IRON ABSORPTION AND TRANSLOCATION

IN SOYBEANS

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Bernard Leo Dronzek

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LIST OF ABBREVIATIONS

ALA............δ amino levulenic acid

ATP............adenosine triphosphate

CoA............coenzyme A

cpm............counts per minute

DNP............-2-4 dinitrophenol

dpm............disintegrations per minute

DPN............diphosphopyridine nucleotide

DPNH...........diphosphopyridine nucleotide reduced

EDDHA..........ethylenediamine di(-o-hydroxphenylacetate)

EDTA...........ethylenediaminetetraacetic acid

HA.............Hawkeye soybean

PEP............phospho-enol pyruvate

PI.............PI 54619-5-1 soybean

PPM............parts per million

tris HCl.......tris (hydroxymethyl) aminomethane

POPOP..........P-Bis/_2-(5-phenyloxazolyl)_7 - Benzene

PPO............2-5 diphenyl-oxazole

ABSTRACT

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Major Professor: L.J. La Croix

The role of organic acids as translocating agents of iron in soybeans was studied. $NaHC^{14}O_3$, and Fe^{59} chelates or $Fe^{59}Cl_3$ were supplied simultaneously to two soybean varieties; PI (chlorosis susceptible) and HA (chlorosis resistant). The C^{14} was fixed into organic acids in the roots and translocated to the tops in similar manners for both varieties. Malic and citric acid were the main organic acids labelled. Fe^{59} supplied as Fe^{59} chelates moved simultaneously with the organic acids and at equal rates for both varieties. When $Fe^{59}Cl_3$ and $NaHC^{14}O_3$ was supplied to the soybean varieties, the C^{14} fixed into organic acids was rapidly translocated to the tops, while Fe^{59} movement occurred at slow rates for both varieties. This evidence suggested that translocation was not the limiting mechanism of iron nutrition in the chlorosis susceptible soybeans.

The uptake of freshly prepared $Fe^{59}Cl_3$ and $Fe^{59}(OH)_3$ in a precipitated state into root segments of the two soybean varieties indicated that the form of the iron fed was important. More iron was taken up from $Fe^{59}Cl_3$ than iron fed in a precipitated form. Equal uptake of iron occurred into the two soybean varieties from $Fe^{59}Cl_3$ while the uptake from $Fe^{59}(OH)_3$ resulted in more Fe^{59} in the root segments of the PI than the HA variety. DNP fed along with Fe^{59} as $Fe^{59}(OH)_3$ over short feeding times resulted in an increased iron uptake into both soybean varieties, although the uptake

was substantially greater in the PI variety.

A significant negative correlation between iron uptake into the roots and size of tops was found in the PI variety, but not in the HA variety. The rate of top growth may have affected iron uptake into the roots of the PI variety.

INTRODUCTION

Iron is one of the essential mineral elements in plant growth. This element has been shown to play an important role in the synthesis of chlorophyll, the photosynthetic pigment found in plants. Although, iron does not make up part of the chlorophyll molecule, it is associated with enzymes which are vital for the growth and development of the plant.

Plants lacking iron develop a typical chlorosis or lack of green color in the leaves. Iron chlorosis sets in rapidly in younger leaves deprived of iron. Unlike other elements such as nitrogen, iron cannot be translocated from the older leaves to alleviate this deficiency. Recent evidence has shown a good correlation between the degree of chlorosis and iron content (32, 59).

Most soils contain an abundance of iron, thus a deficiency of iron in the soil is seldom a limiting factor in plant nutrition. Many different conditions prevalent in the soil induce iron deficiency in the plant. These conditions tend to hinder the plant from either absorbing or translocating the iron present in the soil.

In recent years attempts have been made to study iron absorption and transport in plants. Iron chelates have been used as the main source of iron in these studies. Wallace and co-workers using iron chelates have implicated the translocation mechanism in the blockage of iron nutrition (58). Other authors have implicated iron absorption at the root cells as well as the state of iron in the soil as the controlling factors in iron nutrition (30, 35).

The purpose of this study was to attempt to determine the limiting mechanism in iron uptake using inorganic iron and iron chelates. Two varieties of soybeans PI 54619-5-1 and Hawkeye which differ in their ability to utilize iron from the soil were chosen for this study.

LITERATURE REVIEW

The process of fundamental importance in iron nutrition is the trans-
fer of iron from the soil to specific sites in enzymes within the plant.
This occurs first by the absorption of iron by the plant roots, followed by
a translocation of the iron to the site of utilization. Attempts have been
made for many years to determine the mechanism of absorption and transloca-
tion of iron within the plant.

Investigators have concentrated their efforts on a study of the different
factors that effect the capacity of the plant to utilize iron from the soil.
Many factors such as low iron supply, genetic make up of the plant, high
lime in the soil, temperature, high phosphate etc. (9, 10, 57) may cause the
plant to become iron deficient. Since most soils are not deficient in iron
many conditions or agents seem to affect the absorption and translocation of
iron in the plant.

Iron deficiency is one of the most widespread nutritional disorders in
plants. Plants lacking in iron develop a typical chlorosis (30), the foliar
expression of faulty iron nutrition. The deficiency usually expresses itself
in the expanding new leaves. The area between the viens becomes light green
due to the failure of chlorophyll production while the viens remain green
except in severe cases where the leaves turn white.

Most of the iron in plants has been found in the chloroplasts (9, 32)
where it plays a vital role in chlorophyll synthesis. Marsh et al. (41)
demonstrated that iron deficient chlorotic cow peas produced protoporphyrin
when fed δ amino levulenic acid (ALA). They concluded that iron deficiency

affects the production of the enzyme ALA synthetase which results in the limited formation of ALA. This block was one rate limiting step in chlorophyll synthesis.

In addition to a role in chlorophyll synthesis iron has been found to be associated with numerous enzymes and carriers involved in the respiratory mechanism of living cells (29). These iron compounds, some of which are cytochromes, undergo freely reversible oxidation and reduction reactions in the plant. Other enzymes such as catalase and peroxidase (25) have been reported to be iron containing enzymes which are widely distributed in plants.

A genetic basis of selective iron transport exists in certain plants. In 1943 two varieties of soybeans, Glycine max (L.) Merr., were discovered which differed in their ability to absorb iron from a given nutrient medium (60). One variety HA was found to absorb and translocate iron more efficiently than PI, the other variety, under low iron supply. The difference in efficiency of iron utilization was found to be governed by a single gene. HA soybeans were found to grow sufficiently well while PI developed chlorosis if grown on calcareous soils, on nutrient solution containing 2PPM inorganic iron or on high phosphate or copper in the soil.(13). Grafting studies (14) showed that HA and PI soybeans were non chlorotic on HA rootstock, but both PI and HA developed chlorosis on PI rootstock when grown on calcareous soils. The differences between the varieties suggested that HA had a greater absorptive capacity for iron than PI from relatively low iron supplies.

Work done with chelates suggested that the plant roots had a chelating property by which they competed with the chelating agent for iron in the growth medium (31). The susceptibility to chlorosis in some plant species

may be due to a metabolic process, such as the reduction of iron at the root surface (18). Iron has been reported to be physiologically active in the ferrous state (42). Reductive capacity at the root was measured for HA and PI soybeans (15). It was reported that HA roots had a more reductive capacity than did PI. There was an apparent relationship between reductive capacity of certain plants and the capacity to absorb iron. Differences in reductive capacity has also been demonstrated in mutant corn plants (4). Certain mutants were unable to use ferric iron as a source of iron while others could reduce iron to the ferrous state. Kliman (34) postulated that ferric iron was reduced to the ferrous state by roots of peas and corn before it was absorbed and transported into the plant.

Wallace and co-worker reported that the chlorosis susceptibility of PI soybeans was due to its failure to translocate iron to the leaves (58). Various treatments of high HCO_3^-, Ca^{++}, $PO_4^=$, and Cu^{++} decreased iron translocation in PI greater than in HA. The amount of iron found in the roots remained constant except in the case of $NaHCO_3$ where iron absorption and translocation were decreased in both varieties. Brown (10) reported that the susceptibility of PI soybeans to chlorosis largely disappeared when it was grown under low phosphorous concentrations.

High levels of phosphate in the growth medium reduced iron absorption and utilization especially under neutral or alkaline conditions (44). High levels of phosphate in the soil have been known to precipitate iron as insoluble iron phosphates. The absorption of excess phosphate from nutrient solution results in an accumulation of phosphate in the leaves,

stems and roots and subsequent immobilization of the iron in the plant (5, 23). The P/Fe ratio in plants has been used as an assessment of the iron status of the plant (22). Wallace (56) reported that there could be two effects of phosphorus in inducing iron chlorosis, the decrease of iron translocation to the leaves or an inactivation of iron in the leaf.

Organic materials in the xylem have been proposed as a metal chelator, thus serving as a transporting agent (19). This type of chelation could prevent the precipitation of iron in the plant. It has been proposed that chelates are the natural form in which iron is absorbed from the soil by higher plants (49). Roots could produce a natural chelating agent that could aid iron uptake. Bitcover and Seeling (6) suggested that iron might be taken up by the plant as a ferric organic complex rather than simply as either the ferric or ferrous ion.

Tiffin and co-workers have implicated organic acids in the tranlocation of iron in the plant (53). Stem exudates collected from soybeans fed with $Fe^{59}EDDHA$ contained an iron organic complex of malic and malonic acid. These organic acids were suggested as iron transporting agents in soybean plants. Further work (17) indicated that there was a relationship between citrate and iron in exudate from soybean. The iron and citrate found in the exudate varied according to the degree of iron stress produced within the plant. Brown (12) found that under iron stress, citrate accumulated in the root sap, but there was not an accompanying increase of citrate in the stem exudate. The factors controlling the uptake of iron were found to be unrelated to the citrate concentration in the roots. Increased citric

acid in iron deficient roots might be due to a decrease in aconitase activity (1). Recently, Tiffin (50) reported that citrate, not malate, translocated iron in soybeans. He measured the citrate iron ratio from sunflowers and found it to be between 1 and 3 (51) for exudates of deficient plants while exudates from normal plants gave a citrate / Fe ratio of 15. Further analysis of the citrate iron ratio in plant stem exudates indicated that if iron concentration was raised in the nutrient solution, the increase of iron in the exudate was proportionally greater than that of citrate (52). This indicated that not all the iron trans- located in the plant could be bound by citrate and other iron carriers must exist.

A naturally occurring chelate of iron was recently reported to exist in xylem exudates of tobacco (47). This complexing agent seemed to keep the iron in solution and had a stability constant between 17.0 - 20.7. Chromatographic evidence indicated that this compound was not a amino acid or organic acid chelate. A flavin compound has now been suggested to translocate iron in tobacco plants (24). It was noted that iron deficient tobacco roots excreted a flavin compound which ceased upon the addition of iron.

Certain sugars such as fructose and sorbose have been shown to possess the ability to chelate iron (21). With fructose the chelating ability has been shown to be related to the dihydroxyacetone structure. An iron fructose complex has been demonstrated to move across biological membranes (48) and could be a mechansim by which iron moves across the membrane structure of the root and may then serve as the translocating agent of iron in plants.

Iron deficiency may occur in plants grown on relatively high levels of bicarbonate ion. The problem has been discussed in detail in different contexts (36, 57). One of the first observations of bicarbonate ion induced chlorosis was made by Harley and Linder (28) who investigated the development of iron deficiency in orchards of apple and pear trees. They found that chlorotic orchards received irrigation water that was relatively high in bicarbonate ion. Substitution of water low in bicarbonate resulted in a marked improvement of the chlorosis and removal of the carbonates from the soil. Sodium bicarbonate added to nutrient solution cultures has shown to induce marked chlorosis in some plants (55). A hypothesis was arrived at that bicarbonate ions plays a specific role in inducing iron deficiency in susceptible plants and that it also decreased the iron availability at the root surface. Bicarbonate ion has been postulated to inhibit translocation of minerals more than it does their absorption (27). High bicarbonate ion has been reported to immobilize iron in the plant which leads to chlorotic conditions (2). Conclusions were drawn that increased bicarbonate ion in the plant resulted in a inactivation of the iron in the plant, due to an increase in tissue pH. Wallace believed that the bicarbonate effect in iron translocation was simply due to the increased insolubility of iron (57).

Brown et al. (16) used the split medium to study the effect of bicarbonate in inducing iron chlorosis in plants. Plant roots were extended from a soil medium through an air gap and into a solution culture. Iron was supplied in the soil and the bicarbonate in the nutrient culture. The separation of iron prevented precipitation or competitive effects which might occur in the nutrient solution. High levels of bicarbonate in the

nutrient solution did not result in chlorosis of the susceptible soybean variety. In contrast these plants grown on complete nutrient solution plus bicarbonate developed severe chlorosis. Chlorosis developed in the susceptible variety of soybeans grown on the split medium technique with bicarbonate if the concentration of phosphate increased in the nutrient solution. From these results the effect of bicarbonate on chlorosis was believed to be indirect rather than direct.

High organic acid content in plants has been associated with high bicarbonate levels. La Croix (35) studied the movement of organic acids and iron in soybean plants and found no evidence for linking iron and organic acid translocation. The bicarbonate stimulated organic acid movement to the tops, but did not affect the movement of iron. The results indicated that bicarbonate interferred with the absorption of iron into the roots.

Branton and Jacobson presented evidence that iron transport into the roots was an active metabolic process while the translocation of Fe up the shoot was passive (7). Iron transport in pea plants was reduced in the presence of DNP, but transpiration was not. Evidence in a subsequent paper using radioautography showed that DNP inhibited iron absorption in the roots (8). Further studies with Fe deficient and Fe sufficient plants indicated that iron requirements in the root cells must first be satisfied before iron can be released to the xylem. Inhibitor work with soybeans (11) indicated that iron movement to the top of the plant was more affected than absorption of iron by the roots.

Iron uptake has been proposed to occur in plants by a direct interaction of the root surface with iron particles (26). Iron may be absorbed by

plants roots without being dissolved in the soil solution. Concentration studies with iron by Branton and Jacobson (7) have suggested that iron is taken up from colloidal particles on the root surface.

MATERIALS AND METHODS

Seed Supply

Seed of two soybean varieties, Hawkeye (HA) and a Hawkeye derivative PI 54619-5-1 (PI) supplied by Dr. R. W. Howell, U.S. Regional Soybean Laboratory, Urbana, Illinois were used in this study. These seeds were stored at approximately 5° C until used.

Culture of Soybeans

Plywood boxes lined with polyethylene film containing 50 liters of nutrient solution were used to culture the soybean plants. The boxes were covered with epoxy - painted plywood lids containing sixteen evenly spaced holes, through which plants supported with polyurethane, protruded into the nutrient solution. Aeration was supplied by forcing air through perforated polyethylene tubing in the bottom of each box.

The soybeans were germinated in trays containing vermiculite. After 10 days, seedlings of uniform size were selected and transferred to nutrient solution (table I). The plants were grown in the greenhouse at 75° F, with 16 hours light and 8 hours darkness.

Translocation Studies

The plants were grown in nutrient solution with Fe supplied as $FeSO_4$ for 3 weeks. The nutrient solution was changed at weekly intervals. Prior to the feeding experiment the plants were placed for 36 hours in nutrient solution containing no iron. Radioactive $Fe^{59}EDTA$ or $Fe^{59}EDDHA$ and $NaHC^{14}O_3$ were fed simultaneously to individual plants in 200 ml jars

Table I. Nutrient media for soybean culture

Nutrient	Composition of stock solution (g/liter)	Final Concentration in Nutrient Medium (mg/liter)	Molarity
$Ca(NO_3)_2 \cdot 4H_2O$	236.15	1180	5×10^{-3}
KNO_3	101.1	505	5×10^{-3}
$MgSO_4 \cdot 7H_2O$	246.5	493	2×10^{-3}
KH_2PO_4	136.1	136.1	1×10^{-3}
H_3BO_3	2.86	2.86	4.6×10^{-5}
$MnCl_2 \cdot 4H_2O$	1.81	1.81	9.1×10^{-6}
$ZnCl_2$	0.11	0.11	8.1×10^{-7}
$CuCl_2 \cdot 2H_2O$	0.05	.05	2.9×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	0.025	.025	1×10^{-7}
$FeEDTA$ or $FeSO_4$*			

* Iron was supplied at 5 PPM.

(12)

containing 100 ml of $\frac{1}{2}$ strength nutrient solution buffered at 7.5 with either tris HCl or potassium phosphate. At the end of the feeding experiment the roots were dipped in 0.1 HCl for 10 seconds and then washed with distilled water. The plants were separated into roots and tops by cutting the stem just below the cotyledons. They were dried in an oven overnight at 70° C and dry weights were determined.

Extraction of Tissue

Plant roots and tops were ground in a mortar with sand in hot 80% ethanol (v/v) acidified with formic acid according to MacLennan (39). The plant material was subsequently extracted with hot solutions of 40% ethanol, distilled H_2O and again with 80% ethanol. The tissue remaining after the ethanol extraction contained the Fe^{59}. This material was ashed in a muffle furnace.

The combined alcohol - water extract containing the radioactive organic acids was evaporated to dryness in a flash evaporator at 40° C. The residue was successively extracted 3 times with ethyl ether and water. The ether phase was discarded. The water soluble compounds were adjusted to 25 ml with water and placed on ion exchange columns.

Isolation of Organic Acids

The water soluble extract was passed through a 6 x 1 cm column of Dowex 50-x 8 (H+), then through a 6 x 1 cm column of Dowex 1-x10 (formate). The basic compounds are retained on the Dowex 50-x 8 columns, acidic compounds on the Dowex 1-x10 and neutral components passed through the columns. The acidic compounds, composed mostly of organic acids, were

eluted with 40 ml of 4N formic acid followed by 30 ml of 8N formic acid.

The acidic fraction was evaporated to dryness on a rotary flash evaporator attached to a water bath at 40°C. The organic acids were re-dissolved in 10 ml of distilled H_2O. An aliquot was removed and the radioactivity in the sample determined.

Esterification of Organic Acids

Diazomethane prepared according to the method of Mangold (40) was used as the methylating agent. The organic acids were dissolved in ether – methanol (9:1 v/v). The ethereal diazomethane was incubated with the acids until a yellow color persisted. The excess diazomethane and solvents were removed by evaporation and the residue taken up with methanol.

Gas Chromatography and Peak Collection of Organic Acids

An Aerograph A-90-P2 gas chromatograph equipped with a thermol conductivity detector and supplied with a 150 milliampere current was used in this study. The gas chromatograph was attached to a Brown recorder equipped with a Disc integrator for quantitation of the recorded peaks.

The column used was 10% Reoplex 400 coated on Chromosorb W (60-80 mesh) packed in a copper tube (15 inches x ¼ inch). The gas chromatograph was operated with the injector temperature set at 180°C, detector temperature 190°C and the oven temperature programmed from 55°C to 175°C at 4° per minute after the injection of the sample.

Samples were injected in 40 µl aliquots using a 50 µl Hamilton syringe. The methyl esters were collected in glass tubes attached to the outlet of the chromatograph. They were rinsed from the collection tube with ether into scintillation solution and counted.

Iron Absorption Studies

Root tips from 4 week old plants grown on chelated iron were cut in 1 cm length, 500 mg fresh weight of tissue was added to 15 ml Warburg flasks or 30 ml vials containing Fe^{59} at 5PPM plus 10 ml of standard nutrient solution. The vials were placed on a horizontal shaker.

At the end of the feeding period the roots were washed with distilled water and placed in an aerated solution of $10^{-3}M$ EDDHA (free acid) to remove the non absorbed iron (33). After 1 hour the roots were washed with distilled water and dried overnight at 70°C. The dry weight was determined and the tissue was subsequently ashed at 550°C and the radioactivity determined.

Iron ^{59} Determination

The ashed material was taken up in 6N HCl and made to volume and counted. Radioactivity was determined by a shielded well type Tracerlab ED-8 Sodium Iodide, thallium activated crystal coupled to a photomultiplier tube. Counts were recorded on a Tracerlab Scaler. The overall efficiency of the counting tube was 10.1% with a background of 143 cpm. All results were corrected for decay.

Assay of Radioactive C^{14}

Carbon 14 was assayed on a Nuclear – Chicago Liquid Scintillation Counter, Series 725. Samples were prepared in either non-aqueous scintillator solution containing 4 grams PPO, 100 mg POPOP and 1 liter toluene or an aqueous scintillator solution contained 4 g PPO, 100 mg POPOP, 50 g naphthalene, 167 ml ethylene glycol monoethyl ether and 833 ml dioxane.

Preparation of Radioactive Iron Chelates

Two radioactive chelates $Fe^{59}EDTA$ and $Fe^{59}EDDHA$ were prepared. The acid form of the chelate was converted to the salt by the addition of 1N NaOH. The pH was adjusted to 7.0 and suitable amounts of radioactive and cold iron were added. Air was bubbled through the solution overnight, then, the pH was adjusted to 5.2.

RESULTS AND DISCUSSIONS

Two soybean varieties PI-54619-5-1 and Hawkeye were chosen for this study. These varieties are known to differ in their absorption and translocation of iron. PI soybeans are extremely susceptible to iron chlorosis while HA soybeans are known to be relatively resistant. The difference in iron utilization between the varieties has been reported to be under the control of a single gene (60).

The present study was undertaken to investigate the possible role organic acids play in the translocation of iron in PI and HA soybeans. If the organic acids play an important role in the absorption and translocation of iron, then the two soybean varieties should differ in their organic acid movement to the tops of the plants. Translocation studies led to further examination of the absorptive mechanism of iron into the root system. Iron was fed in different forms and under different conditions to determine if the varieties differed in the absorption of iron at the root surface.

Translocation Studies

Individual HA and PI soybean plants were simultaneously fed 5PPM Fe as $Fe^{59}EDTA$ (276,380 cpm) and .635μ moles $NaHC^{14}O_3$ (7,500,000 dpm) in nutrient solution for various periods of time. The nutrient solution was was at half strength, containing no phosphate and was buffered at pH 7.5 with 0.007N tris HCl. The plants were analyzed for the time course of fixation of $C^{14}O_2$ into organic acids as well as the Fe^{59} absorption into the roots and translocation to the tops.

Fig. 1.   Relation of C^{14} organic acids and Fe^{59} in soybean roots fed Fe^{59} EDTA and $NaHC^{14}O_3$.

C^{14} organic acids (OA) in HA roots   ─── ‒ ───

C^{14} organic acids (OA) in PI roots   ── ── ── ──

Fe^{59} in HA roots   ─────────

Fe^{59} in PI roots   ── · ── · ──

(6τ)

Fig. 2.   Relation of C^{14} organic acids and Fe^{59} in
soybean tops fed Fe^{59} EDTA and $NaHC^{14}O_3$.

C^{14} organic acids (OA) in HA tops ——— · ———

C^{14} organic acids (OA) in PI tops — — — —

Fe^{59} in HA tops   ————————

Fe^{59} in PI tops   —— · —— · ——

Fig. 3. Relation of C^{14} organic acids and Fe^{59} in soybean roots fed Fe^{59} EDDHA and $NaC^{14}O_3$.

C^{14} organic acids (OA) in HA roots — — — —

C^{14} organic acids (OA) in PI roots ___ _ ___

Fe^{59} in HA roots   —————

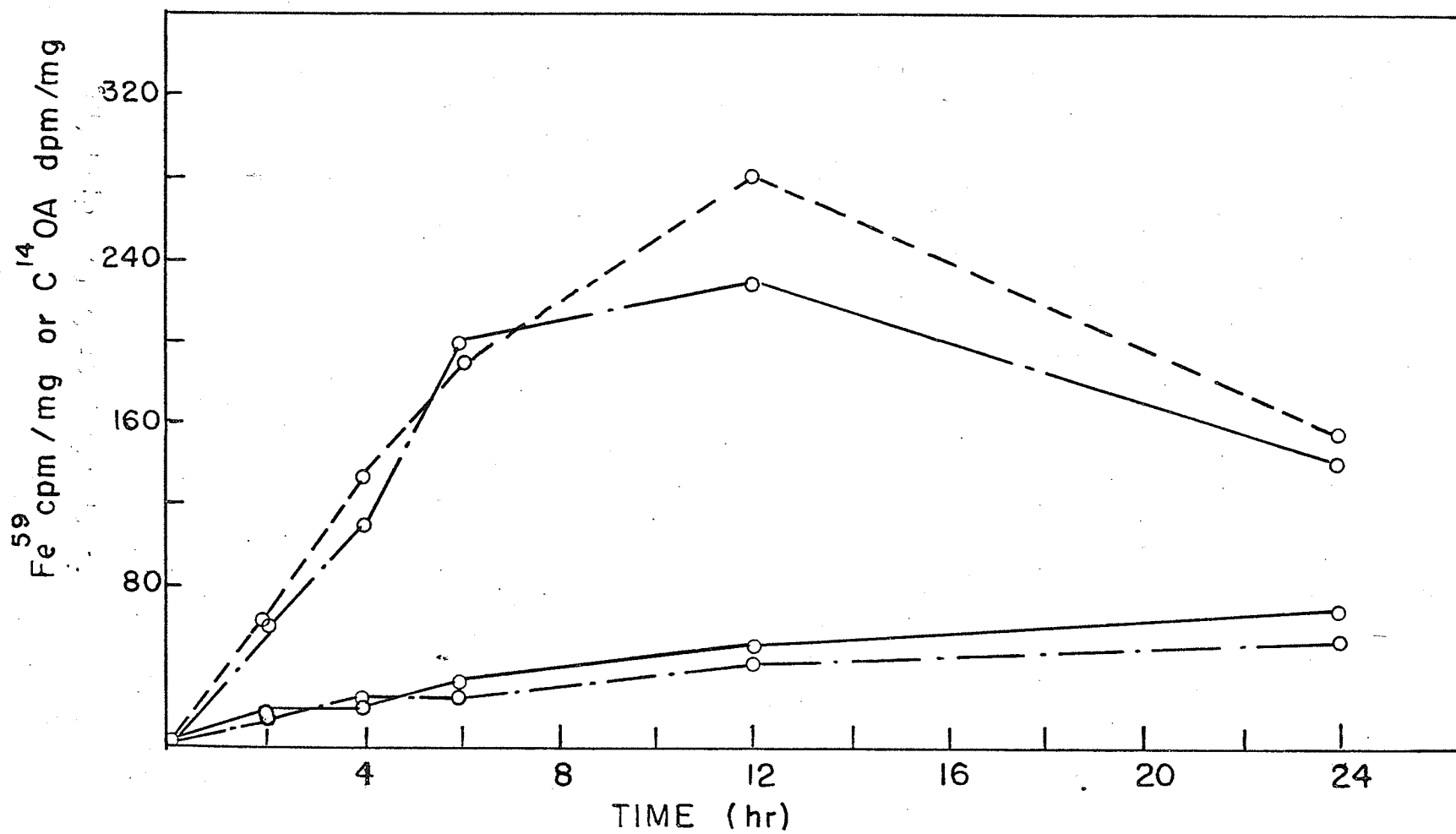Fe^{59} in PI roots   —— · —— · ——

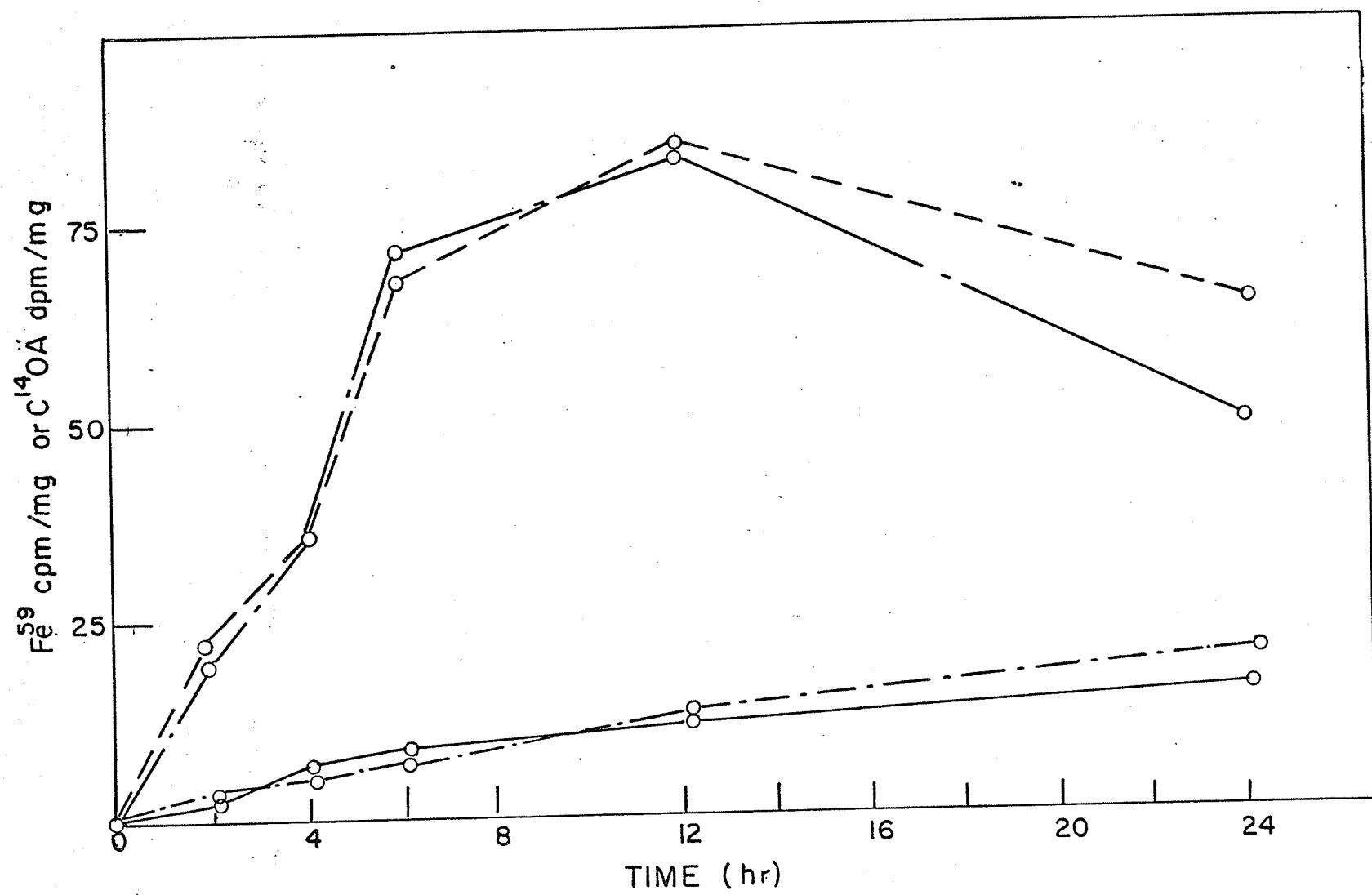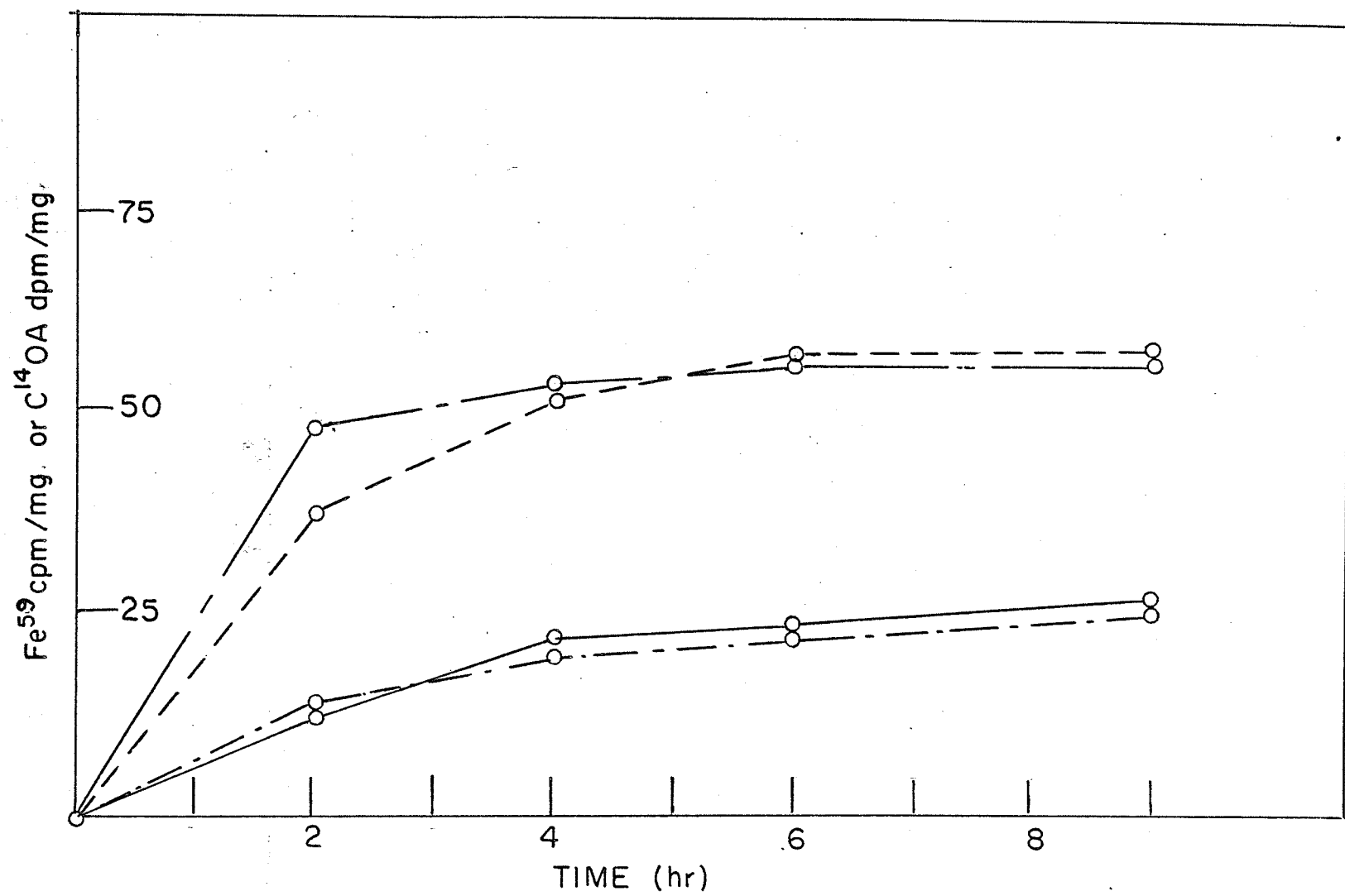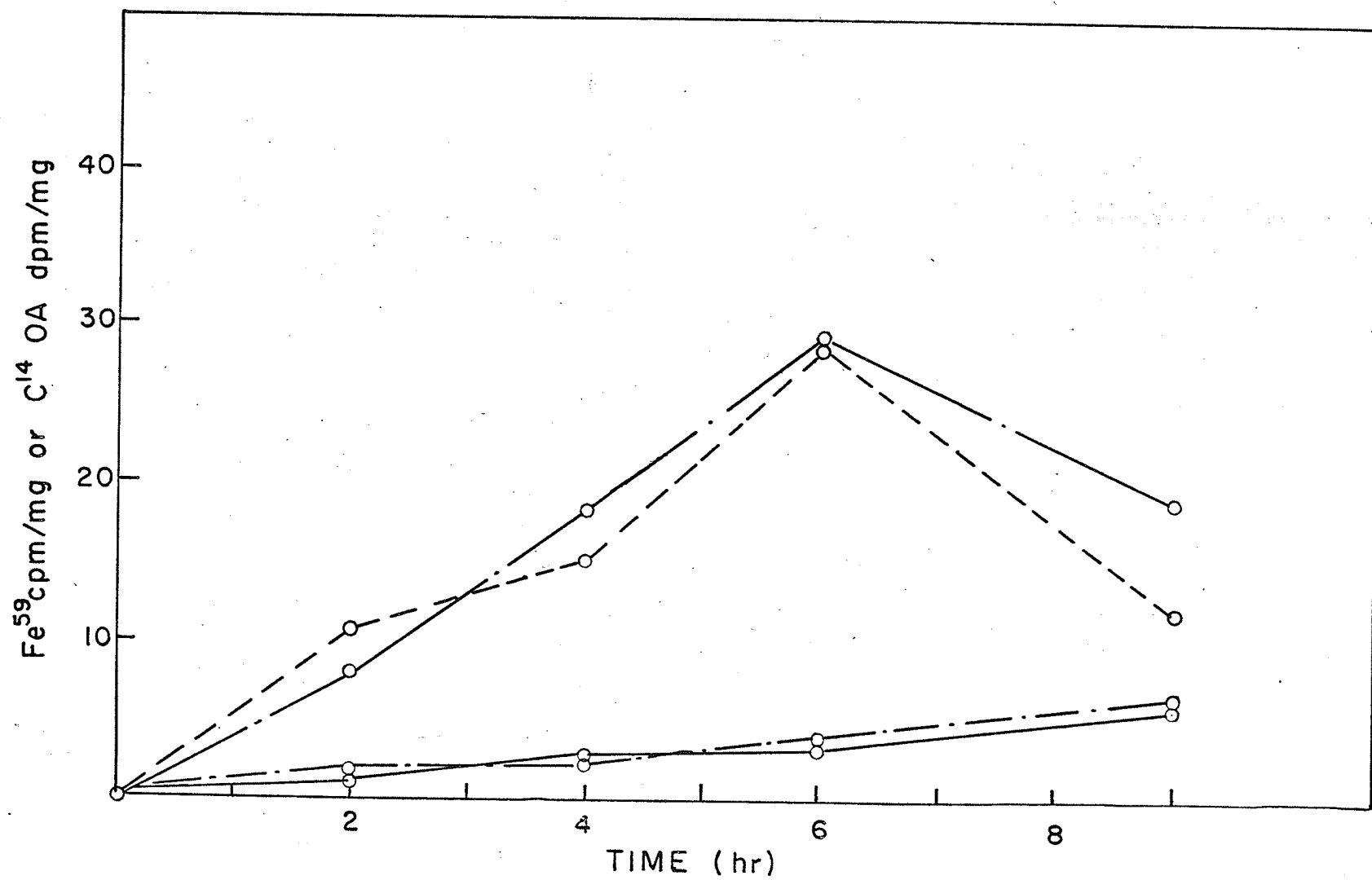Fig. 4. Relation of C^{14} organic acids and Fe^{59} in soybean tops fed Fe^{59} EDDHA and $NaC^{14}O_3$.

C^{14} organic acids (OA) in HA tops ———— . ————

C^{14} organic acids (OA) in PI tops — — — —

Fe^{59} in HA tops ————————

Fe^{59} in PI tops —— . —— . ——

$NaHC^{14}O_3$ was rapidly fixed into organic acids in the soybean roots. A linear increase of C^{14} in organic acids for the first 12 hours occurred with both varieties (Fig. 1). The amount of radioactivity found in the organic acids in the roots over the next 12 hours decreased. This could be due to the complete utilization of the $NaHC^{14}O_3$ fed, movement of the organic acids to the top of the plants or the conversion of the organic acids to other plant constituents in the roots.

Figure 2 shows the amount of C^{14} fixed into organic acids and accumulated in the tops of the soybean plants with time. There was no indication that the movement of label to the top of the plants differed between the varieties. Both varieties over the first 12 hours showed a linear increase in the amount of labelled organic acids found in the tops followed by a slight decline over the next 12 hours. The decline was probably due to the decreased movement of C^{14} to the tops from the roots and the conversion of the organic acids in the tops to other plant compounds. Organic acids are known to be metabolized to protein in plants (25). No attempts were made in this study to trace the fate of the $C^{14}O_2$ except its incorporation into radioactive organic acids.

The Fe^{59} was absorbed and translocated to the top of both varieties in a similar manner (Fig 1,2). The Fe^{59} content increased with time in both the roots and tops. A rapid increase of Fe^{59} was noted in the roots over the first few hours which tended to level off with time. The rapid increase could be due to the fact that the plants were on a medium containing no iron for 36 hours prior to the feeding experiment. To offset the iron deficiency created, an increased rate of iron uptake may have occurred. Over the first 6 hours the HA and PI roots absorbed a similar amount of
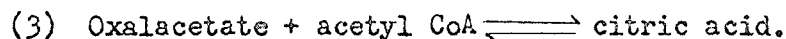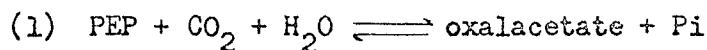
iron. After 12 and 24 hours more iron was absorbed in the HA roots than
in the PI (Fig. 1). The rate of iron movement to the tops was the same
for both varieties for the first 12 hours of feeding. After 24 hours
more iron was found in the PI variety. The amount of iron found in the
tops of both varieties increased with time.

Figures (1,2) show that the uptake and translocation of iron and
C^{14} was similar in both varieties of soybeans. The differences between
the susceptible and resistant varieties in Fe uptake and movement in the
plant was not demonstrated by feeding Fe^{59} chelate. Both varieties
responded equally to Fe fed as iron chelate and the uptake of $C^{14}O_2$ into
the plant as organic acids and subsequent movement to the tops was similar
for both varieties. It was felt that if translocation was limiting in
the susceptible variety as proposed by Wallace (58) and organic acids
were the iron transporting agent in the plant, then a difference in organic
acid movement between the varieties would be expected. This was not
observed. The HA variety did not translocate more iron to the tops than
the PI variety, nor was there more organic acid movement in the HA variety.

Another experiment was conducted where Fe^{59}EDDHA with $NaHC^{14}O_3$ was
fed in a medium buffered at pH 7.5 with phosphate. The iron was supplied
at 5PPM (135,000 cpm) and the $NaHC^{14}O_3$ at (3,030,000 dpm). The Fe^{59}
moved into the roots and was translocated to the tops at equal rates for
both varieties (Fig.3,4). Simultaneously C^{14} was fixed into organic acids
in the roots and translocated to the tops. The rates of C^{14} fixation and
translocation were the same for both varieties. These results further
substantiated the view that the differences between the varieties does
not lie in the lack of organic acid movement in the variety PI.

Analysis of organic acids was carried out by gas liquid chromatography to determine which organic acids were labelled with C^{14} in the two feeding experiments conducted. Citric and malic acids were found to contain more than 75% of the radioactivity in the organic acid fraction of the roots and tops of both varieties.

Plant roots have been known to fix $NaHC^{14}O_3$ into organic acids, which are subsequently translocated to the top of the plants (3, 43, 45). The $C^{14}O_2$ has been shown to be fixed into organic acids by the PEP carboxylase system.

(1) PEP + CO_2 + H_2O ⇌ oxalacetate + Pi

(2) Oxalacetate + DPNH ⇌ malate + DPN^+

(3) Oxalacetate + acetyl CoA ⇌ citric acid.

A higher specific activity of malic acid was found in the tops than in the roots in the $Fe^{59}EDDHA$ and $NaHC^{14}O_3$ feeding experiments (tables II and III). The specific activity of malic acid was higher than that of citric acid in the tops of both varieties of soybeans. The specific activity of malic and citric acids increased with time in the roots and tops for 4 to 6 hours in both varieties, then declined with time. There was no indication in these experiments that citrate movement to the tops differed between varieties or that the susceptible variety translocated less citrate than the resistant variety.

Table II. Specific activity of the organic acids isolated from soybean tops fed $NaHC^{14}O_3$ and $Fe^{59}EDDHA$ at the roots for various periods of time.

Specific Activity dpm/mg organic acid

Time hours	PI		HA	
	Malic	Citric	Malic	Citric
2	469	104	378	116
4	723	143	828	165
6	555	204	500	217
9	437	170	382	287

Table III. Specific activity of the organic acids isolated from soybean roots fed $NaHC^{14}O_3$ and $Fe^{59}EDDHA$ at the root for various periods of time.

Specific Activity dpm/mg organic acid

Time hours	PI		HA	
	Malic	Citric	Malic	Citric
2	288	300	318	545
4	325	346	374	609
6	354	235	394	579
9	254	238	275	392

Experiments were carried out in which $Fe^{59}Cl_3$ (5PPM Fe) was fed simultaneously with $NaHC^{14}O_3$. Results indicated that the movement of Fe^{59} to the top of the plants was extremely slow while the $C^{14}O_2$ was rapidly fixed into organic acids and moved to the top of the plants. Over a 24 hour period the HA was able to translocate twice as much Fe^{59} to the top of the plants than the PI variety (table IV). In experiments where $Fe^{59}Cl_3$ or Fe^{59} chelates were fed simultaneously with $NaHC^{14}O_3$ to resistant and susceptible soybeans, results indicated that the varieties differed in their ability to take up different forms of iron (precipitation of iron occurred from $FeCl_3$, but not from the chelated iron). The varieties differed in their ability to translocate iron depending upon the form of iron fed, yet the movement of organic acids was found to be similar between varieties. There did not appear to by any link between the movement of organic acids and the movement of iron in the resistant and susceptible soybean plants.

Table IV. Uptake of $Fe^{59}Cl_3$ fed at 5PPM to HA and PI soybeans for 24 hr

	Fe^{59} (cpm/mg dry wt)	
	Roots	Tops
HA pH 7.5	162.6	3.82
PI pH 7.5	156.7	2.02

The form of the iron fed played an important role in determining the availability of iron to the plant. Synthetic iron chelates have been known for years to effectively supply iron in a soluble form to the plant.

In this form the iron is available at the root surface for uptake into the plant. Iron can be fed in inorganic forms to susceptible varieties of plants providing the pH is such that iron availability is maintained. As the pH increases in the soil or in nutrient culture the iron becomes oxidized and precipitates. The oxidation of iron from the ferrous to the ferric form and the precipitation of iron as insoluble hydroxides occurs rapidly in slightly acid, neutral and alkaline solutions.

Iron fed as $FeSO_4$ to HA and PI soybeans in standard nutrient medium precipitated rapidly in a few hours. Both varieties were able to grow on iron supplied in this form providing fresh iron was added at weekly intervals. If fresh iron was not added to the nutrient solution, the PI variety started to show visual signs of chlorosis in the leaves a week and a half later. The HA variety could remain for longer periods of time with no chlorosis developing. This indicated that the HA variety could utilize iron more efficiently from the precipitated form than PI.

When PI and HA soybeans were fed $NaHC^{14}O_3$ and $Fe^{59}EDTA$ or $Fe^{59}EDDHA$ there was no evidence to suggest that translocation was limiting to the iron nutrition of either variety Figures (1,2,3,4). When $NaHC^{14}O_3$ and $Fe^{59}Cl_3$ were fed to the soybean varieties, C^{14} organic acids were rapidly translocated to the tops while Fe^{59} movement occurred at extremely slow rates. This evidence suggested that translocation was not the limiting mechanism to iron nutrition of the chlorosis susceptible soybean. Our attention was turned to study the absorptive mechanism at the root surface.

Absorption Studies

The uptake with time of Fe^{59} from precipitated Fe^{59} and freshly

prepared $Fe^{59}Cl_3$ into root segments of HA and PI plants was studied. The unabsorbed iron on the root segments was removed by placing the roots after the feeding time into 10^{-3} M EDDHA (free acid) for 1 hour (33). Precipitated Fe^{59} was prepared by dissolving 5PPM $Fe^{59}Cl_3$ in half strength nutrient solution minus phosphate and adjusting the pH to 8.0 and then to 6.0. This medium was used after 24 hours. All the iron precipitated out of solution during this time. The suspension of iron was stirred vigorously and 5 ml aliquots were pipetted into 15 ml Warburg flasks containing 500 mg of root segments. Freshly prepared Fe^{59} was prepared by the addition of $Fe^{59}Cl_3$ (5PPM) to $\frac{1}{2}$ strength nutrient solution minus phosphate. The pH was adjusted to 6.0 and the iron solution was fed immediately to 500 mg of root segments in Warburg flasks at 30°C.

Freshly prepared Fe^{59} was taken up into the root segments of both varieties more rapidly than iron fed in the precipitated form (Fig 5). The uptake of freshly prepared Fe^{59} followed an identical pattern of uptake for both avarieties. The PI variety took up Fe^{59} more rapidly from the precipitate than the HA variety.

Another experiment was run over a short period of time to check if PI did take up more Fe than HA in root segments from precipitated forms of iron. The procedure was the same as the previous experiment except the roots were fed in 30 ml vials. These vials were placed on a more vigorous shaking apparatus to distribute the precipitated Fe more uniformly throughout the medium. PI took up more iron into the root segments than the HA variety (Fig 6). The uptake was extremely rapid, iron absorption reached saturation in approximately 15 minutes and very little further absorption occurred up to 60 minutes.

Fig. 5. Uptake of Fe^{59} into root segments of PI and HA
soybeans.

Fe^{59} in HA roots fed $Fe^{59}Cl_3$ —————

Fe^{59} in PI roots fed $Fe^{59}Cl_3$ —————

Fe^{59} in HA roots fed precipitated Fe^{59} — — — —

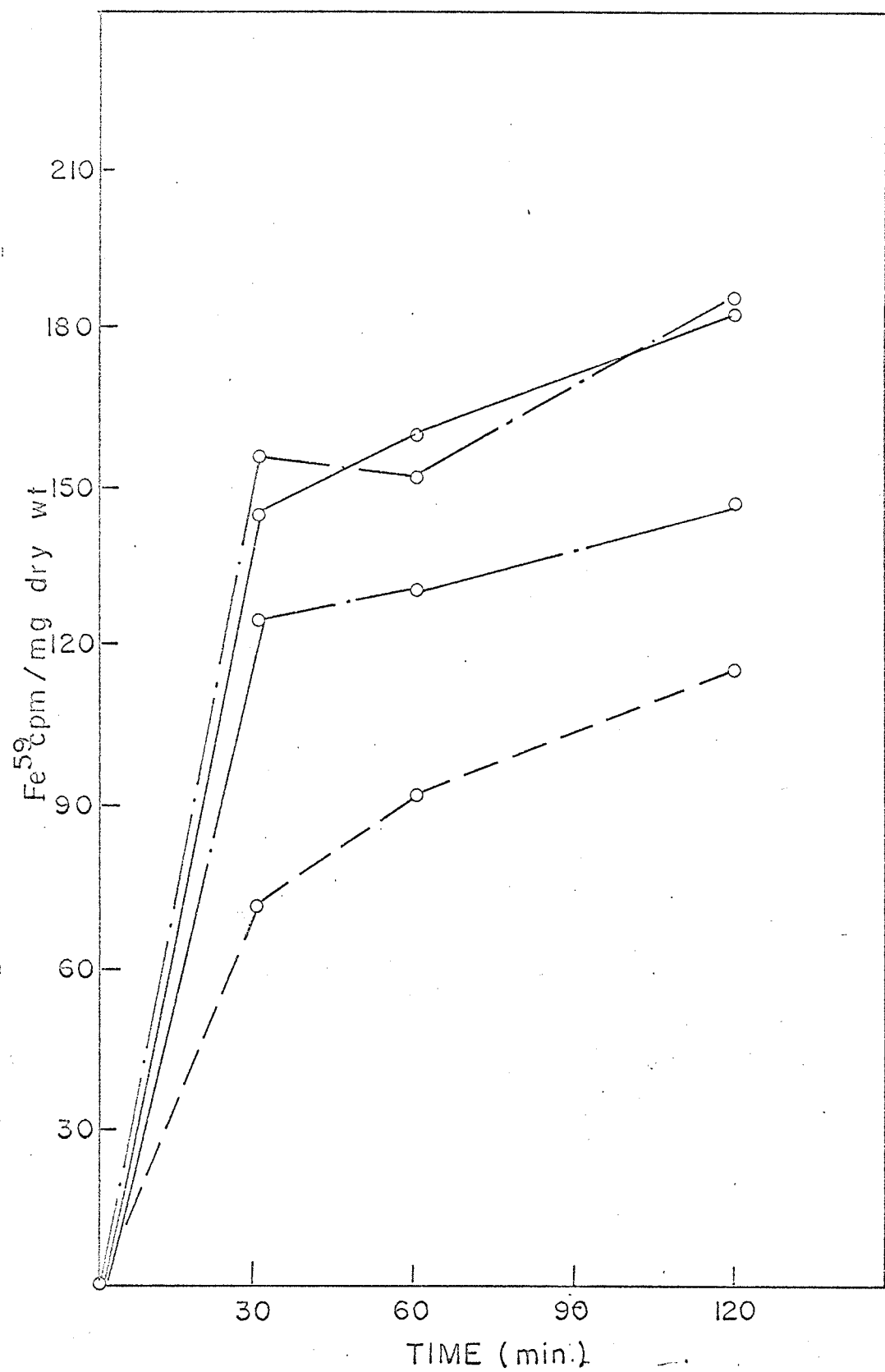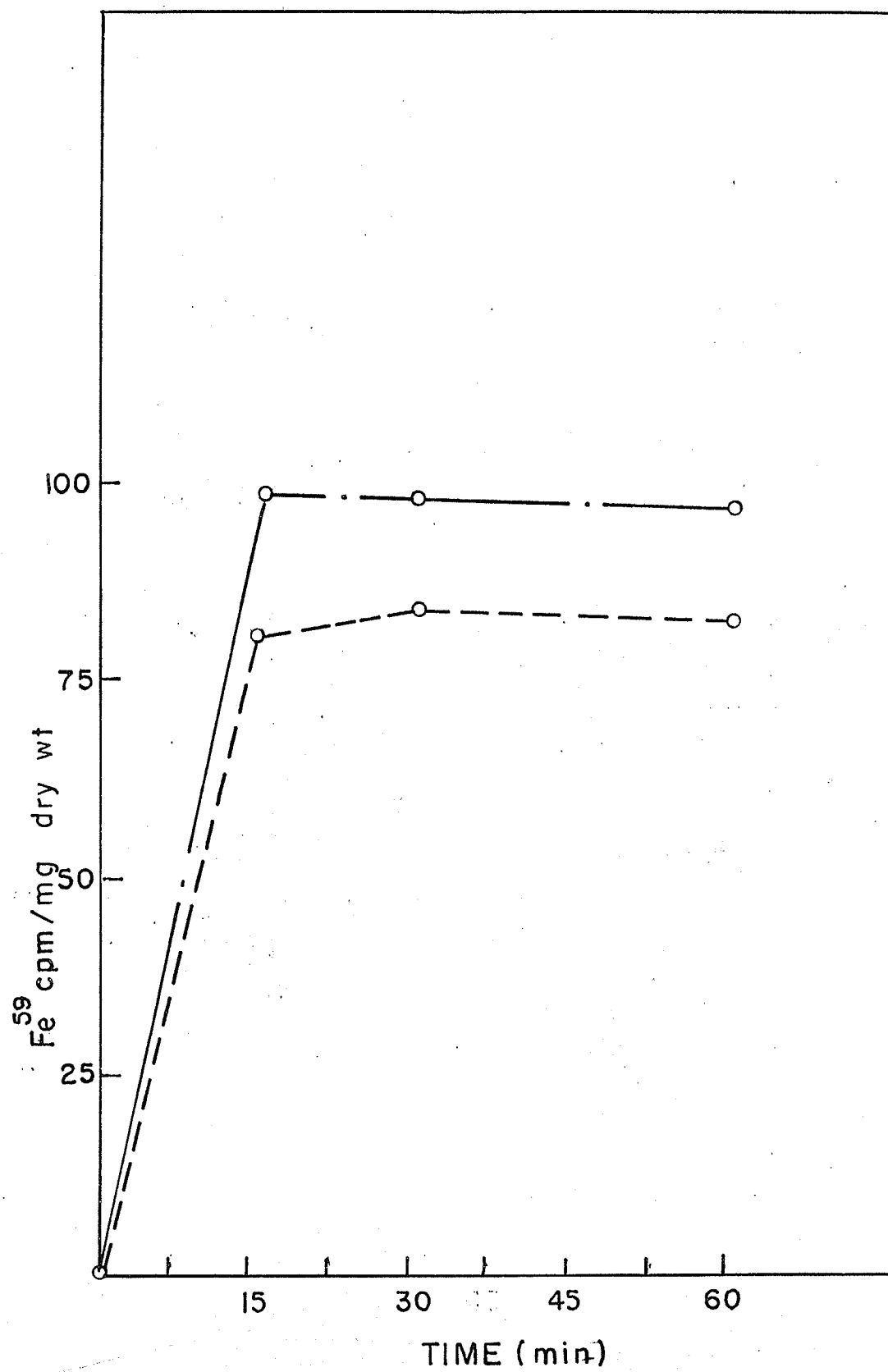Fe^{59} in PI roots fed precipitated Fe^{59} —————

Fig. 6. Uptake of Fe^{59} from precipitated iron into root segments of PI and HA soybeans.

Fe^{59} in HA root segments — — — —

Fe^{59} in PI root segments ——— - ———

Both HA and PI root segments absorbed the same amount of iron from freshly prepared $FeCl_3$ solution (Fig.5). Parallel results were found in the translocation studies where iron chelates were fed (Fig. 1,3). Results indicated that if iron was fed in a soluble form both varieties absorbed an identical amount of iron into the roots with time.

The susceptibility of the PI variety to chlorosis in this study was not found to be related to the lack of absorptive ability of iron at the root surface. More iron was absorbed into the roots from precipitated forms of iron in the PI variety than in the HA. Translocation studies indicated that the form of the iron fed was important. The HA variety translocated more iron from the precipitated form than the PI. A block in iron movement seemed to occur somewhere in the root system in the PI variety.

The overall process of iron absorption and translocation in plants may occur in a number of steps. The iron is first made available at the root surface of the plant. The iron then moves across a lipoprotein membrane and into the root cells. After entering the root cells the iron is transported across the cells in the root towards the xylem. Finally the iron is transported into and through the conducting system in the plant, the xylem. Any limitation on iron movement through these steps could be the controlling mechanism in the iron nutrition of the plant.

The uptake of iron or any ions into plants occurs by a passive or active mechanism or a combination of the two. Active uptake depends upon metabolism to move ions into the cells against concentration gradients or any other barrier which might block ion movement. The

passive process of iron uptake could be considered a closed system, energetically self contained where iron is taken up by straight diffusion or a Donnan equilibrium type mechanism and transported in the plant by the simple process of mass flow. The overall passive process is not affected by temperature or metabolic inhibitors.

Inhibitor studies

Inhibitor studies were undertaken to separate the active and passive uptake of iron into the root segment of the two varieties of soybeans. DNP was the inhibitor selected for the work since it is known to uncouple oxidative phosphorylation and block the energy producing system in the cells. A preliminary experiment was carried out to determine the concentration of DNP which uncoupled phosphorylation in the root segments of PI and HA soybeans.

The effect of different concentrations on respiration was studied. Oxygen uptake at 30°C was used as a measure of respiration. The gas exchange was measured by standard manometric techniques (54). Root segments, 1cm in length were obtained from HA and PI soybeans grown for two weeks in nutrient solution. Samples of 500 mg were incubated in 15 ml Warburg flasks. The incubation medium consisted of 5 ml of half strength nutrient solution minus phosphate and 5 PPM iron. DNP at different concentrations was added from the side arm. Carbon dioxide evolved was trapped in 20% NaOH in the center well of the flask. For a number of samples of a given variety the oxygen uptake was found to be uniform for the first hour. The HA root segments had a higher respiration rate 161.1μl O_2/hr as compared to 116.6μl O_2/hr for PI. After one hour the

DNP was tipped into the main compartment and oxygen uptake was measured

for the next two hours. The highest concentration of DNP (10^{-4}M)

inhibited respiration of the root segments of both varieties (table V).

After 2 hours the respiration in the HA variety had decreased to 35.8%,

while the PI had decreased to 29.8% of the control. At the concentration

of 10^{-5}M DNP, the respiration rate increased above the control. DNP is

known to uncouple phosphorylation. Tissues in which the capacity of

phosphorylation limits respiration will show an increased respiration

rate when DNP is added (37). At 10^{-6}M DNP the respiration rate was less

affected than at the higher concentrations of 10^{-5}M DNP. From these results

10^{-5}M DNP was selected as the concentration of DNP to be used in the

inhibitor studies.

The effects of 10^{-5}M DNP on the absorption of iron in root segments of

HA and PI soybean was studied. Root segments, 1cm in length were obtained

from HA and PI soybeans. Half of the root segments of each variety were

pretreated in half strength nutrient solution minus phosphate and iron

plus DNP at the concentration of 10^{-5}M DNP while the control contained root

segments in nutrient solution with no DNP added. After one hour the root

segments were washed 3 times with distilled water and 500 mg samples of DNP

treated root segments were placed in 30 ml vials containing 10 ml of $\frac{1}{2}$

strength nutrient solution minus phosphate, 10^{-5}M DNP and Fe^{59} (prepared as

a precipitate see p. 32) at a concentration of 5 PPM (47,800 cpm). The

control samples contained no DNP. The vials were placed on a horizontal

shaking apparatus.

An absorption time course study of four replications for each time

period was run for 15, 30 and 60 minutes for each variety. At the end of the

Table V. Effect of DNP on the oxygen uptake into root segments of HA and PI soybeans

| DNP (M) | Oxygen uptake (μl/hr/500 mg) | | | | % of control | | | |
| | 1 hr. | | 2 hr | | 1 hr | | 2 hr | |
	PI	HA	PI	HA	PI	HA	PI	HA
0	104.0	161.9	96.0	149.2	100.0	100.0	100.0	100.0
10^{-6}	124.0	176.4	111.4	161.9	119.2	109.0	116.0	108.5
10^{-5}	152.2	183.8	126.4	166.7	146.3	113.5	131.7	111.7
10^{-4}	78.3	137.0	28.6	53.4	75.2	84.6	29.8	35.8

(47)

absorption period, the roots were washed in 50 ml of 10^{-3}M EDDHA for one hour to remove the iron from the free spaces. The uptake of Fe^{59} into the root segments was rapid, most of the Fe^{59} was taken up in 15 minutes (table VI). DNP increased the uptake of iron into the root segments of both varieties. In the PI variety the DNP treatment resulted in a 49% increase in iron absorption above the control, while in the HA variety the DNP treated root segment absorbed 9.% more iron than the control after 60 minutes.

Table VI. Time course of Fe^{59} absorbed from precipitated Fe^{59} in 10^{-5}M DNP in PI and HA root segments.

	Absorption (cpm/mg dry wt)					
	DNP added		No DNP		% of control	
	PI	HA	PI	HA	PI	HA
15	83.9	41.4	54.4	36.8		
30	99.7	51.6	63.3	43.7		
60	99.2	45.3	67.5	41.6	149.0	108.9

Another experiment was performed on a different lot of PI and HA grown plants. The procedure was identical except iron absorption was measured at only 60 minutes. DNP treated root segments of PI took up 62.1% more iron than the control while the HA variety absorbed only 17.8% more iron than the control.

Previous reports (37, 46) have shown that DNP inhibits oxidative phosphorylation. Robertson reported (46) that DNP inhibited the accumulation of salt in carrot tissues while the respiration either remained constant or increased. These results indicated that coupled phosphorylation was

necessary for salt accumulation in certain tissues.

Our studies with iron absorption into DNP treated root segments of two varieties of soybeans showed that respiration increased upon the addition of DNP at the concentration of 10^{-5}M and this increase was associated with an increase Fe uptake into the root segments. The PI variety responded more to the DNP treatment than the HA variety. In the PI variety, the control of iron uptake seemed to be linked more to the electron flow through the cytochrome system than the HA variety.

Similar results were obtained by Cormack and Wallace (20). They found that DNP fed along with Fe^{59}EDDHA to intact plants stimulated iron uptake into the roots of both varieties at pH 4.0. Iron absorption by the roots increased more in the PI than the HA variety. However, they found that DNP decreased transfer of Fe to the leaves in HA while a substantial increase was noted in the PI variety. Brown (11) working with HA soybeans reported that DNP inhibited the translocation of iron to a greater extent than the absorption of iron by the roots.

Our results showed that increased electron flow in the root segments due to DNP resulted in an increase of iron uptake in the PI variety which was substantially greater than in HA. Since DNP is known to uncouple oxidative phosphorylation this should result in an decrease in ATP production. Our inhibitor studies were conducted over a short period of time and the ATP supply might not have been depleted. ATP could still be produced by substrate phosphorylation. If ATP is not lacking, then the PI increase in iron uptake could be related to an increase in glycolysis and Krebs cycle activity.

This could occur since DPNH, which supplies the electrons for the cytochrome system, is an important cofactor for the Krebs and glycolytic pathways. Since it is reoxidized more rapidly when an increased electron flow occurs more DPN would then be available and this could stimulate an increased glycolytic and Krebs cycle activity.

DNP might affect the permeability of the individual root cells as well as affecting oxidative phosphorylation in the root. This effect could be greater in the PI variety than in HA. This increased permeability of the cells in PI may result in a greater uptake of iron into the root cells.

Lundegårdh's cytochrome pump (30) could explain our increase in iron uptake into the roots of PI. The increased electron flow resulting from the DNP treatment could cause an increase movement of anions into the cell. Cations such as iron could then passively move into the cell to balance the charge produced by the anion accumulation in the cell.

Root - Shoot Competition

Observations were made in previous experiments that there appeared to be a relationship between the size of top and the amount of iron in the roots of the soybean varieties. An experiment was performed to measure the amount of Fe^{59} accumulated in the roots after 6 hours of feeding and to attempt to relate these values to the dry weight of tops. $Fe^{59}Cl_3$, 5PPM (250,402 cpm) freshly prepared was fed to 9HA and 9PI plants in half strength nutrient solution minus phosphate. The plants were four weeks old and had been growing on iron supplied as $FeSO_4$. Forty-eight hours prior to the feeding experiment the plants were placed on a medium containing no iron.

In the PI variety a correlation coefficient "r" = -.7085 was found between the dry weight of tops and the amount of iron taken up into the roots (Fig.7). This "r" value was found to be significant at the 5% level. No significant correlation was found between the dry weights of tops and iron uptake into the HA variety.

Our results indicated that the size of the tops in the PI variety may control the uptake of iron into the roots. An active uptake of iron seemed to be involved. Larger tops in the PI variety was related to less iron accumulation in the roots on a dry weight basis. It is proposed that the larger tops may control the growth of the roots which results in less growth, decrease in respiration and less iron uptake into the roots. Since no correlation between size of tops and iron uptake into the roots was found in HA a passive uptake of iron independent of top growth could occur.

Figure 7.   Regression lines of Fe^{59} content in soybean roots
vs. dry weight of tops

PI soybeans ⎯⎯⎯⎯⎯

HA soybeans ⎯ ⎯ ⎯ ⎯ ⎯
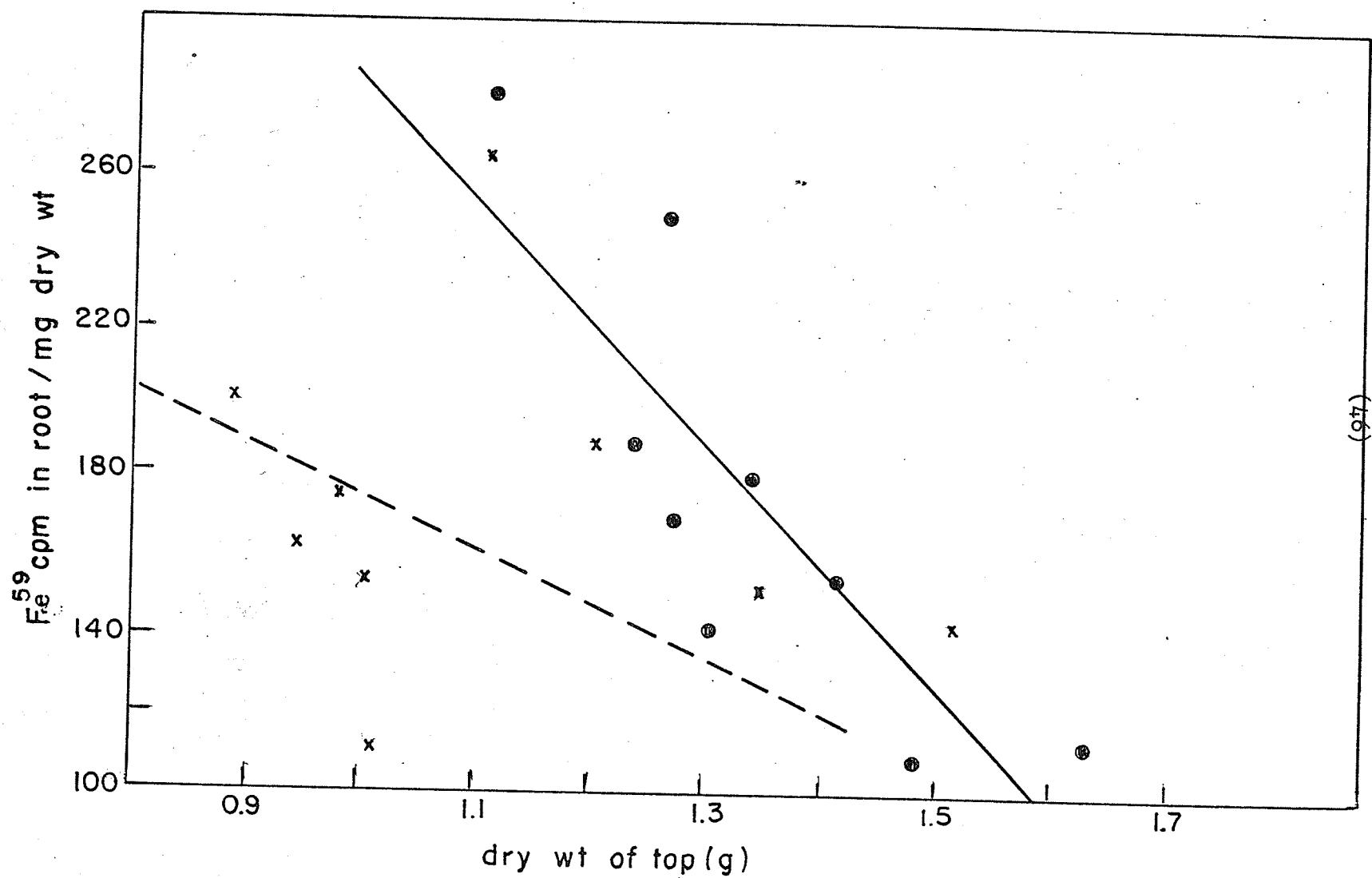
SUMMARY

1. When $NaHC^{14}O_3$ was supplied to chlorosis susceptible and resistant soybean varieties, C^{14} was rapidly fixed into organic acids and translocated to the tops in a similar manner in both varieties.

2. Fe^{59} fed as Fe^{59} chelates was absorbed into the roots and translocated to the tops at equal rates for both varieties. If iron was supplied as $Fe^{59}Cl_3$, the movement of iron occurred at slow rates for both varieties, although HA translocated twice as much Fe to the tops as PI in a 24 hour period.

3. The evidence suggested that organic acid movement was not connected to the movement of iron in the resistant and susceptible soybean plants.

4. Uptake of iron into root segments was found to be dependent upon the form of the iron fed. Freshly prepared $Fe^{59}Cl_3$ was taken up in larger quantities than iron fed in a precipitated form $Fe^{59}(OH)_3$. The uptake from $Fe^{59}Cl_3$ was found to be similar for both varieties. Fe^{59} uptake from $Fe^{59}(OH)_3$ was found to be greater in the PI variety.

5. Uncoupling of phosphorylation with DNP resulted in an increased oxygen uptake into the root segments which was associated with an increased iron uptake into the soybean varieties. More Fe^{59} was taken up into the root segments of the PI variety than into the HA variety treated with DNP.

6. A negative correlation was found between the dry weights of tops and the Fe^{59} uptake into the roots in the PI variety, but not in the HA variety.

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