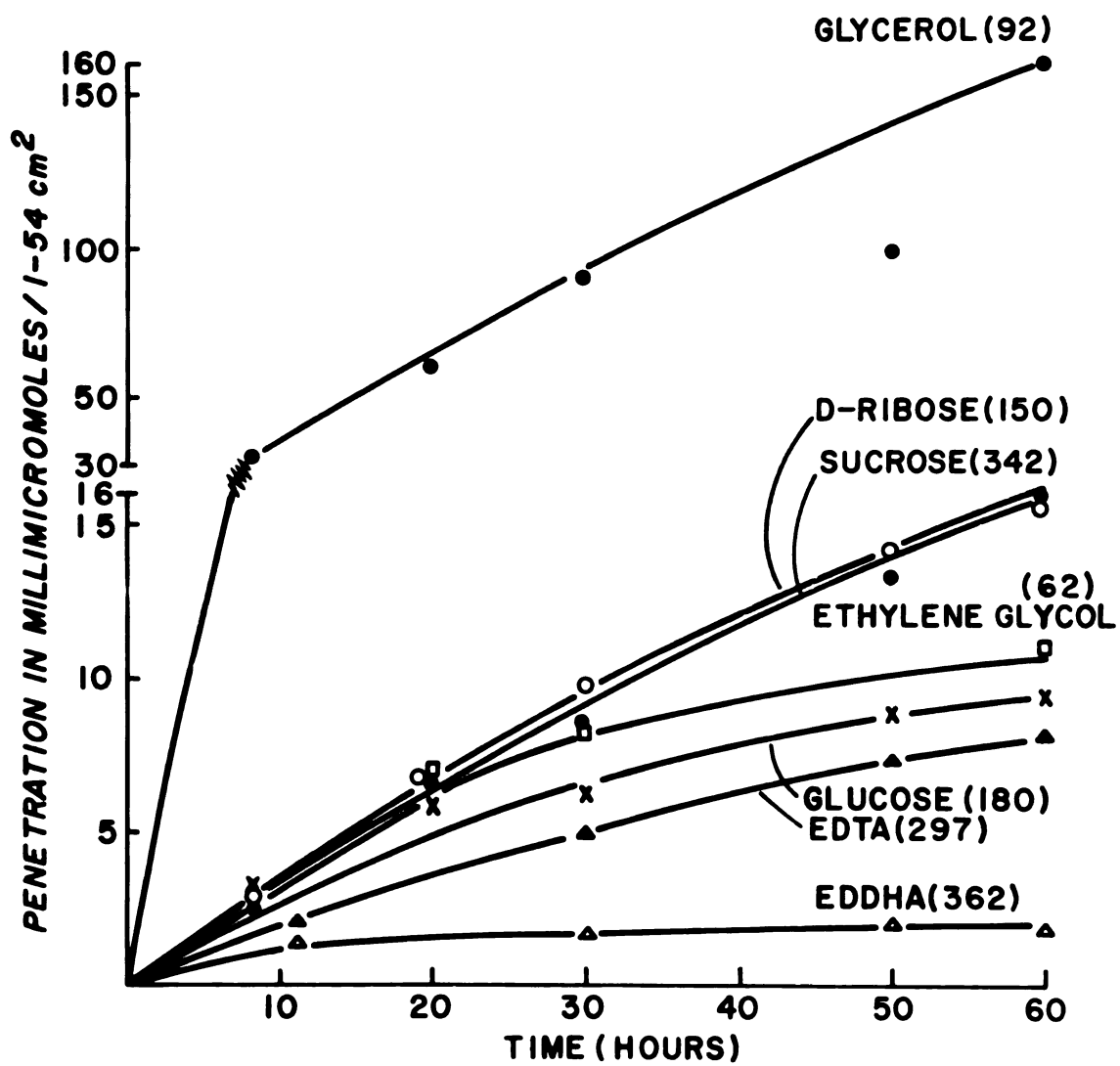


Figure 4

Rates of penetration of ^{14}C -labeled organic substances through cuticular membranes enzymically isolated from the dorsal leaf surfaces of Euonymus japonicus. The concentration of each was 0.1mM. (Figures in parentheses are molecular weights).



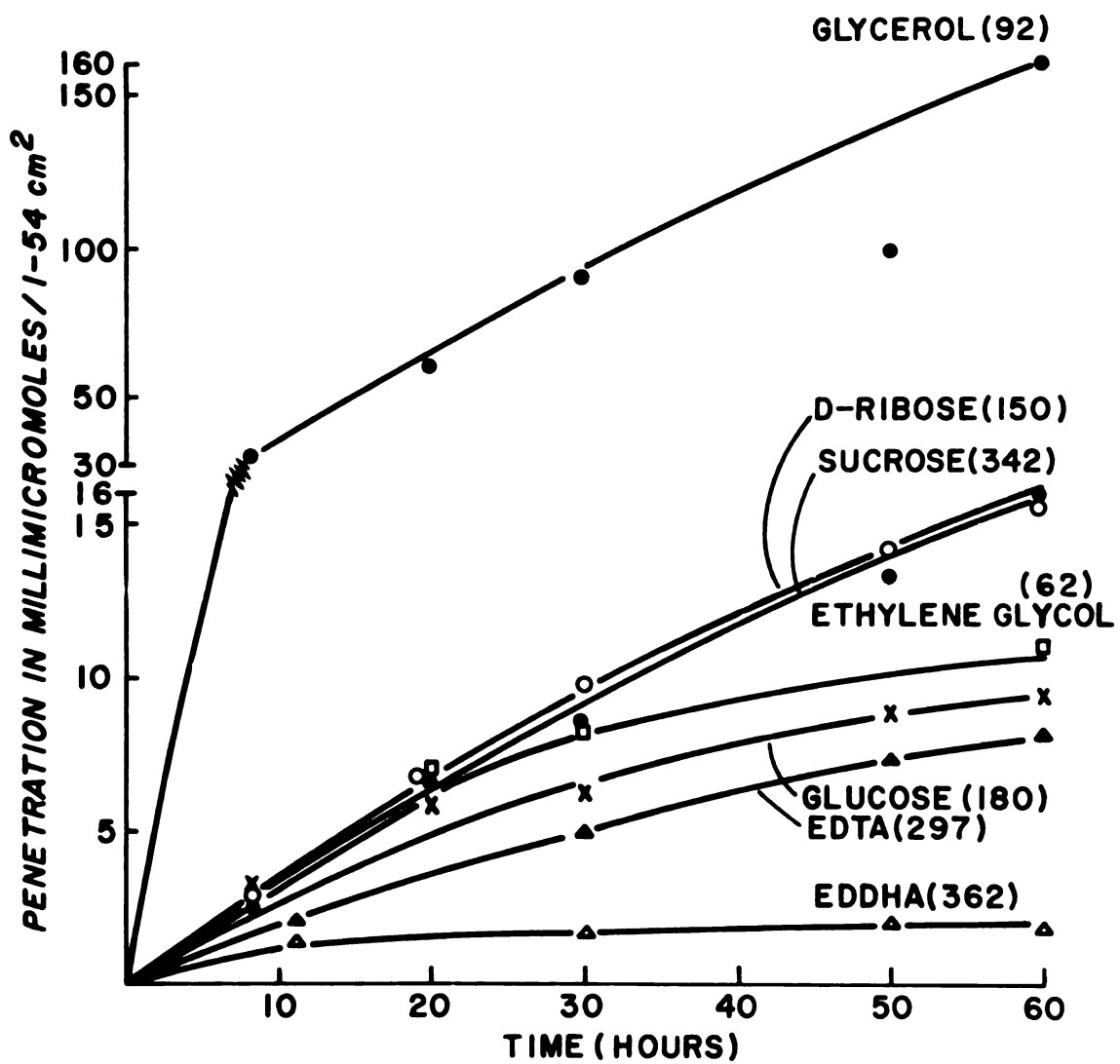


Figure 5

Rates of penetration of ^{14}C -labeled organic substances through cuticular membranes enzymically isolated from ripe tomato fruits. The concentration of each was 0.1mM. (Figures in parentheses are the molecular weights).

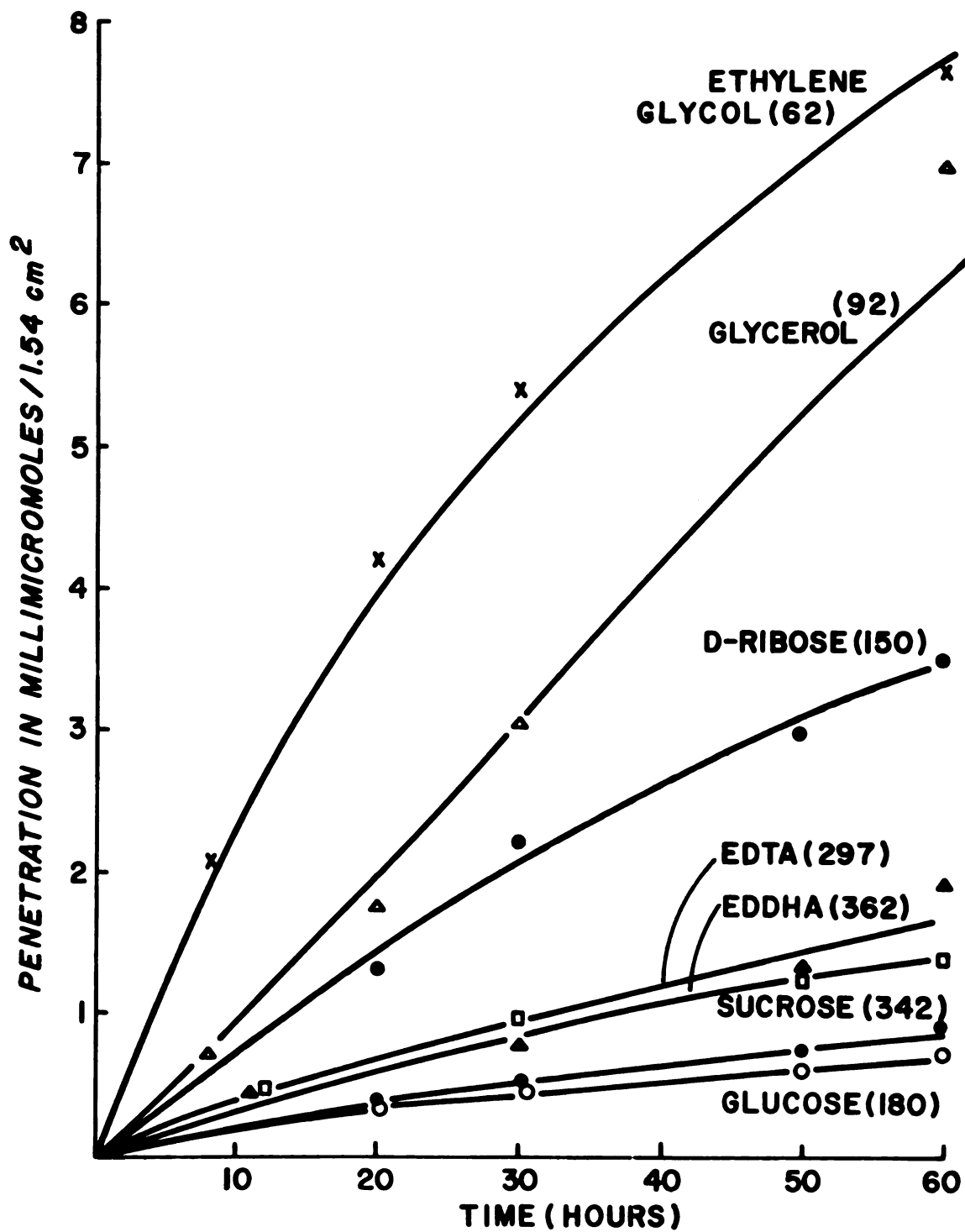
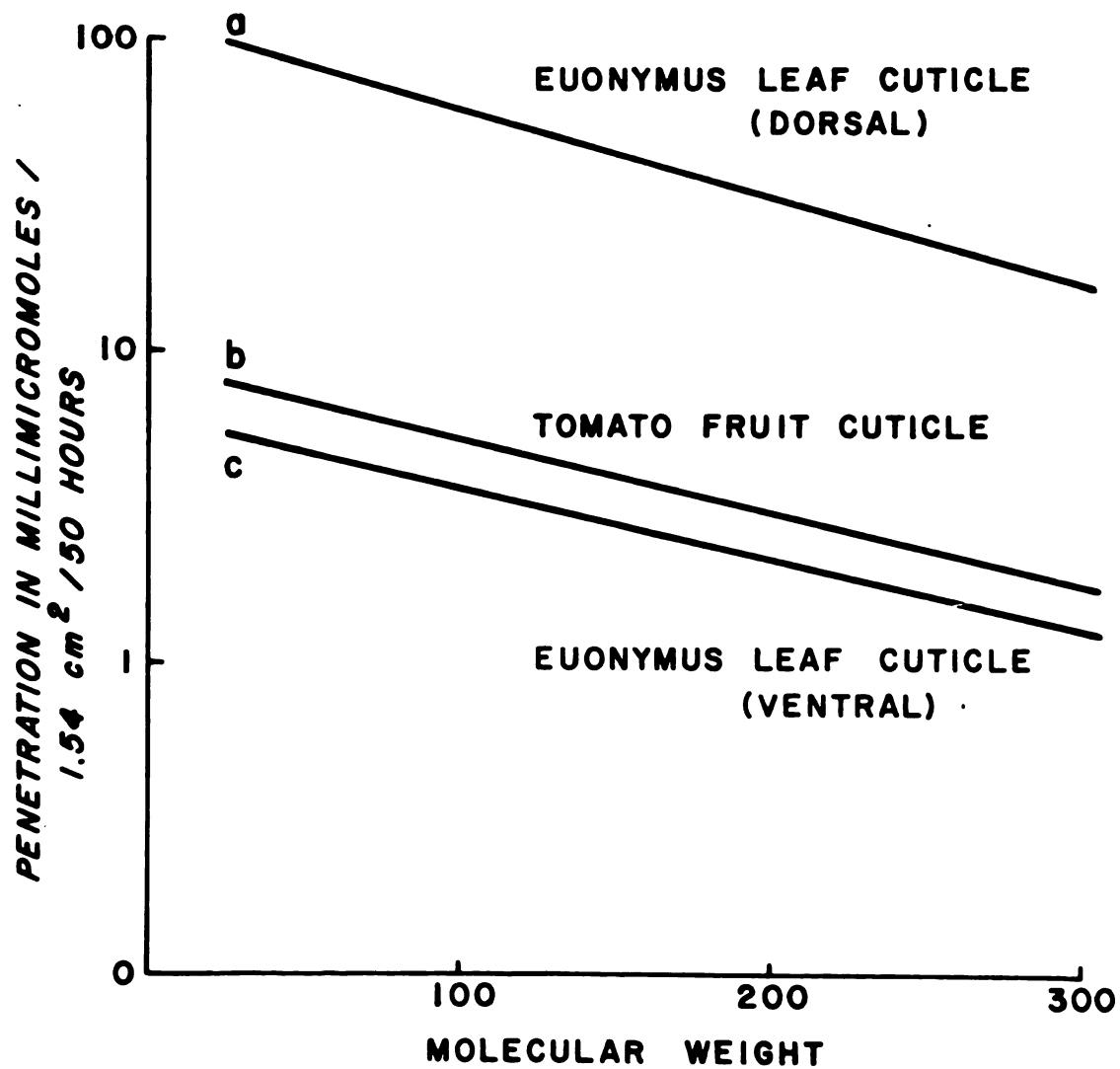


Figure 6

The relation of molecular weight of organic substances and penetration through cuticular membranes as revealed by regression analysis. Molecular weight (x axis) and amount of penetration at 50 hours (y axis). Regression lines: a, cuticular membranes from dorsal leaf surfaces of Euonymus japonicus, $y = 77.15 - 0.21x$; ($r = -.45$, significant at $P = .05$); b, cuticular membranes from ripe tomato fruits, $y = 7.53 - 0.019 x$, ($r = -.75$, significant at $P = .01$); and c, cuticular membranes from the ventral leaf surfaces of Euonymus japonicus, $y = 4.82 - 0.012 x$, ($r = -.61$, significant at $P = .01$). The regression lines are drawn with the above equations with the y values plotted on the log scale.



Permeability Coefficients

In cuticular penetration studies, it would be useful for comparative data if the rates of penetration could be mathematically expressed in terms of a constant. If penetration of solutes through a cuticular membrane is an example of passive transport or free diffusion (165), then Fick's equation applies, namely,

$$\frac{ds}{dt} = -D \cdot \frac{dc}{dx},$$

where ds is the amount of substance diffusing across an area 1 cm.^2 , in time dt , for a concentration gradient of $\frac{dc}{dx}$, with D as a constant.

By substituting P (permeability coefficient) for D , (71), the equation would then be,

$$\frac{ds}{dt} = P (C_1 - C_2).$$

and the movement or flux across the cuticular boundary would be,

$$\phi = P (C_1 - C_2),$$

Or,

$$P = \frac{\phi}{(C_1 - C_2)}.$$

where, ϕ is the amount moving per unit area per unit time, and C_1 and C_2 are the internal and external concentrations, respectively (difference in concentration across the boundary). The dimensions of P are $\text{cm.}/\text{hour}$, provided that the concentration is in millimicromoles per cm^3 .

The permeability coefficients for different substances were calculated using the above equation, and are given in Table III. These values for different substances suggest the importance of molecular size on penetration. The permeability is generally higher for ethylene glycol, glycerol and ribose than for other compounds. It is also fairly high for the inorganic ion, Fe, which has an atomic weight of 55. As in the case of Fick's constant, D , the permeability coefficient also is a property of both the boundary (cuticular membrane) and the solute.

Mechanism of Absorption of Fe by Leaf Cells

Absorption of Fe as Related to Cell Frequency in the Incubation Solution

The absorption of Fe by leaf cells varied greatly with the concentration of cells in a constant volume of incubation solution. An experiment was designed with various amounts of leaf cells in a given volume of incubation mixture to determine the optimum cell mass per unit volume of incubation solution. The absorption time was 4 hours. The incubation mixture, in addition to the cells, contained 1170 umoles sucrose, and 0.5 umoles ^{59}Fe labeled FeSO_4 in a final volume of 10 ml. The contents were shaken under a light intensity of 500 ft. c. and at temperature of $20^\circ \pm 2^\circ \text{C}$. The results are summarized in Figure 7.

The relationship between the amount (mass or frequency) of cells and

Table III

Permeability coefficients (cm/hour) for ^{59}Fe from $^{59}\text{FeSO}_4$ and several organic compounds for enzymically isolated cuticular membranes from the Euonymus leaf and tomato fruit.

	Cuticle from ventral leaf surface	Cuticle from dorsal leaf surface	Cuticle from ripe tomato fruits
FeSO_4	3.3×10^{-4}	1.3×10^{-3}	8.8×10^{-4}
Ethylene glycol	6.1×10^{-4}	1.8×10^{-3}	1.1×10^{-3}
Glycerol	7.4×10^{-4}	2.9×10^{-2}	6.6×10^{-4}
D-Ribose	2.4×10^{-4}	2.1×10^{-3}	4.8×10^{-4}
Glucose	0.4×10^{-4}	1.3×10^{-3}	1.1×10^{-4}
EDTA	1.7×10^{-4}	1.1×10^{-3}	1.9×10^{-4}
Sucrose	0.4×10^{-4}	2.0×10^{-3}	1.1×10^{-4}
EDDHA	2.4×10^{-4}	3.3×10^{-4}	1.9×10^{-4}

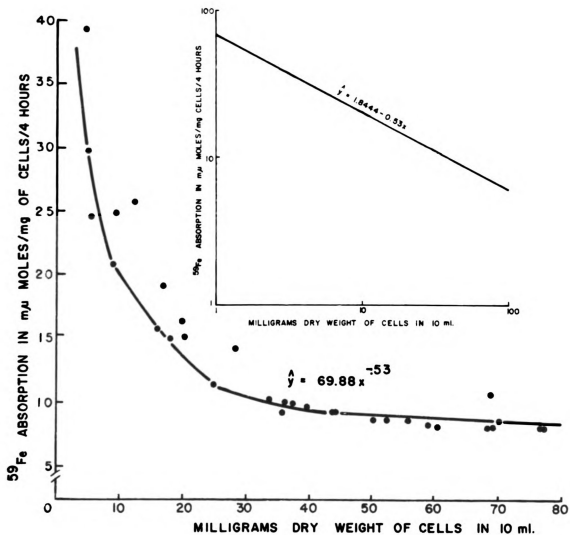
Figure 7

Absorption of ^{59}Fe by enzymically isolated cells from tobacco leaves, as a function of the amount of cells in the incubation mixture.

For details see text.

(Insert). Linear transformation of the curve showing correlation between the weight of cells and absorption of ^{59}Fe by cells.

($r = -.83$, significant at $P = .01$).



Factors Influencing the Absorption of Fe by Tobacco Leaf Cells

External Factors

Light, succinate, and bicarbonate:

The incubation mixture used in the experiments contained the following umoles in 40 ml.: sucrose 4680, tris-maleate (pH 6.4) 400, ^{59}Fe labeled FeSO_4 2.0, and cells equivalent to 200 mgs. dry weight. Na_2 -succinate (80 umoles) was included in all treatments except the minus succinate. The contents were shaken at $20^\circ \pm 2^\circ \text{C}$ and in light ca. 500 ft. c. in all treatments excepting the minus light. The samples were taken at various time intervals and comparative rates of absorption determined. Light and succinate substantially increased the absorption of Fe by the tobacco leaf cells (Figure 8).

The effects of an interaction between succinate and bicarbonate are shown in Table IV. Unlike in the case of Rb and phosphate absorption by tobacco leaf cells (76), bicarbonate reduced Fe absorption significantly in dark. While succinate counteracted the effect of bicarbonate in dark, it enhanced the inhibitory effect of bicarbonate in light. This difference was however not statistically significant.

Citrate, malate, and succinate:

Fe forms chelates with some organic acids and is translocated as a chelate in the intact plant (140). Accordingly, the effects of citrate,

Figure 8

- (a) Effects of succinate on the rate of absorption of ^{59}Fe by cells enzymically isolated from tobacco leaves in the light.
- (b) Effects of light on the absorption of ^{59}Fe by cells enzymically isolated from tobacco leaves.

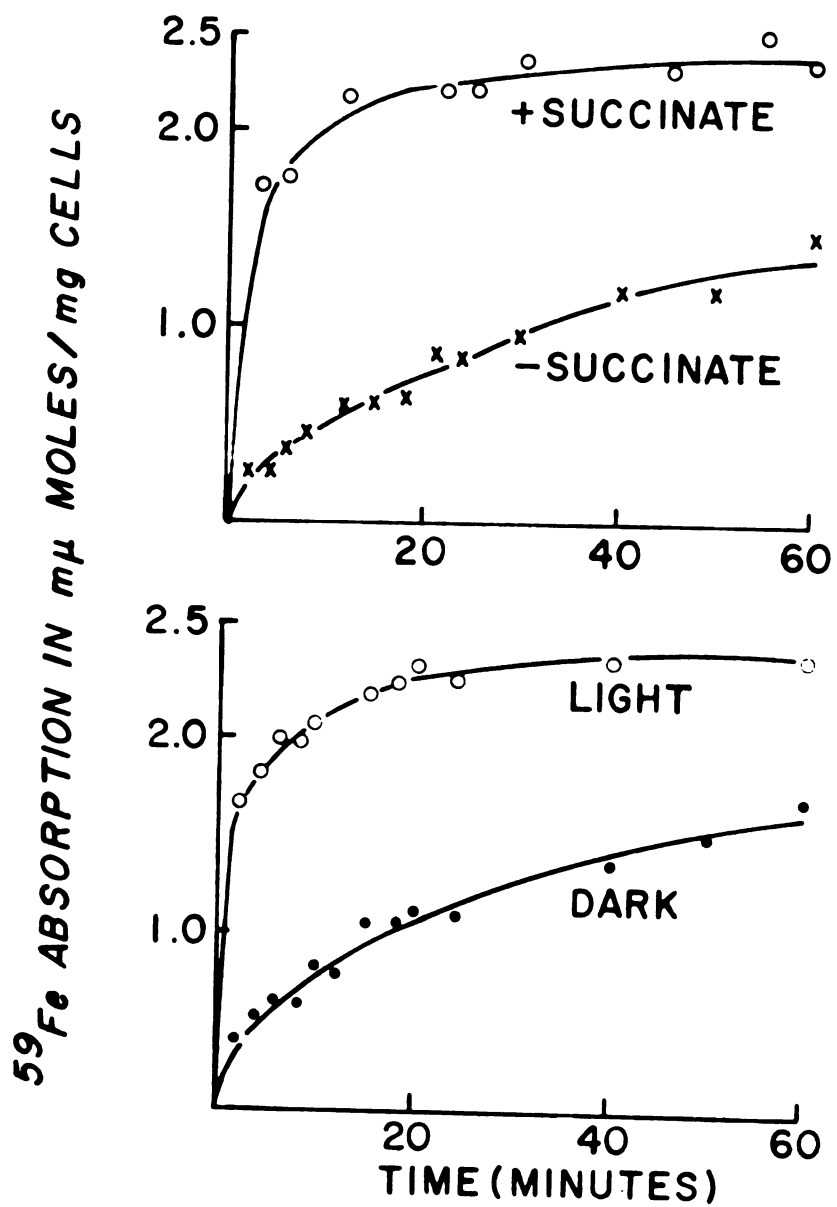


Table IV

Effects of succinate and bicarbonate on the absorption
of ^{59}Fe by tobacco leaf cells.

Treatment * Chemical	^{59}Fe absorption in mumoles/mg. cells/4 hrs. ^{1/}	
	Light (500 ft. c.)	Dark
Na_2 -succinate ($2 \times 10^{-3}\text{M}$)	5.96 ^a	3.26 ^d
NaHCO_3 ($2 \times 10^{-3}\text{M}$)	5.71 ^{ab}	2.05 ^c
Na_2 -Succinate + NaHCO_3	4.93 ^b	2.55 ^{cd}

^{1/} Means not followed by the same letters were significantly different at odds of 19:1.

* The basic incubation mixture contained the following in umoles in the final volume of 10 ml., sucrose 1170, tris-maleate (pH 6.4) 100, ^{59}Fe labeled FeSO_4 0.5, and cells 50 mgs. Contents were shaken in water bath in light at ca. 500 ft. c., and at $20^\circ \pm 2^\circ \text{C}$.

malate and succinate on Fe absorption were studied. The results are summarized in Table V. There was no enhancement of uptake, and citrate significantly reduced the absorption of Fe.

Temperature:

The time course of Fe absorption by tobacco leaf cells, as influenced by temperature is illustrated in Figure 9, and data on temperature coefficients and activation energies are summarized in Table VI. Both Q_{10} and E_a values decrease with increasing temperature. Absorption of Fe is temperature-dependent, and the optimum temperature for ^{59}Fe uptake by cells appears to be about 25°C .

Metabolic inhibitors:

The results of a study utilizing metabolic inhibitors viz., NaN_3 , and 2,4-DNP (dinitrophenol), in the absence and presence of ATP are summarized in Table VII. Absorption of Fe was significantly reduced by NaN_3 , DNP, and even ATP. It is probable that externally applied ATP might compete with Fe and form a Fe-phosphate complex, which might preclude absorption by cells.

Effects of Ca on absorption of Fe and Mn:

Esperiments with barley roots on the absorption of, and interactions with Na, K, and Rb (33) have indicated the essential role of calcium in selective cation transport in plant cells. The results in Table VIII show the effects of

Table V

Effects of tris-maleate, Na_2 -succinate, K_3 -citrate, and Na-bicarbonate
on absorption of ^{59}Fe by tobacco leaf cells.

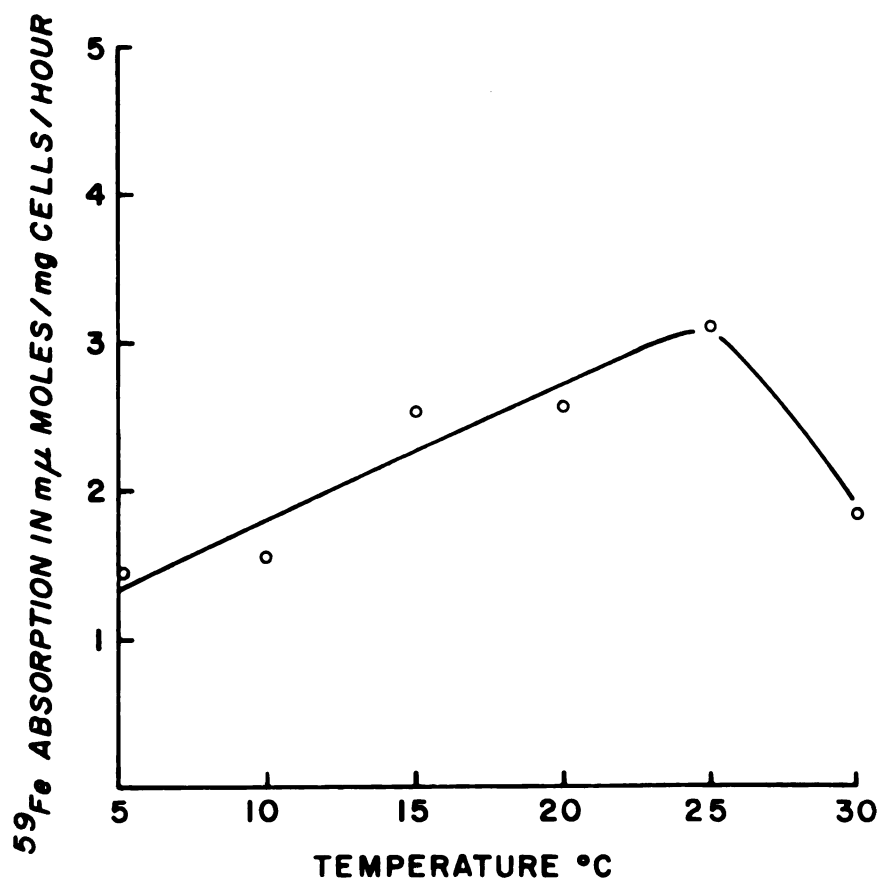
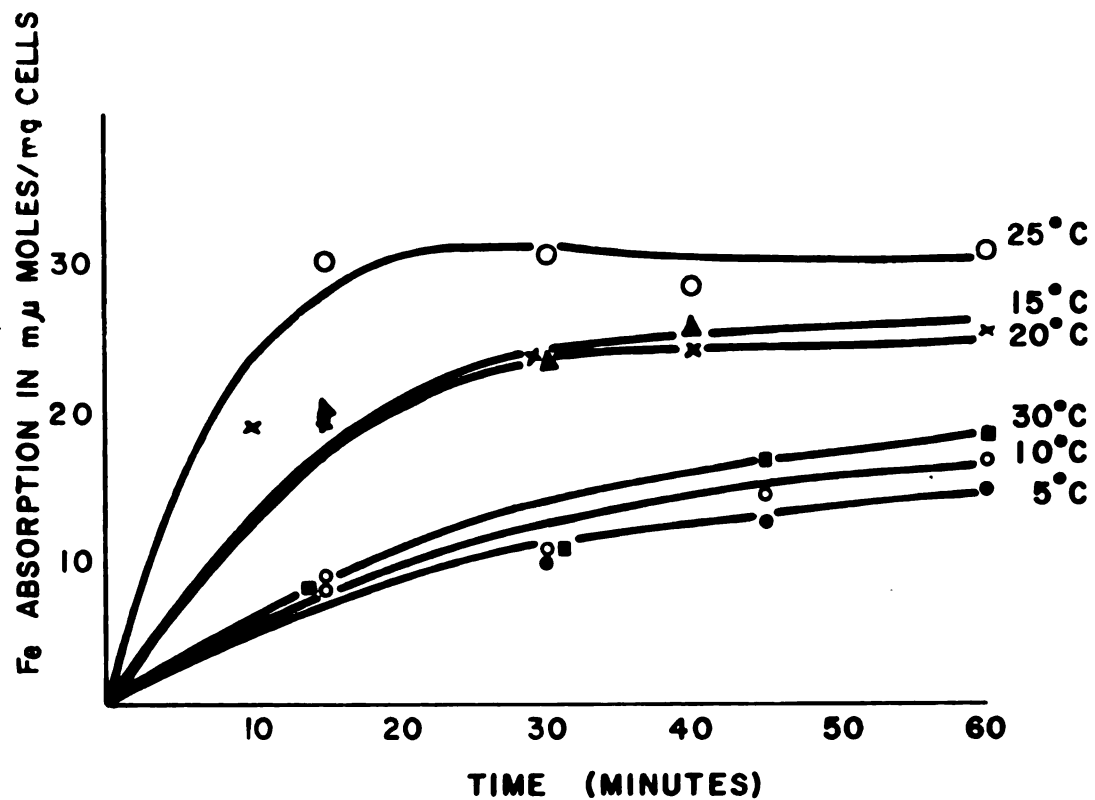
Treatment Chemical*	^{59}Fe absorption in mumoles/mg. cells/5 hrs. ^{1/}
None	5.20 ^a
Tris-maleate	4.99 ^a
Tris-maleate + Na_2 -succinate	5.20 ^a
Tris-maleate + K_3 -citrate	4.48 ^b
Tris-maleate + Na_2 -succinate + K_3 -citrate	4.56 ^b
Tris-maleate + Na_2 -succinate + K_3 -citrate + Na-bicarbonate	4.55 ^b

^{1/} Means not followed by same letters were significantly different at odds of 19:1.

* The basic incubation mixture contained sucrose 1170 umoles, ^{59}Fe labeled FeSO_4 0.5 umole, and cells 50 mgs., in a final volume of 10 ml. The chemicals included in the treatments were tris-maleate (pH 6.4) 100 umoles, and others 20 umoles each. Contents were exposed to light 500 ft. c., and temperature $20^\circ \pm 2^\circ\text{C}$.

Figure 9

- (a) The effects of temperature on the rate of absorption of ^{59}Fe by cells enzymically isolated from tobacco leaves.
- (b) Absorption of ^{59}Fe by cells enzymically isolated from tobacco leaves, as a function of temperature.



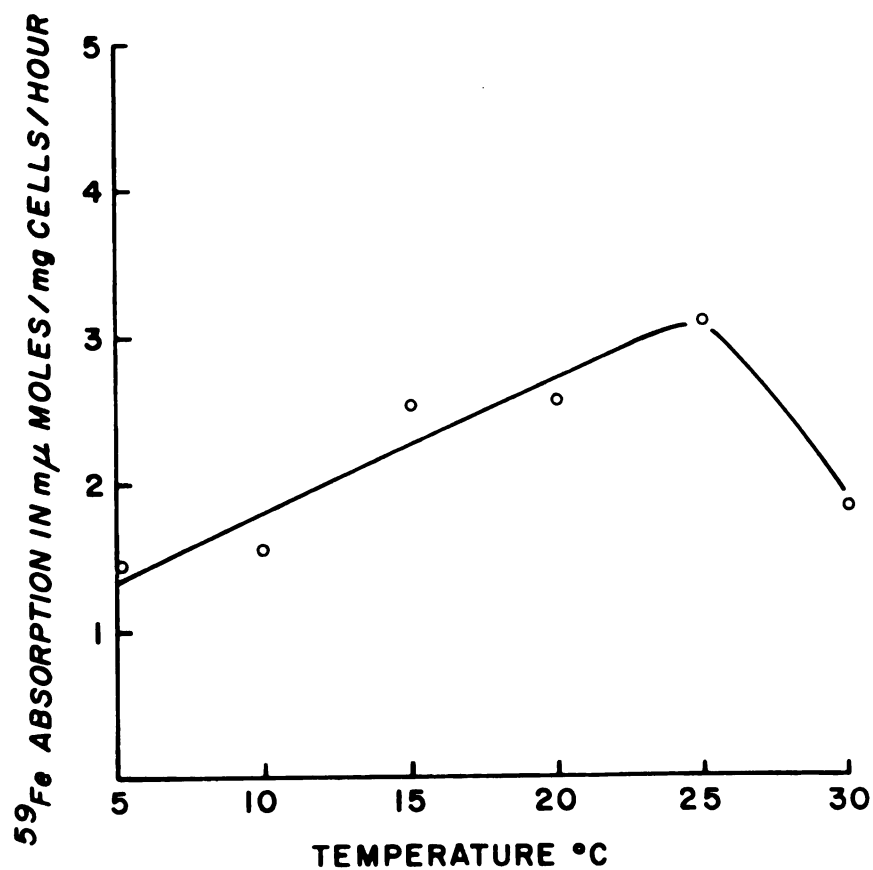
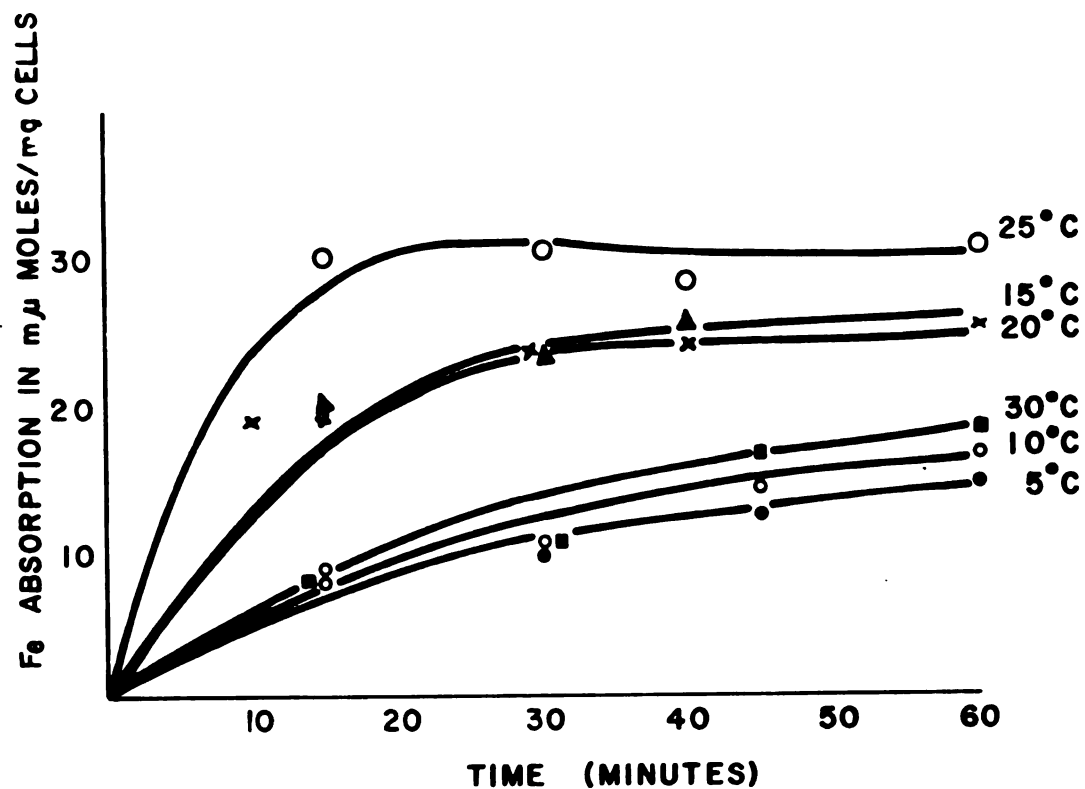


Table VI

Effects of temperature on ^{59}Fe absorption by tobacco leaf cells.

Temperature ($^{\circ}\text{C}$)	^{59}Fe absorption (mumoles/mg. cells/hr.)	Temp. coeff. (Q_{10})	Active Energy (E_a -Kcal.mole $^{-1}$)
5	1.45	1.77 ($5^{\circ} - 15^{\circ}$)	9.08
10	1.55	1.65 ($10^{\circ} - 20^{\circ}$)	
15	2.57	1.19 ($15^{\circ} - 25^{\circ}$)	
20	2.56	0.71 ($20^{\circ} - 30^{\circ}$)	
25	3.10		
30	1.82		

Experimental conditions are the same as those in Table IV excepting for temperature.

Table VIII

Effects of NaN_3 , 2,4-DNP and ATP on ^{59}Fe absorption by tobacco leaf cells.

Treatment		^{59}Fe absorption	Inhibition (%)
Chemical	Conc. (M)	mumoles/mg. cells/2 hrs. ^{1/}	
Control		4.13 ^a	--
NaN_3	10^{-3}	2.30 ^b	44
2,4-DNP	10^{-4}	2.04 ^b	51
ATP	10^{-4}	1.75 ^b	58
2,4-DNP + ATP	10^{-4}	1.74 ^b	58

^{1/} Means followed by different numbers are significantly different at odds of 99:1.

Experimental conditions same as Table IV.

Table VIII

Effects of Ca on ^{59}Fe and ^{54}Mn absorption by tobacco leaf cells.

Concentration of CaSO_4 (M)	Absorption in mumoles/mg. cells/4 hrs. ^{1/}	
	^{59}Fe	^{54}Mn
Control	17.4 ^a	5.2 ^a
2×10^{-4}	--	4.9 ^a
5×10^{-4}	18.3 ^a	3.6 ^b
8×10^{-4}	17.0 ^a	--
1×10^{-3}	8.3 ^b	2.8 ^b
2×10^{-3}	4.8 ^b	--
1×10^{-2}	--	1.0 ^c

^{1/} Within each column, means followed by different letters were significantly different at odds of 19:1.

Experimental conditions the same as in Table IV.

calcium on the absorption of Fe and Mn. Calcium either had no effect or reduced the absorption of both Fe and Mn by leaf cells. The physiological concentration of Ca, viz., 0.5 mM, used in studies of cation transport in root tissues by many workers, was perhaps too high for the enzymically isolated leaf cells employed in the present study.

Interactions of Fe and Mn in absorption and the role of calcium on Fe-Mn transport are portrayed in Figure 10. These data suggest that absorption of Fe and Mn is mutually antagonistic. The inhibitory effects of Fe on Mn absorption is much greater than that of Mn on Fe uptake. While calcium partially reverses the inhibition of Mn on Fe absorption, it increases the inhibitory effect of Fe on Mn uptake.

The inhibition of foliar absorption of Fe, Mn, Zn, and Rb by calcium was further studied using tobacco leaf disks (0.8 cm^2). Four disks weighing about 100 mgs. were shaken in a flask containing sucrose 2340 umoles, and 1.0 umole of isotopically labeled FeSO_4 , MnSO_4 , ZnSO_4 or RbCl in a volume of 20 ml. All experiments were conducted in the light at ca. 500 ft. c. and a temperature $20^\circ \pm 2^\circ \text{C}$. At the end of four hours the disks were rinsed in deionized water and radioassayed. The means of 3 values were used to plot the graph in Figure 11. The inhibiting effect of calcium on absorption of Fe, Mn, Zn was observed even with intact leaf disks. There was little effect on Rb.

Figure 10

- (a) Absorption of ^{59}Fe by cells enzymically isolated from tobacco leaves, as a function of FeSO_4 concentration, and the presence of Mn, and Mn + Ca. (Mn, 20mM; Ca, 10mM).
- (b) Absorption of ^{54}Mn by cells enzymically isolated from tobacco leaves, as a function of MnSO_4 concentration, and the presence of Fe and Fe + Ca. (Fe, 20mM; Ca, 10mM).

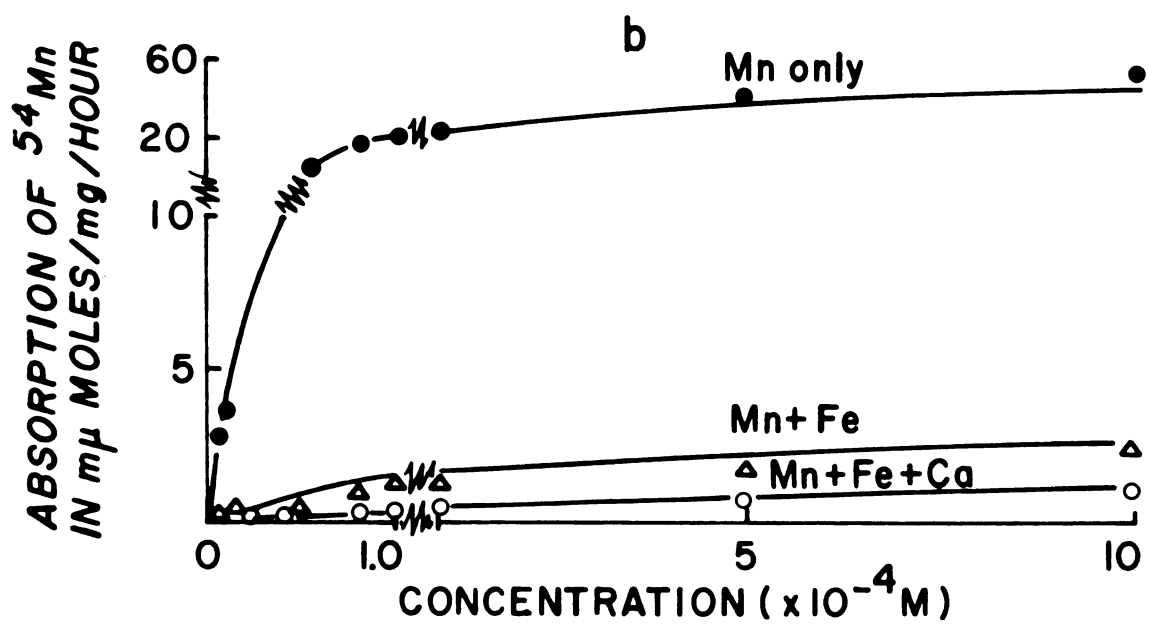
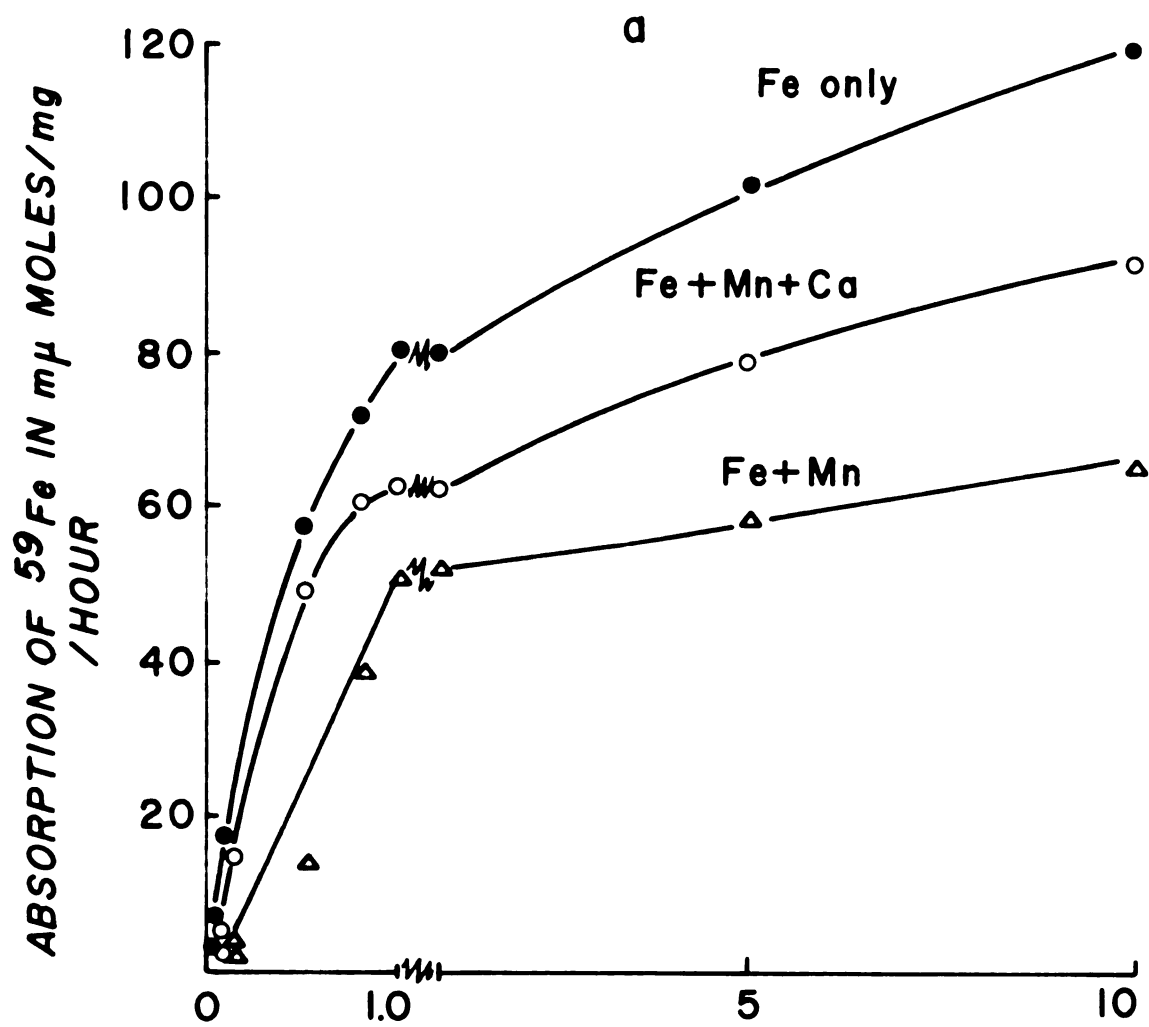
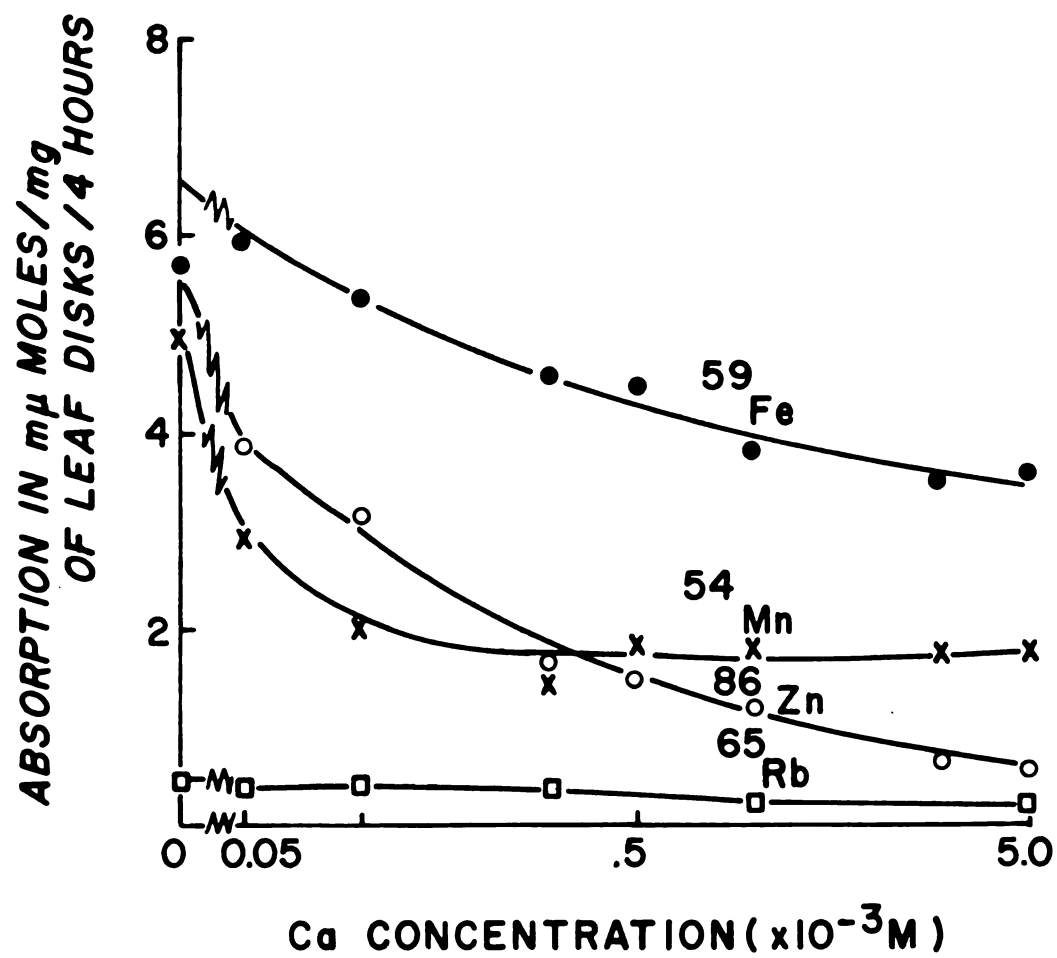


Figure 11

Effects of increasing levels of calcium on the absorption of ^{59}Fe , ^{54}Mn , ^{65}Zn , and ^{65}Rb by tobacco leaf disks.



Internal Factors

The age of the leaf may be a significant factor in foliar absorption of nutrients. Accordingly, leaf number beginning from the apex to the base of the tobacco plant was correlated with the rate of Fe absorption by cells isolated from them. With increasing leaf age (leaf number) cell absorption of Fe was significantly reduced (Figure 12). Cells from the youngest leaves absorbed the greatest amount of Fe.

The relationship between the chlorophyll content of tobacco leaves and the ability of the leaf cells to absorb ^{59}Fe is portrayed by Figure 13. Total chlorophyll was estimated by the method of Benne *et al.* (5). The absorption rate was directly correlated with the chlorophyll content.

Fe chlorosis was induced in Hawkeye variety (chlorosis non-susceptible), and P1 54619-5-1 variety (chlorosis susceptible) soybeans by growing the seedlings in minus and plus Fe nutrient solutions. Leaf cells were then isolated from chlorotic and non-chlorotic (normal) first trifoliate leaves and the rate of absorption of ^{59}Fe recorded (Table IX). Cells from chlorotic leaves of P1 54619-5-1 absorbed less Fe than those isolated from healthy leaves of the same variety. There was, however, no significant difference in ^{59}Fe absorption of cells derived from chlorotic and normal leaves of the Hawkeye soybean.

Figure 12

Effects of tobacco leaf age (designated by leaf number from apex to base) on the absorption of ^{59}Fe by cells enzymically isolated from them. Regression equation, $y = 4.61 - 0.18 x$, $r = -.67$, significant at $P = .01$. Absorption in millimicromoles of $^{59}\text{Fe}/\text{mg. cells}/\text{hour}$ is plotted on log scale.

Figure 13

Relationship between the rate of ^{59}Fe absorption by enzymically isolated leaf cells and the chlorophyll content of the leaves. Regression equation, $y = 1.90 + 0.28 x$, $r = +.61$, significant at $P = 0.1$. Absorption in millimicromoles of $^{59}\text{Fe}/\text{mg. cells}/\text{hour}$ is plotted on log scale.

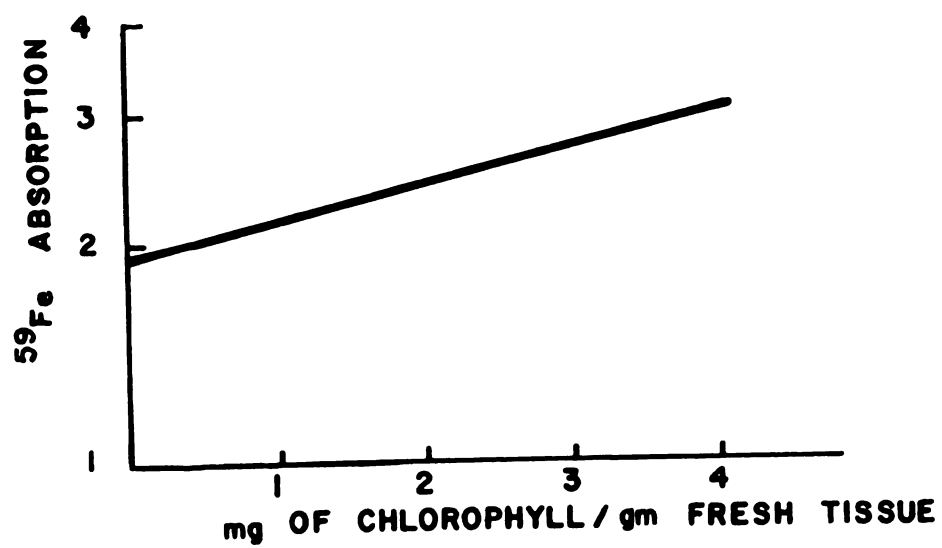
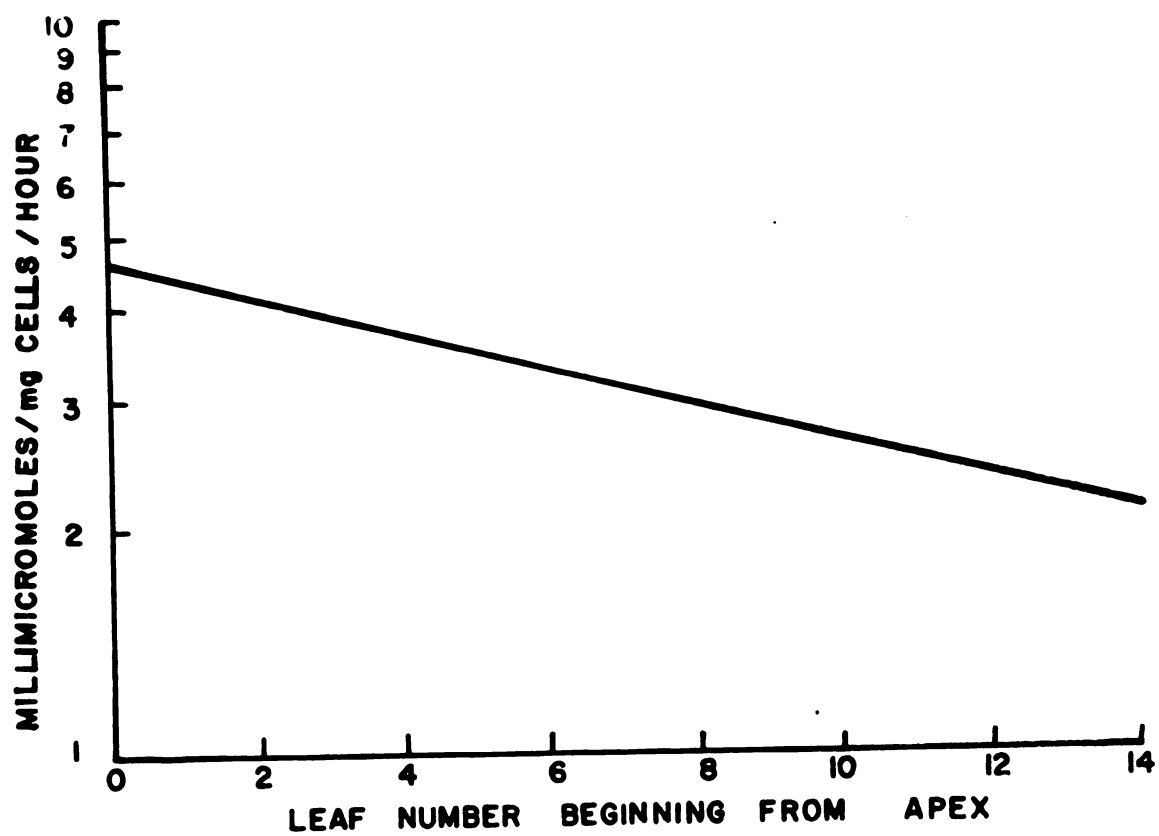


Table IX

Comparative absorption of ^{59}Fe by cells removed from chlorotic and healthy leaves of two varieties of soybean.

Condition of the leaves	Absorption of ^{59}Fe in mumoles/mg. cells/1 hour. ^{1/}	
	Hawkeye (chlorosis nonsusceptible)	PI 54619-5-1 (chlorosis susceptible)
Chlorotic	4.36 ^a	2.41 ^b
Healthy	3.64 ^a	5.10 ^a

^{1/} Within each column, means followed by different letters were significantly different at odds of 19:1.

Experimental conditions are the same as in Table IV.

Absorption of ^{59}Fe from FeSO_4 , FeEDTA , FeEDDHA ,
and FeSO_4 in the Presence of Urea.

Absorption of ^{59}Fe by Leaf Cells

The effects of two chelates namely, EDTA and EDDHA, and urea on the absorption of Fe by cells isolated from tobacco and bean leaves are summarized in Table X. Chelation reduced, while urea enhanced uptake.

Uptake and Transport of ^{59}Fe in Intact Plants

Intact primary leaves of bean plants were utilized to measure specific absorption and subsequent transport of ^{59}Fe according to methods of Jyung et al. (74). The effects of chelation and urea on both uptake and translocation are given in Table XI. Urea enhanced uptake but less of the absorbed ^{59}Fe as percentage was translocated. On the other hand, chelation inhibited absorption but similarly enhanced the translocation of the absorbed ^{59}Fe .

Effects of Pre-treatments with EDDHA and Urea on ^{59}Fe Absorption by Bean Leaf Cells

The effects of pre-treatments with EDDHA and Urea, on the subsequent absorption of Fe from different sources, were studied and the results are summarized in Table XII. Urea combined with FeSO_4 was necessary for

Table X

Comparative absorption of ^{59}Fe from different sources, and in the presence of urea, by cells enzymically isolated from green tobacco and bean leaves.

Source of ^{59}Fe	Uptake in mumoles/mg. cells/8 hrs. ^{1/}	
	Tobacco leaf cells	Bean leaf cells
FeSO_4	14.5 ^a	33.8 ^a
Fe-EDTA	6.2 ^b	29.3 ^a
Fe-EDDHA	4.3 ^b	5.8 ^b
FeSO_4 + Urea	24.4 ^c	45.2 ^c

^{1/} Within each column, means followed by different letters were significantly different at odds of 19:1.

The incubation mixture contained sucrose 1170 umoles, ^{59}Fe labeled FeSO_4 , FeEDTA, and FeEDDHA, 0.5 umoles each, and urea 50 umoles, in a final volume of 10 ml., in treatments where these are indicated. Contents were exposed to 500 ft. c. light and to $20^\circ \pm 2^\circ\text{C}$, in a shaking water bath.

Table XI

Uptake and transport of ^{59}Fe by intact primary leaves of the
bean plant as affected by different carriers
(Leaf immersion technique).

Carrier*	Uptake** mumoles/mg./12 hrs.	Translocation** (%)
FeSO_4	12.8 ^b	0.5 ^c
FeEDTA	9.1 ^c	2.5 ^b
FeEDDHA	1.3 ^d	6.0 ^a
FeSO_4 + Urea	30.7 ^a	0.6 ^c

** Within each column, means followed by different letters were significantly different at odds of 19:1.

* The concentration of all the chemicals used was 0.1mM., excepting urea, which was 10 mM. The leaves were exposed to 500 ft. c. of light, and $20^\circ \pm 2^\circ\text{C}$.

Table XII

Effects of pre-treatments with EDDHA on the absorption of ^{59}Fe from FeSO_4 , in the absence and presence of urea, and pre-treatment with urea on the absorption of ^{59}Fe from FeSO_4 and FeEDDHA by isolated bean leaf cells.

Treatment		Absorption of ^{59}Fe in mumoles/mg. cells/4 hrs. ^{1/}
FeSO_4		28.8 ^b
FeEDDHA		7.1 ^c
FeSO_4 + Urea		39.3 ^a
FeEDDHA + Urea		8.8 ^c
Pretreated with		
EDDHA	FeSO_4	30.8 ^b
	FeSO_4 + Urea	39.4 ^a
Urea	FeSO_4	29.0 ^b
	FeEDDHA	6.1 ^c

^{1/} Means followed by different letters were significantly different at odds of 19:1.

Experimental conditions are the same as in Table X. In pre-treatments, the cells were treated for 2 hours. in solutions containing sucrose 1170 umoles, EDDHA 0.5 umoles, or urea 50 umoles, in a total volume of 10 ml., and treatments were given to the cells after separating them by centrifugation.

enhancing the absorption of Fe. Urea had no effect on Fe absorption from FeEDDHA. Pre-treatment with EDDHA had no effect on the subsequent absorption of Fe from FeSO_4 , either in the absence or presence of urea.

Absorption of ^{59}Fe from FeSO_4 in Absence and Presence of Urea, ^{14}C -EDTA, ^{14}C -EDDHA, and ^{14}C -Urea by Tobacco Leaf Cells

The absorption rates of ^{59}Fe as the sulfate and chelated with EDDHA are portrayed in Figure 14. The enhancement of ^{59}Fe uptake by urea is also given along with absorption of ^{14}C labeled urea and EDDHA. Absorption of ^{14}C urea is less than ^{59}Fe from FeSO_4 ; and ^{59}Fe absorption is greatly reduced by chelation with EDDHA. Absorption of ^{14}C -EDDHA by cells is negligible. Absorption of the ligand is also reduced when combined with the metal (Figure 15). These data reveal that the metal and ligand are absorbed differentially by enzymically isolated leaf cells. Conceivably, the two separate at the cell surface prior to absorption.

Determination of Affinity Constant, and Application of Enzyme-Kinetics to Absorption of ^{59}Fe by Leaf Cells

The velocity of ^{59}Fe absorption by isolated tobacco leaf cells as a function of the concentration of ^{59}Fe labeled FeSO_4 is illustrated in Figure 16. The affinity constant $1/K_m$, for the metal-carrier complex was calculated and found of low order.

Figure 14

Comparative absorption rates by isolated tobacco leaf cells of ^{59}Fe from FeSO_4 alone and in combination with urea; also ^{59}Fe absorption when chelated with EDDHA, and the absorption of ^{14}C labeled urea and EDDHA. The basic incubation mixture contained sucrose 1170 umoles, cells 50 mgms, Fe (as in FeSO_4) and EDDHA 0.5 umoles, each, and urea 50 umoles, in a final volume of 10 ml. Each point is a mean of three values.

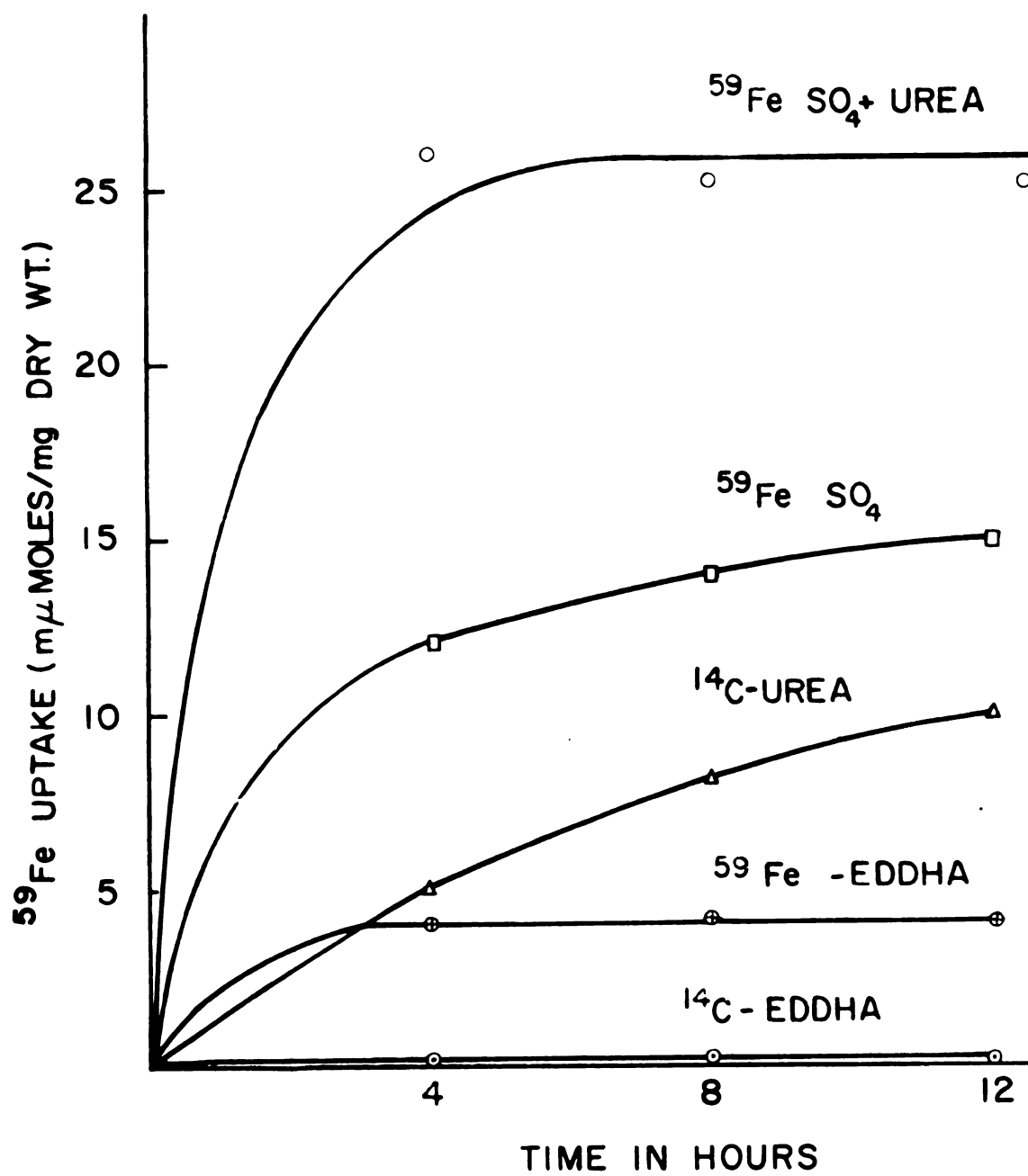


Figure 15

Absorption rates for ^{14}C labeled EDTA and EDDHA, separately and in combination with Fe, by cells enzymically isolated from tobacco leaves. The incubation mixture contained sucrose 1170 umoles, cells 50 mgms, Fe (as in FeSO_4), EDTA, and/or EDHA, 0.5 umoles, in a final volume of 10 ml. Each point represents a mean of three values.

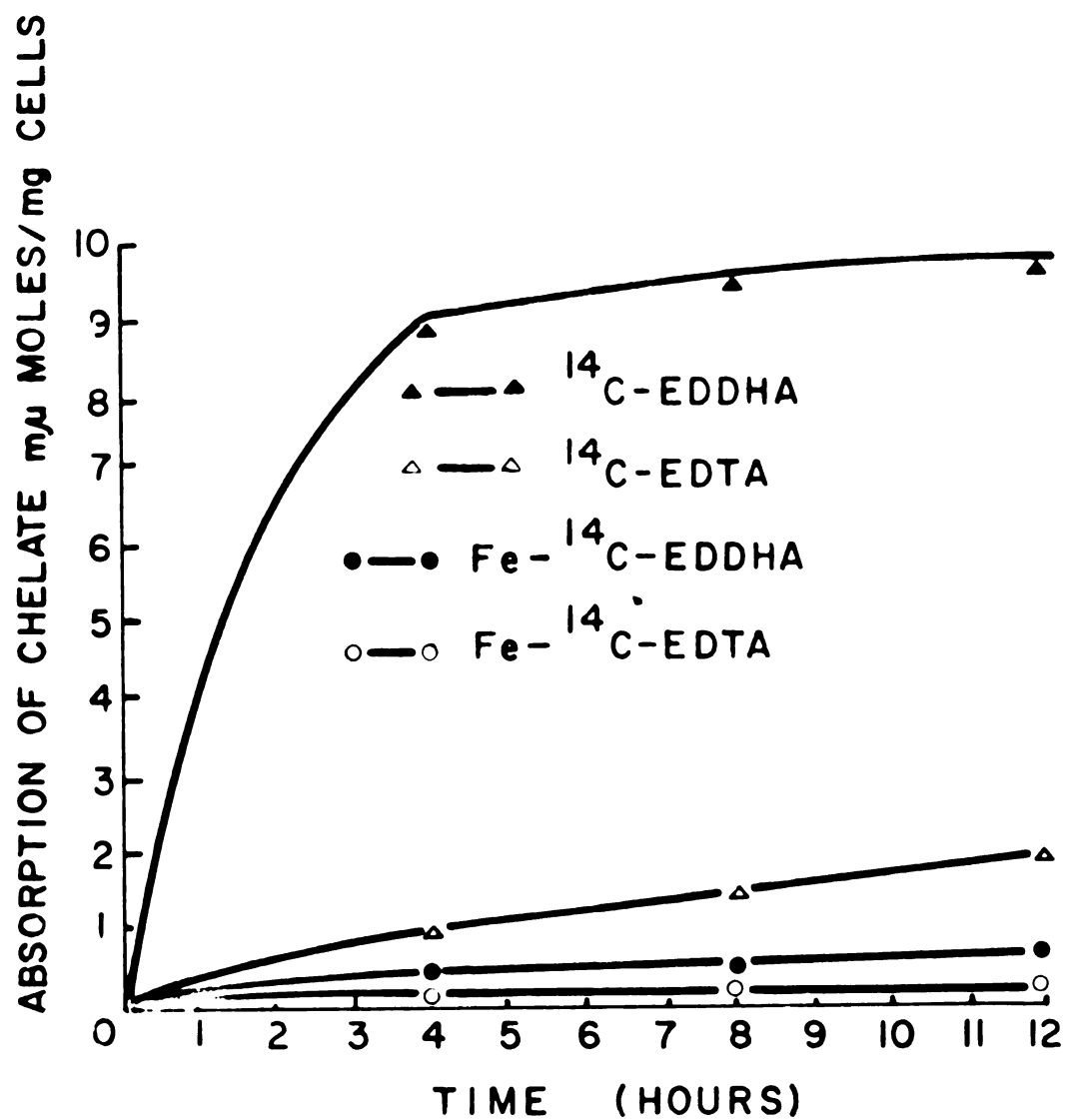
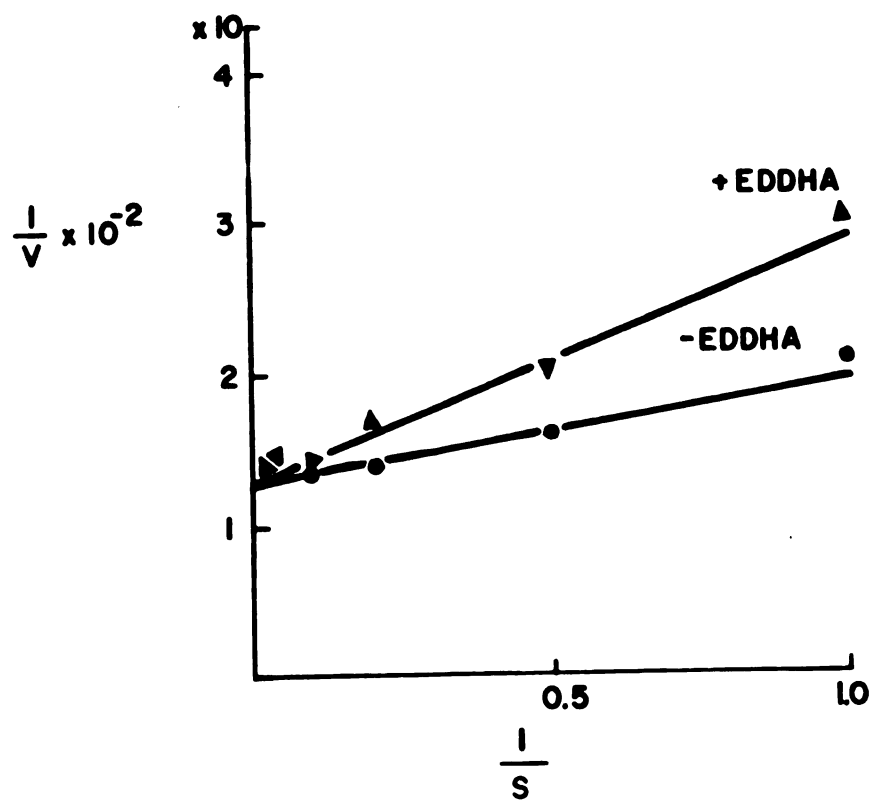
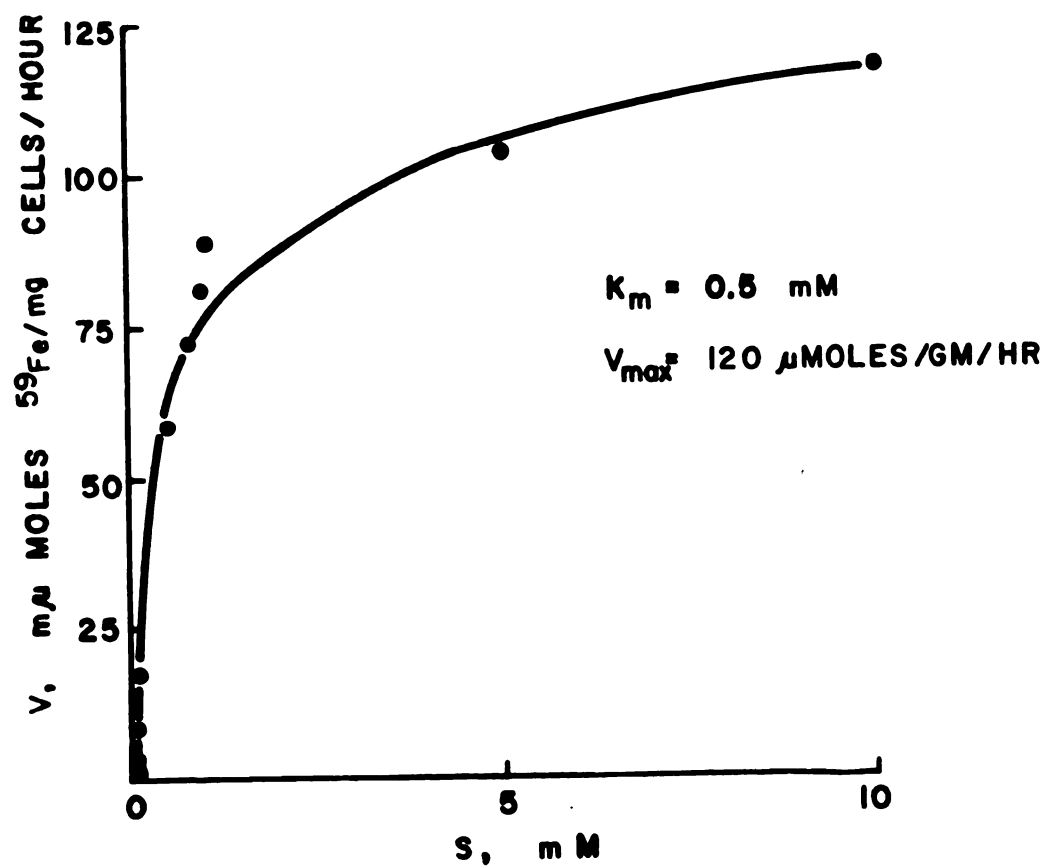


Figure 16

- (a) Absorption of ^{59}Fe by cells enzymically isolated from tobacco leaves as a function of the concentration of ^{59}Fe labeled FeSO_4 .
- (b) Lineweaver-Burk plot of ^{59}Fe absorption by cells enzymically isolated from tobacco leaves, in the presence and absence of EDDHA. The incubation mixture contained sucrose 1170 umoles, cells 50 mgs., and EDDHA 1.0 umole where this was included in a final volume of 10 ml. The absorption was measured at the end of one hour.



The Lineweaver and Burk plot (93, 134) of the data (Figure 16b) illustrates a competitive inhibition of Fe absorption by EDDHA. It appears at the cellular level that EDDHA competes with some unknown natural chelate (carrier), for Fe.

Ion Absorption by Leaves -- A Multistep Process

In the sequence of events leading to ion uptake by the leaf, a number of phenomena participate. Over-all, the process is multistep, in which various phases may be rate-limiting, depending on external and internal conditions. The latter variables are especially related to physiological processes concerning cell activity and energy supply.

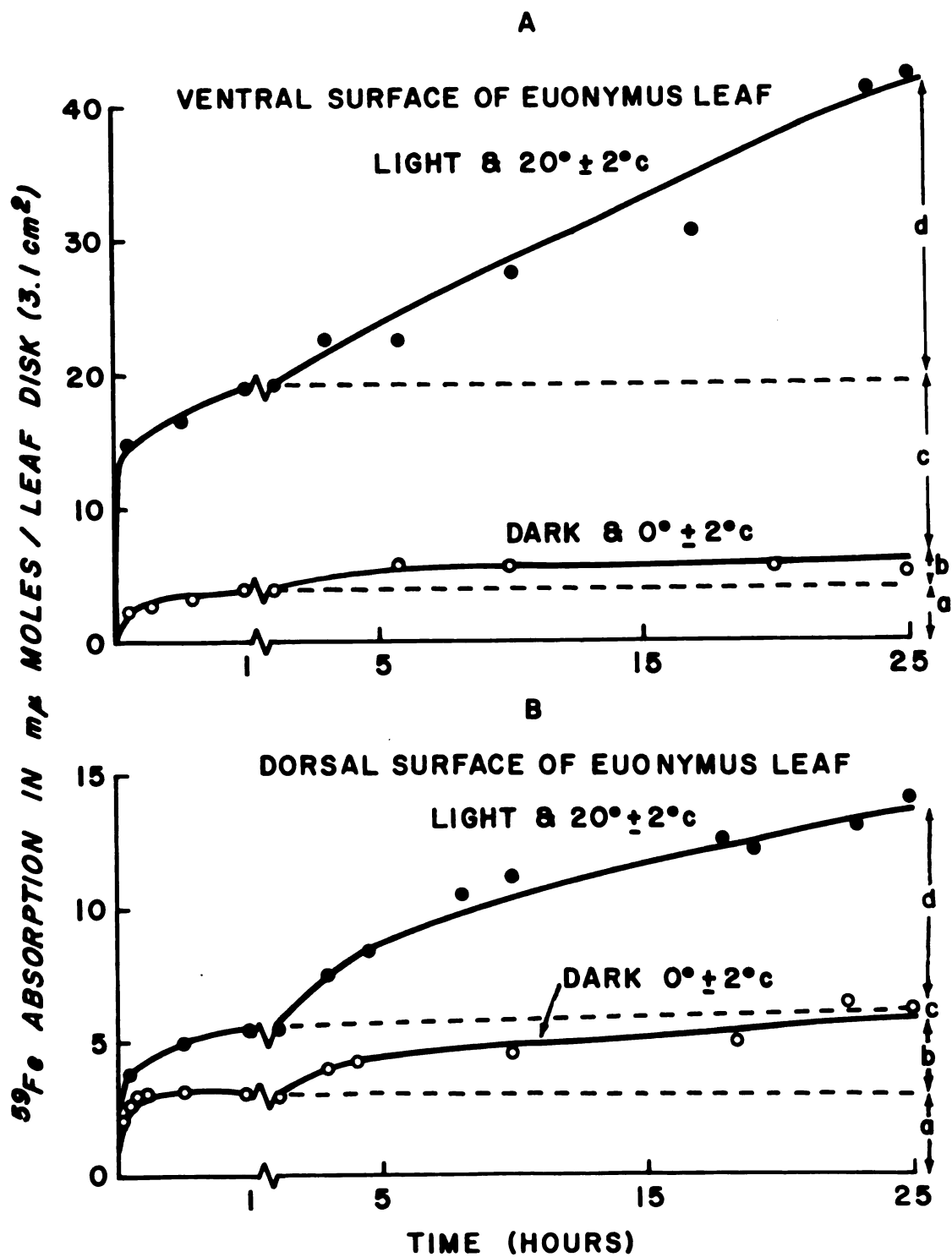
The phases concerned with the absorption of Fe or ions by the leaf can be distinguished as (1) initial adsorption, which may be on the surface of the cuticle, (2) penetration through the cuticle which is possibly a diffusion process, not requiring energy, and (3) the active or metabolic uptake of the ions, which have diffused through the cuticle by living cells. Adsorption on and diffusion through the cuticle are related to the physical properties of the leaf which vary with plant species. These processes, however, may be partially controlled by the environment.

Euonymus japonicus leaves of uniform size and age were selected from plants growing on the University campus during mid-summer. The cut ends of the petioles were sealed with rubber cement, and floated in a

solution containing 0.1 mM ^{59}Fe labeled FeSO_4 . They were oriented such that only the dorsal or ventral surface was in contact with the isotopic solution. Leaves were then removed at different time intervals, rinsed in deionized water for 30 seconds. Disks (3.1 cm^2) were then punched by means of a cork borer from the middle of the leaves, and the radioactivity measured. Results are summarized in Figure 17. The experiments were conducted both at a temperature of $20^\circ \pm 2^\circ \text{C}$ and light of 500 ft. c., and at $0^\circ \pm 2^\circ \text{C}$ in the dark. With leaves exposed to light, the surface in contact with the isotopic solution was opposite the side receiving light, and no orientation was made such that the same surface could be simultaneously exposed to light and to the isotopic solution. Thus, as the dorsal (stomatous) surface was illuminated, uptake was from the ventral (astomatous) surface, and vice versa.

Figure 17

- (a) Absorption isotherm for ^{59}Fe from a solution of 0.1 mM of ^{59}Fe labeled FeSO_4 by the ventral surfaces of detached leaves of Euonymus japonicus as a function of time.
- (b) The same as above, by the dorsal surfaces of the detached leaves of Euonymus japonicus.
- a indicates the probable uptake by adsorption.
 - b indicates the probable uptake by diffusion.
 - c indicates the probable uptake by "activated diffusion".
 - d indicates the probable uptake by active absorption.



DISCUSSION

Permeability of Fe Through Cuticular Membranes:

Information on the permeability of Fe through cuticular membranes is inadequate, hence, penetration rates were studied utilizing three different cuticular membranes. The effects of chelation and urea were included in the study, since facilitation of penetration of ions has already been indicated, particularly for urea (75), probably owing to its non-ionic properties (147). The results show that the rate of penetration of Fe from FeSO_4 was greater than from FeEDDHA. The addition of 10 mM urea also reduced the penetration of Fe through all cuticular membranes. The reductions caused by both EDDHA and urea were significant, especially through the cuticular membranes derived from the dorsal surface of Euonymus leaves. The rate of penetration of Fe from FeSO_4 was generally comparable to that of Ca and Rb through tomato fruit and onion leaf cuticles (169). Although the results indicated a reduction in Fe penetration by EDDHA, the possibility exists that FeEDDHA, because it is more stable in solution, may be a superior source of Fe for foliar sprays. Experiments with intact plants might reveal a greater availability of Fe over a long period of time from FeEDDHA sprays. Also, Fe applied as FeEDDHA might be transported more expeditiously from leaves than the Fe applied as a FeSO_4 spray.

Penetration of Organic Compounds and the Relation of Molecular Weight

The importance of molecular weight has been indicated by Wittwer et al. (166). The results herein (Figures 3, 4, 5, 6) also suggest a high correlation between molecular weights and permeability, particularly through astomatous cuticles, and for organic solutes. Similar relationships between hydrated ion size and penetration rates through astomatous cuticles have recently been obtained for Ca, Sr, Ba, Na, K, Rb, and Cs (49). It has been suggested that permeability for liquids may be related to the pores in the cuticle (95). "Lipoidal" and "aqueous" pathways for cuticular penetration of solutes have often been mentioned (27, 32). Pores extending up to the surface of the cuticle have also been identified, Trifolium repens, Brassica oleracea and Poa colensoi (51).

The permeability properties of cuticular membranes may be analogous to those of cell membranes. The classical work of Collander (23, 24, 25) showed that the permeation of non-electrolytes into Nitella protoplasts was inversely proportional to the molecular weight $\times 1.5$ which was evidence of a "molecular sieve" principle.

Mechanism of Absorption of Fe by Enzymically Isolated Leaf Cells

Influence of External Factors:

The enhancement of Fe absorption by light is evident from Figure 8b. The stimulatory effects of light on ion uptake have been observed in several

instances (59, 83), and a direct effect of light on ion absorption by leaves has been suggested (10). An energy source derived from the photosynthetic process might explain the effect of light. Jyung et al. (76) obtained a similar enhancement of Rb uptake by light. The stimulation of Fe absorption by succinate also has as its origin a supply of energy which serves as a respiratory substrate. Inhibition of ^{59}Fe uptake by NaHCO_3 is contrary to the promotive effect for Rb uptake (76). Bicarbonate has been attributed as a causative factor in Fe chlorosis. Its effect is presumably indirect, however (15), in that it increases the amount of phosphate and calcium in solution cultures. The results obtained herein suggest that the bicarbonate effect may also be direct and perhaps at the cellular level.

Fe supplied through the roots is transported in soybeans, as the chelate of malate and malonate. Thus, organic acids might function in the translocation of Fe in this and other plants (140). The inhibition of Fe absorption by externally supplied citrate, observed in the present experiments, might be the result of the citrate competing with the cells for Fe.

The absorption of Fe by cells is markedly influenced by temperature (Figure 9). Rb and phosphate absorption by tobacco leaf cells was also temperature-dependent (76). Temperature Coefficients (Q_{10}) and energies of activation are used extensively as criteria for enzyme catalyzed reactions.

A Q_{10} of more than 2 is generally associated with an active uptake process. A Q_{10} of 3 for K accumulation between 0.5° and 20° C (59), and 2.2 of 2.45 for K uptake by wheat roots (98) have been reported. Robertson (119) demonstrated that the Q_{10} varied from 2 to 2.1 for accumulation of KCl by carrot tissue. For Ca uptake by pear roots (69) it was 0.9 between 0° and 10° C, and 2 between 10° and 20° C. A Q_{10} of slightly less than 2 was obtained for Rb (76) uptake by enzymically isolated tobacco leaf cells. A value of 5 Kcal./mol. for E_a is generally indicative of a diffusion system (38), and about 13 Kcal./mol. for many metabolic reactions (151). The E_a values for Fe absorption are summarized in Table VI. This would suggest that the absorption of Fe by enzymically isolated tobacco leaf cells is also essentially an active metabolic process, involving energy consumption.

A relationship between respiration and salt uptake by plants is well established (84, 97, 98, 120) and salt uptake is generally linked with metabolism. Absorption of Fe by tobacco leaf cells is suppressed by metabolic inhibitors, viz., NaN_3 , and DNP (Table VII). Inhibition of oxidative phosphorylation caused by DNP (20, 96) increased the rate of cytochrome-mediated electron transport, but simultaneously depressed salt accumulation (121). Briggs et al. (10) therefore suggested that it was not simply the electron transport system in the cell that was associated with salt accumulation, but that coupled phosphorylation was necessary.

Metal oxidase inhibitors like cyanide and azide did not effect Sr uptake and the uncoupler DNP decreased Sr absorption only to some extent (151). The inhibitory effects of DNP on salt uptake and respiration may be a function of rendering the mitochondria "leaky" (62).

The significance of Ca for maintaining the integrity of the selective absorption mechanism for K, Rb and Na (33), and on the apparent permeability of cell membranes (143) has recently been emphasized. On the other hand, Ca does not appreciably promote Fe-Mn absorption by enzymically isolated tobacco leaf cells, and high levels of Ca are distinctly inhibitory to Fe-Mn absorption. Ca decreased Fe uptake by soybean plants from nutrient solution (94). Calcium stimulation of K uptake in maize roots and a depression in soybean roots, are both reported by Kahn and Hanson (78). Handley et al. (52) found that Ca exerted 2 antagonistic effects on ion uptake. The first was inhibitory and the second stimulatory. Perhaps extremely low concentrations of Ca are required to induce the stimulatory effect on Fe and Mn absorption by leaf cells. The inhibitory effects of high concentrations of Ca were evident on the absorption of Fe, Mn, Zn, and Rb by tobacco leaf disks (Figure 11).

Chemically related ions have virtually equal affinities for common carrier sites, and are mutually and almost equally competitive. This has been established for K and Rb (34, 35). Similar affinities between Fe and Mn, which are also chemically related, have been observed in their

absorption by tobacco leaf cells (Figure 10). Although they are mutually competitive, they are not equal. The inhibition of Mn absorption by Fe is greater than that of Fe by Mn. Calcium unlike its role in K, Rb and Na transport (33), has apparently no effect in the Fe-Mn absorption by tobacco leaf cells.

Effects of Internal Factors:

Age of the leaf is an important factor affecting absorption of nutrient elements. The cells obtained from younger leaves of the tobacco plant showed greater absorption of Fe than those from the older leaves. Age of the leaf was indexed by leaf number from the apex to base of the plant. Age was reflected by a greater physiological activity of the younger than the older leaves.

A reverse effect on absorption as related to leaf age, however, has been observed. Kamimura and Goodman (79) found that apple leaves near the apex absorbed less leucine than those near the base. The presence of discontinuities and cracks in the cuticles of the older leaves is offered as a possible explanation for greater uptake.

The amount of chlorophyll in the leaf may be also highly correlated with the rate of Fe absorption by its cells (Figure 13). This was particularly striking in the chlorosis susceptible Pl 54619-5-1 variety of soybean.

The Role of Chelates and Urea

The absorption of Fe by tobacco leaf cells is considerably reduced by EDTA, and EDDHA, as compared to FeSO_4 and greatly enhanced by combining urea with FeSO_4 (Figure 14). Cellular absorption of the ligand from FeEDDHA is negligible with a ratio of 1000:1 for Fe:ligand.

The question as to whether or not the ligand is absorbed by plant cells along with the metal, has not been fully resolved. Does FeEDDHA enter the cell as an intact molecule? The following hypotheses have been proposed as mechanisms of absorption of FeEDDHA (147):

- (i) $\text{FeEDDHA} + \text{Cell} \text{-----} \text{Fe-Cell} + \text{EDDHA}$ (EDDHA remains outside the cell)
- (ii) $\text{FeEDDHA} + \text{Cell} \text{-----} \text{EDDHA-Fe-Cell complex} \text{-----}$
 $\text{Fe-Cell} + \text{EDDHA}$ (EDDHA diffuses out of cell).
- (iii) $\text{FeEDDHA} + \text{Cell} \text{-----} \text{EDDHA-Fe-Protein}.$

The results obtained herein with single cells enzymically isolated from tobacco leaves suggest the validity of the first mechanism.

The enhancing effect of urea on ^{59}Fe uptake by separated leaf cells was further evaluated by pre-treatments with urea or chelate. Pre-treatments of EDDHA and urea had no effect on subsequent uptake of Fe. Urea increased the absorption of ^{59}Fe only if it was included along with FeSO_4 . The effect of urea may arise from an activation of carriers of Fe, or on the permeability of cell wall membranes.

The Use of Enzyme Kinetics and the Competition by Chelate

Analysis of the data of absorption rates by tobacco leaf cells of Fe in the absence and presence of EDDHA, utilizing an enzyme-kinetic model, reveals that the carrier for Fe has a very low affinity constant, and the inhibition of Fe absorption by EDDHA is probably of a competitive nature. There appears to be a competition for Fe, between EDDHA and a presumed Fe-carrier, which is likely a natural chelating substance within the cells. Recently some enzymes which could incorporate metals have been identified (111). An enzyme prepared from rat liver mitochondria has been found to catalyze the chelation of iron by protoporphyrin. Porra and Jones (114) have further suggested the presence of more than one ferrochelatase (pophyrin-iron-chelating enzyme) in Thiobacillus X. These findings suggest that there may be an iron transporting mechanism in the plant which is chelate-like.

Steps Involved in the Absorption Process by Intact Leaf

Ion absorption by a leaf begins with initial adsorption by the cuticular surface, followed by diffusion through the cuticular membranes and further uptake by the leaf cells underlying the cuticle. In the absence of cellular activity in the leaf, the quantity of ions entering the leaf would essentially be through the process of diffusion. An attempt was made to differentiate these processes, by plotting the absorption isotherms. The results

suggest that adsorption was instantaneous, and that it was not possible to distinguish adsorption from diffusion. Diffusion was also very rapid and reached a maximum in 15-30 minutes (Figure 17). Diffusion was markedly influenced by light and temperature, and may be accordingly designated as an activated diffusion, prompted perhaps by a rapid removal of the diffused ions by the cells beneath the cuticle. This was particularly characteristic of uptake by the ventral (astomatous) surface. Under the present experimental conditions, the uptake by ventral surface seems to be greatly influenced by the illumination of the dorsal (stomatous) surface. This perhaps is an evidence for the implication of the role of stomates in foliar absorption.

SUMMARY

Mechanisms of foliar uptake of Fe were investigated with a view to elucidate the factors relative to the diffusion of Fe through cuticles, absorption by leaf cells, and transport from the leaf to other parts of the plant.

Enzymically isolated cuticular membranes from ventral and dorsal leaf surfaces of Euonymus japonicus and from ripe tomato fruits were employed for studying the rates of penetration of Fe. Cuticular membranes from dorsal surfaces of Euonymus japonicus leaves were characteristically stomatous, and those from ripe tomato fruits and from the ventral surfaces of the Euonymus leaves were astomatous. The course of penetration of ^{59}Fe from ^{59}Fe labeled FeSO_4 revealed that Fe penetrated at rate comparable to that of Rb and Ca. ^{59}Fe penetration was reduced by combining with EDDHA and/or urea. Penetration rates were generally higher through the cuticular membranes isolated from the dorsal (stomatous) leaf surface.

The penetration properties of ^{14}C -labeled organic compounds, varying in molecular weights, were studied using the three types of cuticles. Diffusion was greater for solutes of small molecular weight. The negative correlation between molecular weight and permeability was demonstrated by regression analysis. This correlation was

especially high for astomatous cuticles, and is in accord with the relationship obtained between hydrated ion size and penetration through cuticles. These observations are in further accord with theories of "aqueous pathways" and "lipoidal pathways" for cuticular penetration.

Enzymically isolated leaf cells were employed for resolving mechanisms of Fe absorption at the cellular level. Light and succinate stimulated the uptake, while metabolic inhibitors, viz., NaN_3 and DNP reduced it. Temperature had a significant effect on Fe absorption. These results, along with those of temperature coefficient (Q_{10}) and activation energies (E_a), strongly support the involvement of an active mechanism for the absorption of Fe by leaf cells.

The effects of Ca on absorption of Fe and Mn were inhibitory, especially at concentrations higher than 0.5mM, which is the physiologically optimum concentration for the K-Rb, Na transport mechanism in other systems. The absorption of chemically similar ions, Fe and Mn, was mutually antagonistic at the cellular level. If there is a common carrier for K-Rb uptake, it is also possible that Fe- and Mn are transported by a common carrier. Results also show that such a carrier has a greater affinity for Fe, than for Mn, as indicated by a more efficient inhibition of Mn uptake by Fe than of Fe by Mn.

The chlorophyll content and age of the leaf are vitally related to the Fe uptake mechanism. Healthy condition of the leaf is a prerequisite for efficient absorption of Fe. So, also, younger leaves appeared more efficient in ion uptake.

The absorption of Fe from FeSO_4 by enzymically isolated cells, and also by intact leaf, was higher than from FeEDTA or FeEDDHA. The enhancing effects of urea on Fe uptake were significant in leaf cells from both tobacco and bean and also in intact leaves. The role of chelates, on the other hand, was evident in the greater transport of Fe from the site of application to other parts of the plant.

The ligands, EDTA and EDDHA, from FeEDTA and FeEDDHA, were not absorbed by the enzymically isolated leaf cells from tobacco. The ratio of absorption between the ligand and metal was about 1:1000. This indicated that the absorption of Fe by the cells from Fe-chelates was selective; the metal alone was absorbed and the ligand remained outside the cell.

Enzyme-kinetics analysis revealed that the inhibition of Fe absorption by EDDHA was competitive. The affinity constant for Fe was low.

The absorption of Fe from FeSO_4 by intact Euonymus japonicus leaves involved a number of processes--initial adsorption by the cuticular surfaces, diffusion of penetration through stomatous or astomatous

cuticular membranes, which might be "activated," and absorption by the leaf cells.

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