QUANTITATIVE RELATION BETWEEN CHLOROPHYLL AND IRON IN GREEN AND CHLOROTIC PEAR LEAVES

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(WITH SIX FIGURES)

I. Introduction

Lime-induced chlorosis is known to be curable by iron, and for this reason is attributed to iron deficiency. Chlorotic leaves should accordingly contain less iron than green leaves. It has been found by several investigators, however, that chlorotic leaves may contain more iron than green leaves. Similar results were obtained also by the writer, regardless of whether the iron content was reported on the dry or on the fresh-weight basis. No positive correlation could be observed between the iron content and the amount of chlorophyll of leaves obtained from chlorotic trees (table II, columns 4 and 5), although iron was the limiting factor in these leaves in so far as chlorophyll formation was concerned. This fact may be explained by either of the following hypotheses:

1. All the iron present in the leaves is equally (or nearly equally) active in chlorophyll formation. The efficiency of its activity, however, may vary considerably in leaves of the same tree. The green leaves would accordingly be those leaves which may contain small amounts of iron, but in which the efficiency of the iron in chlorophyll formation is very great.

2. Only a fraction of the iron in the leaves is active in chlorophyll formation. This active fraction is more abundant in the green leaves than in the chlorotic ones, although the reverse may be true in the case of the total amount of iron in the leaves.

In regard to the first hypothesis, it may be stated that a wide range of variations in the efficiency of the iron in leaves is logically not impossible. On the other hand, the assumption that all the iron in leaves is active in chlorophyll formation seems improbable, since not all the iron is present in one form. Thus BOUSSINGAULT (2) could extract with alcohol only about one-fourth to one-half of the iron content in leaves. SERGER (11) also found that not all the iron in spinach leaves could be extracted with alcohol, or with a mixture of benzene, chloroform, and ether. The investigations of SUZUKI (13), GRIESSMEYER (6), and INGALLS and SHIVE (7) indicate also that the iron in leaves is present in more than one form.

This evidence favors the assumption that a specific form of iron is active in chlorophyll formation. This form of iron is designated in this paper *active iron*. In the following pages, the attempts which were made to determine the amount and the nature of this active iron are discussed.

II. Material and methods

Pear trees of two varieties were used, Hardy and Bartlett. The trees grew on soil rich in lime. Most of the samples were taken from two orchards which were badly affected by chlorosis. The trees in these orchards varied greatly in the chlorophyll content of their leaves. It was not uncommon to find individual trees which bore leaves of all shades of color, ranging from cream-yellow to deep green, and often green and chlorotic trees stood side by side.

Repeated treatments over a period of four years showed that the trees in these orchards always responded to application of iron, when applied in any of the following ways: spraying of leaves with iron salt solutions; injection of iron salt solutions into the trunk and limbs; and application of powdered iron salts into holes bored in the lower end of the trunk or branches.[1] Positive results were obtained regardless of the acid radical attached to the iron, provided the iron compounds were fairly soluble. The following compounds were found to induce greening of chlorotic leaves: ferric sulphate, ferric citrate, ferric chloride, ferric oxalate, ferrous sulphate, and ferrous citrate. On the other hand, application of citric acid, tartaric acid, cupric sulphate, manganese sulphate, and magnesium salts failed to give positive results. It is thus obvious that the plants dealt with in this investigation suffered from a typical lime-induced chlorosis due to a deficiency of iron or an abnormal iron metabolism.

Preliminary work had shown that failure to wash the leaves before analysis may vitiate the iron determination by more than 100 per cent. Thus all the leaves were washed well in distilled water before being analyzed. After washing, the leaves were dried at 50°–60° C., and then ground in a porcelain mortar or in a brass mill specially built for the purpose. Care was taken to avoid contact between iron and the samples.

The leaf powder was ashed in porcelain or silica crucibles, and the iron in the ash determined colorimetrically by the thiocyanate method as modified by WALKER (**14**). Care was taken to keep the standard and samples at about the same acidity, namely 0.25 N. The determination of iron in apricot and peach leaves, and some pear leaves, however, was carried out in 1.0 N HCl solutions. Frequent blank determinations were made with porcelain and silica crucibles, and the values for the iron content of the samples were corrected accordingly.

In the presence of small amounts of iron, for example, of 1.0 parts per million or less, in the solution to be analyzed, a modified method was employed similar to that used by STOKES and CAIN (**12**), the method being

[1] The trees were treated by Dr. J. P. BENNETT, and the writer is indebted to him for the use of the data thus obtained. For details concerning the treatments, see BENNETT (**1**).

based on the property of ethyl acetate and amyl alcohol to extract the red iron thiocyanate compound from aqueous solution. To one volume of the acid solution containing iron and ammonium or potassium thiocyanate, one-half to two-thirds' volume of ethyl acetate (or amyl alcohol) was added. The mixture was shaken in a separatory funnel and allowed to stand for a few minutes. The ethyl acetate was then separated from the aqueous solution and the color of the ethyl acetate solution compared with that of a standard solution treated in the same way. It was found that the ethyl acetate intensified the color, made it more stable, and was particularly suitable for the determination of small amounts of iron in the presence of small amounts of copper.

A direct contact was avoided between corks, rubber stoppers, ordinary filter paper, and the acid iron solution, since it was found that these objects may contain sufficient amounts of acid-soluble iron to vitiate the results. The solutions to be analyzed were filtered through acid-washed filter paper, and were kept in glass-stoppered flasks.

Practically all the iron values here reported represent the averages of duplicate or triplicate determinations.

When leaf material was extracted with various solvents, the following procedure was used: to about 4.7–6.6 gm. of dry powdered leaf material in a glass-stoppered flask, 50–70 cc. of solvent were added in proportion to the weight of the sample. The flasks were put in a shaker for about 24 hours.[2] The suspension was then centrifuged for about 20–25 minutes, and to the solid residue about 20–30 cc. of the solvent were added, mixed with a glass rod and centrifuged again for 10–15 minutes, decanted, 20–25 cc. of the solvent added once more, the solution stirred with a glass rod, centrifuged again for 10 minutes, and decanted. The decanted portions from each sample were combined, the liquid evaporated in porcelain or silica crucibles, the residue ashed, and the amount of iron in the ash determined as described.

Chlorophyll was determined always on *fresh leaves* according to the method of WILLSTÄTTER and STOLL (**16**, pp. 2–3). The color of potassium chlorophyllin of the samples was compared with that of a standard solution of potassium chlorophyllin prepared from pure chlorophyll isolated from fresh grass according to the method of WILLSTÄTTER and his co-workers (**16**, pp. 30–32).

[2] While it is not essential to adhere closely to this period of time (24 hours), it is very important when dealing with 1.0 N HCl extraction that the period of shaking should be the same for all samples of a given series, that is, leaves of equal age, collected from the same trees, and on the same date.

III. Active iron in pear leaves

It was believed that in samples of leaves in which active iron was the limiting factor in chlorophyll formation, the amount of green pigments should bear a positive correlation to the amount of active iron they contain. In an attempt to isolate the active iron, green and chlorotic pear leaves of the same age were extracted with various solvents, and the amount of iron in these extracts was compared with the chlorophyll content of the samples to ascertain whether a direct relation existed between them. No such correlation was found when the leaves were extracted with distilled water or with 0.05 N HCl. Similar results were obtained also in regard to the iron in the vacuolar sap of leaves, which was secured by a method similar to that used by CHIBNALL (3): the fresh leaves were washed in water, then dried with a clean towel or filter paper, dipped in ether for 5–10 minutes, spread on filter paper to dry for 10–20 minutes, then pressed in a Buchner press between porcelain or copper plates. The sap obtained in this manner is termed in this paper *vacuolar sap* merely for convenience, since proof is lacking that the liquid obtained is necessarily pure vacuolar sap. The data relating to the extraction of pear leaves with 1.0 N HCl are presented in table I. This table shows that in all samples, with the exception of samples 17 and 18, the amount of iron extracted is higher in the green leaves than in the corresponding chlorotic leaves. This holds true also in the case of samples 13 and 14, in which the total amount of iron present in the green leaves is smaller than that contained in the yellow leaves. Table I thus clearly indicates that a positive correlation exists between the amount of iron extracted from leaves with 1.0 N hydrochloric acid and with their chlorophyll content. It should be emphasized, however, that the two samples (17 and 18) which show exception to this rule were collected late in the season. This fact will be further discussed later.

The data in column 6, however, show also that the total iron in all cases, except samples 13 and 14, is higher in the green leaves than in the corresponding chlorotic ones. This gave rise to the supposition that the amount of iron extracted with 1.0 N hydrochloric acid stood in direct relation to the total iron present in the sample, and that consequently the values presented in column 7 depended on the total iron present, but did not stand in direct correlation to the chlorophyll content of the samples.

In order to test this assumption, a series of pear leaves was collected, and their chlorophyll content, the total amount of iron present, and the amount of iron extracted[3] with 1.0 N hydrochloric acid determined. The

[3] Most of the samples in this series were extracted for 24 hours. While it is not essential to adhere strictly to this period of shaking, it is very important that samples belonging to the same series (*e.g.*, collected from the same trees at a given date) should be extracted for the same length of time.

TABLE I

TOTAL AMOUNT OF IRON IN PEAR LEAVES AND AMOUNT OF IRON EXTRACTED FROM THEM WITH 1.0 N HCl

No. of sample	Condition of leaves	Description of sample	Variety	Date collected	Total iron in leaves	Iron in 1.0 N HCl extract
					In p.p.m. of dry weight of leaves	
1	Green	Spur leaves from one-year-old wood	Bartlett	Apr. 20/29	70	26
2	Chlorotic			"	42	16
3	Green			May 16/29	100	39
4	Chlorotic			"	97	23
5	Green			Aug. 15/29	117	60
6	Chlorotic			"	73	29
7	Green	Leaves from base of shoots	Bartlett	May 29/29	98	47
8	Chlorotic			"	63	27
9	Green	Leaves from middle of shoots	Bartlett	Aug. 17/29	92	47
10	Chlorotic			"	69	32
11	Green	Spur leaves from wood older than one year	Hardy	Apr. 17/29	42	20
12	Chlorotic			"	25	8
13	Green			May 28/29	49	23
14	Chlorotic			"	70	16
15	Green			Aug. 5/29	79	42
16	Chlorotic			"	76	26
17	Green			Aug. 16/27	76	33
18	Chlorotic			"	120	41

sampling was done as follows: severely chlorotic, moderately chlorotic, and green leaves of the same age were collected from the same chlorotic trees. The samples designated as "green, treated with iron in 1928" were obtained from chlorotic trees which were treated with iron in December, 1928, and in consequence of which bore very green leaves in the 1929 and 1930 seasons.

The results of the analyses are presented in table II, columns 6 and 7, from which it is concluded that no correlation exists between the total iron content of these samples and the quantity of iron which is extracted from them with 1.0 N hydrochloric acid. On the other hand, this table reveals

TABLE II

ACTIVE IRON, CHLOROPHYLL CONTENT, AND AMOUNT OF IRON EXTRACTED FROM HARDY PEAR LEAVES WITH 1.0 N HCl

No. of sample	Description of leaves	Leaves taken from	Date of collecting sample	Chlorophyll content in % of dry weight of leaves	Total iron in leaves	Total iron in 1.0 N HCl extract	Inactive iron in 1.0 N HCl extract	Active iron
					p.p.m. of dry weight of leaves			
1	Severely chlorotic	Spurs	April 29	0.084	33	14.6	13.2	2.4
2	Moderately chlorotic		"	0.18	40	19.4	13.2	5.1
3	Green, from chlorotic trees		"	0.53	33	27.1	13.2	15.0
4	Severely chlorotic		May 13	0.11	37	9.2	6.4	2.7
5	Moderately chlorotic		"	0.18	27	10.7	6.4	4.5
6	Green, from chlorotic trees		"	0.28	36	12.8	6.4	7.1
7	Green, from trees treated in 1928		"	0.65	51	23.2	6.4	16.2
8	Severely chlorotic		May 27	0.073	32	12.0	10.0	1.2
9	Moderately chlorotic		"	0.15	29	12.0	10.0	2.6
10	Green, from chlorotic trees		"	0.34	32	16.4	10.0	5.9
11	Green, from trees treated in 1928		"	0.77	54	22.5	10.0	13.3
12	Severely chlorotic		June 16	0.061	49	14.9	12.3	1.0
13	Moderately chlorotic		"	0.18	47	15.9	12.3	2.9
14	Green, from chlorotic trees		"	0.42	48	17.5	12.3	6.9
15	Green, from trees treated in 1928		"	0.93	63	27.3	12.3	15.2
16	Severely chlorotic		July 9	0.058	62	15.8	14.7	1.4
17	Moderately chlorotic		"	0.18	63	18.9	14.7	3.6
18	Light green from chlorotic trees		"	0.31	64	22.1	14.7	7.2
19	Deep green from chlorotic trees		"	0.44	78	26.1	14.7	10.8
20	Severely chlorotic	Base of shoots	"	0.071	76	22.3	19.1	2.0
21	Moderately chlorotic		"	0.22	66	24.0	19.1	6.0
22	Light green from chlorotic trees		"	0.29	.....	26.4	19.1	8.0
23	Deep green from chlorotic trees		"	0.68	74	37.4	19.1	18.3
24	Severely chlorotic	Middle of shoots	"	0.093	.....	21.1	17.7	1.8
25	Moderately chlorotic		"	0.20	48	19.6	17.7	3.8
26	Deep green from chlorotic trees		"	0.65	57	29.9	17.7	12.2
27	Severely chlorotic	Terminal of shoots	"	0.063	59	17.8	16.7	1.0
28	Moderately chlorotic		"	0.086	54	17.8	16.7	1.2
29	Deep green from chlorotic trees		"	0.60	62	25.0	16.7	8.3

a striking *positive correlation between the amount of iron extracted from the leaves and their chlorophyll content* (see figure 1).

This fact strongly suggests that the active iron, or its hydrolysis product, is contained in the 1.0 N hydrochloric extract. It remains to be determined whether this extract contains *only* the active iron, or whether it contains in addition some other fractions of iron which are inactive.[4]

Since iron in these samples is the limiting factor in so far as chlorophyll formation is concerned, it was reasoned that if all the iron in the extract were active iron, then a more or less direct proportionality should exist between the amount of chlorophyll in samples of each series and the total amount of iron extracted from them by 1.0 N hydrochloric acid. On the other hand, if the 1.0 N hydrochloric extract contained also inactive iron, then such a proportionality should exist only between the active iron fraction and the chlorophyll content. The data of table II are plotted in figure 1, where the chlorophyll content is represented by the ordinate, and the total amount of iron extracted from the leaves with 1.0 N hydrochloric acid is plotted on the abscissa. This figure shows that no direct proportion exists between the *total amount* of extracted iron and the chlorophyll content; hence it is concluded that the 1.0 N hydrochloric acid extract contains some inactive iron in addition to the active iron.

It could have been argued that the iron in the 1.0 N hydrochloric acid extract was active iron only, but that this extract contained merely part of the total active iron present in the leaves. If this were true, then sample 14 (table II), for example, which contains about 6.9 times more chlorophyll than sample 12, should contain also about 6.9 times more active iron than sample 12; in other words, it must contain at least $6.9 \times 14.9 = 102$ p.p.m., which is greatly in excess of the total iron present in sample 15, namely 48 p.p.m.

Figure 1 also indicates that all samples in each series (*e.g.*, samples of the same age, collected from the same trees, and on the same date) lie on a straight line, or their distance from a straight line drawn through them is within the experimental error involved in the determination of their iron content. For most samples in table II the difference between duplicate iron determinations of 1.0 N hydrochloric extracts lies within 5 per cent. of the average value. The error involved in the chlorophyll determination for the values given in this table may therefore amount to as much as 10–15 per cent. This is due in part, presumably, to the variation in chlorophyll content within leaf material of a given sample.

[4] The terms *active iron* and *inactive iron* used in this paper refer to the iron fractions which are active or inactive in the formation of chlorophyll only. These terms do not imply anything in regard to the activity of these fractions in other physiological processes, *e.g.*, respiration.

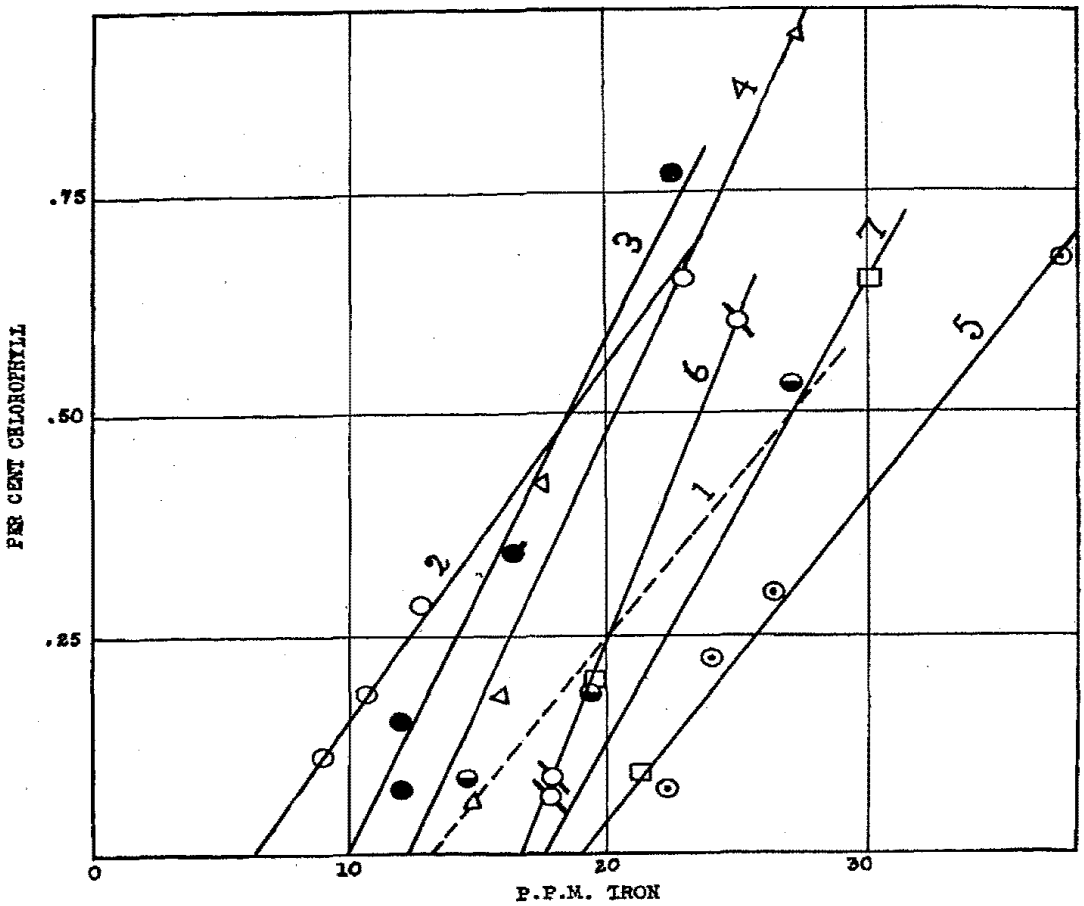

FIG. 1. Chlorophyll content and iron extracted with 1.0 N HCl in 24 hours: 1, ◒ = spur leaves collected April 29; 2, ○ = spur leaves collected May 13; 3, ● = spur leaves collected May 27; 4, △ = spur leaves collected June 16; 5, ⊙ = leaves from base of shoots collected July 8; 6, ⊖ = leaves from terminal shoots collected July 9; 7, □ = leaves from middle of shoots collected July 7.

This fact may be readily explained on the assumption that *all 1.0 N hydrochloric acid extracts of samples belonging to the same series contain the same amount of inactive iron, and differ only in the amount of active iron they contain.* (The validity of this statement will be discussed later on, and it will be shown that it does not always hold true. This, however, does not affect the conclusions drawn here, since this assumption holds true for the samples given in figures 1 and 5.) Thus the difference in the amount of extracted iron of two samples belonging to the same series corresponds to the difference in the amount of active iron.

If, in figure 1, a straight line be passed through points belonging to samples of one series, then the point of intersection of this line with the abscissa will correspond to a hypothetical sample of chlorotic leaves of which the chlorophyll content is equal to 0. It is evident that the amount of active iron in such a sample must be extremely small, or equal to 0. It

then follows that the distance between this intersection point and the origin represents the amount of the *inactive iron* which a 1.0 N hydrochloric extract of such a sample would contain. But this amount of inactive iron represents, according to our assumption, the amount of inactive iron in the extracts of all other samples of this series; hence the amount of active iron in a sample of this series is given by the expression: $Fe_a = Fe - Fe_i$, where Fe_a is the active iron; Fe_i is the inactive iron in the 1.0 N HCl extract; and Fe represents the total iron in the same extract.

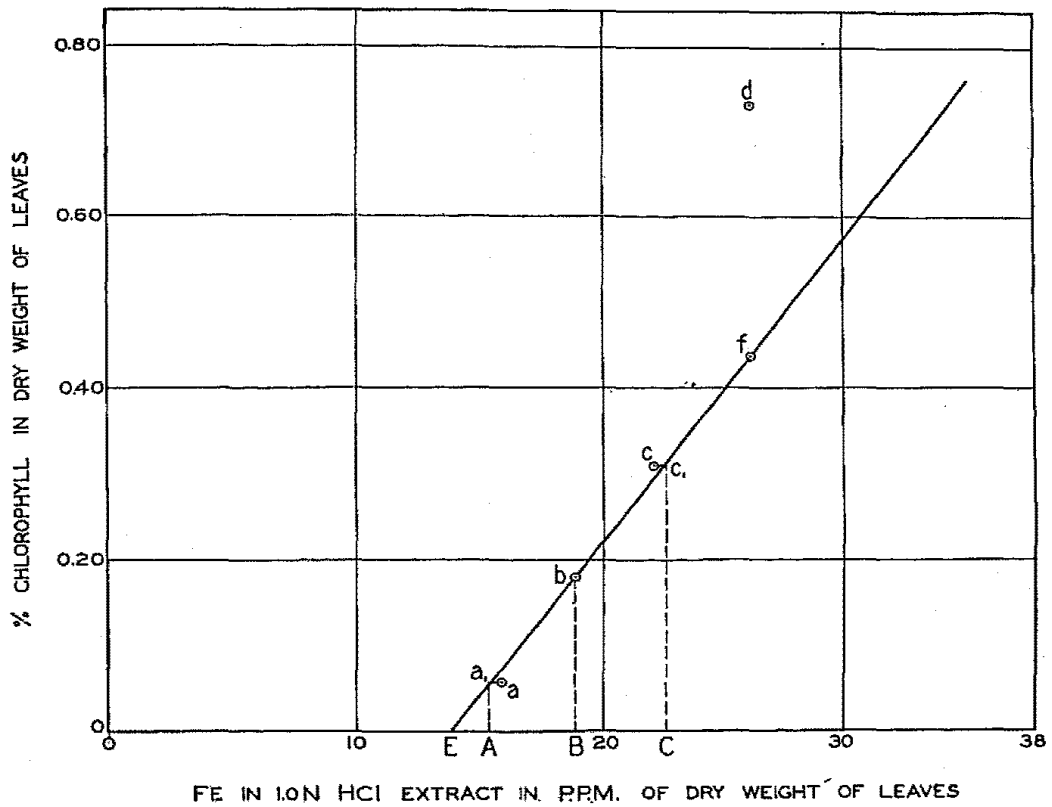

FIG. 2. Estimation of active iron in 1.0 N HCl extract of leaves; Hardy spur leaves collected July 9, 1930.

An example will serve to illustrate the method of estimating the active iron in pear leaves. In figure 2 the points *a*, *b*, *c*, and *f* represent severely chlorotic, moderately chlorotic, slightly green, and very green leaves from chlorotic trees respectively, collected on July 9, 1930. These samples were spur leaves collected from the same trees, and therefore belong to the same series; while sample *d* was collected at the same time and in the same orchard, but from a different group of trees which were treated with iron in 1928. The line $a_1 f$ is the straight line of closest fit to pass between the points *a*, *b*, *c*, and *f*. This line intersects the abscissa at the point *E*. Thus *OE* represents the inactive iron for this series of samples. The active iron for

sample a is obtained by drawing from a a line parallel to the abscissa; this line intersects a_1f at a_1. From a_1 a perpendicular line to the abscissa is drawn which intersects it at A; EA is thus the active iron for a, while EB and EC represent the active iron for b and c respectively. The values of Fe for several series of Hardy pear leaves are plotted in figure 1 against the chlorophyll content, and straight lines are drawn through points belonging to the same series, which allow the estimation of the active iron. The values thus obtained are presented in table II, column 9.

In figure 3 the values of Fe_a are plotted on the ordinate against the value of $(Fe - Fe_l)$ on the abscissa on the same scale. The points on figure 3 represent 29 samples comprising 8 series and collected at different times of the season between April and July. These samples include spur leaves as well as shoot leaves,[5] all of which were collected from Hardy trees in a chlorotic orchard. If Fe_a were equal to $(Fe - Fe_l)$, then all the points

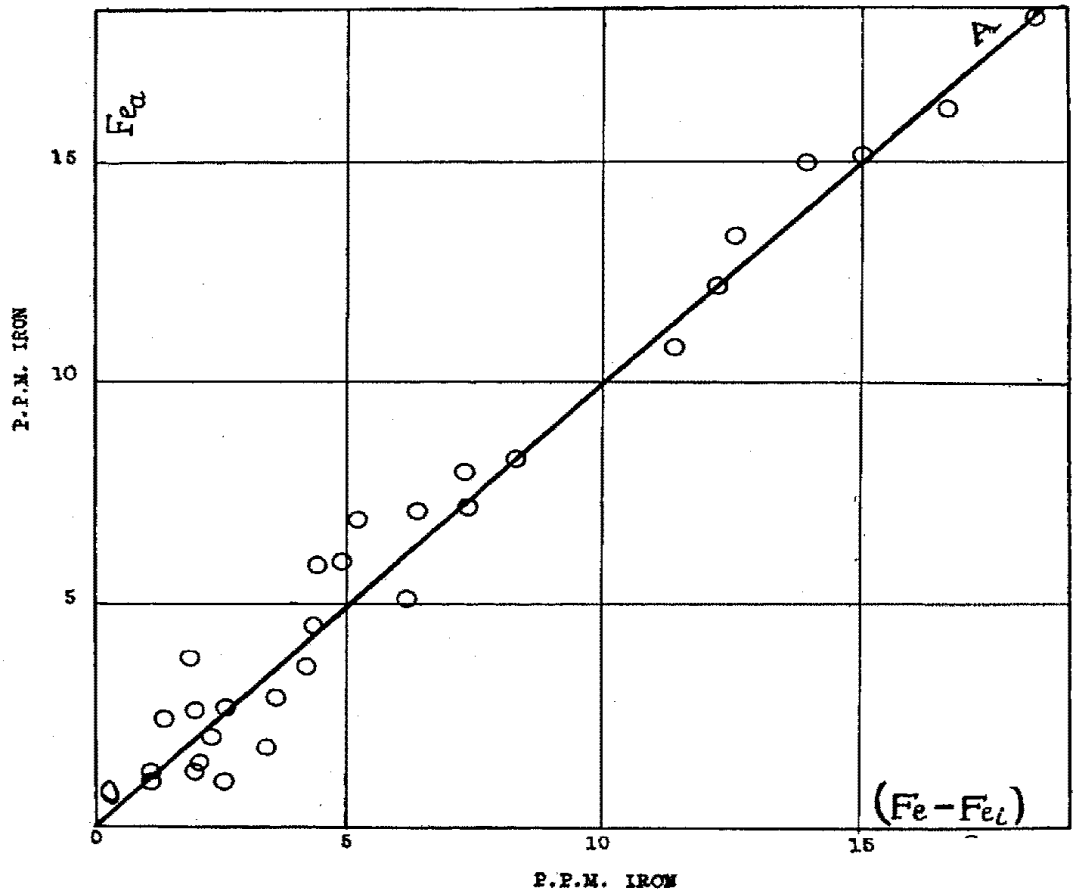

FIG. 3. Active iron (Fe_a) and values of ($Fe-Fe_l$) for Hardy pear leaves.

[5] In the case of shoot leaves, each series comprised leaves of nearly the same age. This was accomplished by segregating the leaves from the base, middle, and top of the shoots to make separate samples.

in figure 3 should lie on a straight line, *OA*, passing through the origin and making an angle of 45° with either one of the axes. The positions of the points on figure 3 afford a means of gauging the agreement between the calculated values of the active iron (that is, Fe_a) and the observed values. This figure shows that out of 29 samples there is not a single one for which the value of $\triangle$ Fe, as defined by the expression $\triangle Fe = Fe_a - (Fe - Fe_i)$, equals or exceeds 2 p.p.m., and for 22 samples $\triangle$ Fe is less than 1 p.p.m. This is very good agreement, since the values of $\triangle$ Fe are well within the limit of the error involved in the determination of Fe.

The data plotted in figures 1 and 2 pertain only to the samples which were collected up to July 9; samples collected later do not show a consistent correlation between the amount of iron extracted in 24 hours with 1.0 N HCl and their chlorophyll content, as can be seen from table III and from figure 4 which represents the results obtained for Hardy samples collected on August 7. This finding is in agreement with the data reported in table I (samples 17 and 18), in which it was shown that the 1.0 N HCl

TABLE III

CHLOROPHYLL CONTENT, AND THE IRON EXTRACTED WITH 1.0 N HCl FROM PEAR LEAVES COLLECTED FROM CHLOROTIC TREES LATE IN GROWING SEASON

DESCRIPTION OF LEAVES	VARIETY	ORCHARD	DATE OF COLLECTING SAMPLES	CHLOROPHYLL CONTENT IN % OF DRY WEIGHT OF LEAVES	TOTAL IRON EXTRACTED WITH 1.0 N HCl IN P.P.M. OF DRY WEIGHT OF LEAVES
Severely chlorotic	Bartlett	Mc	July 20	0.087	53.3
Moderately chlorotic	"	"	"	0.27	43.3
Light green	"	"	"	0.42	39.5
Deep green	"	"	"	0.68	54.1
Severely chlorotic	Hardy	M	"	0.10	16.4
Moderately chlorotic	"	"	"	0.26	25.0
Light green	"	"	"	0.43	22.5
Deep green	"	"	"	0.96	36.1
Severely chlorotic	"	"	August 7	0.056	28.1
Moderately chlorotic	"	"	"	0.20	22.0
Light green	"	"	"	0.25	30.0
Deep green	"	"	"	0.66	34.2
Severely chlorotic	"	B	"	0.045	43.1
Moderately chlorotic	"	"	"	0.14	38.5
Light green	"	"	"	0.38	40.4
Deep green	"	"	"	0.73	54.8

extract of a chlorotic sample collected in August contains more iron than a similar extract from green leaves.

An explanation for this fact may be furnished by the observations of SACHS (10) and other workers. SACHS noticed that chlorotic leaves did not respond to treatment of iron when applied late in the season. ZIMMERMAN (17) and ROUX (9) reported that the chloroplasts of severely chlorotic leaves showed marked signs of injury, which finally resulted in the disintegration of the plastids. These observations indicate that some profound changes take place in chlorotic leaves late in the season, which may affect the solubility of the various iron compounds of the leaves. It is likely that the hydrochloric acid extract of chlorotic samples collected late in the season may contain amounts of inactive iron different from those of the extracts from green leaves. The active iron cannot be determined in samples collected late in the season, since the method of its estimation, previously described, can be used only when all the samples of a series contain in their hydrochloric acid extract the same amount of inactive iron.

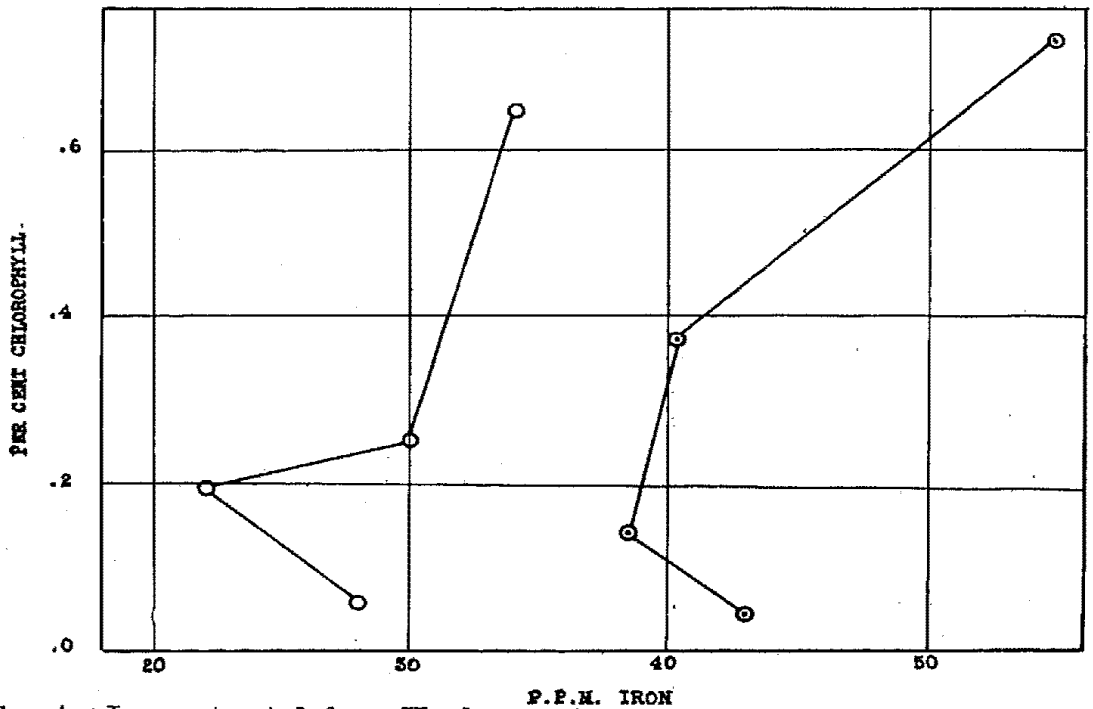

FIG. 4. Iron extracted from Hardy spur leaves with 1.0 N HCl; samples collected August 7: ——○——, leaves from orchard M; ——⊙——, leaves from orchard B.

Again, the injury to the leaf caused by prolonged chlorosis may impair the efficiency of the active iron, until the chloroplasts are injured beyond recovery, when the active iron, even if abundant, may fail to bring about the formation of chlorophyll. If this were the case, then the amount of

active iron in leaves of the same age, collected from the same trees, would not be proportional to their chlorophyll content.

From the foregoing it is obvious that the method of determining the active iron is limited in its application for the following reasons:

1. In order to estimate the active iron in any one sample, it is necessary to determine the chlorophyll content and the acid-extractable iron of a whole *series* of samples (the series should consist of at least three samples).

2. The leaf samples in each series must be of the same age and grown on the same trees; they must, however, differ markedly in their chlorophyll content.

3. The active iron must be the limiting factor in each sample of a series, in so far as chlorophyll formation is concerned.

4. The method cannot be applied to samples collected late in the season (*e.g.*, August or later).

On account of these limitations, a method which could be of wider application is being investigated at the present time.

IV. Active iron in peach and apricot leaves

Green and chlorotic peach and apricot leaves were collected from chlorotic trees. The chlorophyll content, total amount of iron, and iron extracted from the leaves with 1.0 N HCl were determined by the same procedure as used with pear leaves. The data obtained are presented in table IV and figure 5. It may be noted that while the total amount of iron in the chlorotic leaves is smaller than that present in the green leaves,

TABLE IV

ACTIVE IRON IN PEACH AND APRICOT LEAVES FROM CHLOROTIC TREES. SAMPLES COLLECTED JULY 20 FROM MIDDLE OF SHOOTS

DESCRIPTION OF LEAVES	CHLOROPHYLL CONTENT IN % OF DRY WEIGHT	TOTAL IRON IN LEAVES	IRON IN 1.0 N HCl EXTRACT	ACTIVE IRON
		IN P.P.M. OF DRY WEIGHT OF LEAVES		
Severely chlorotic peach leaves.....	0.21	41	14.0	3.8
Moderately chlorotic peach leaves...	0.68	48	22.3	10.1
Light green peach leaves.....	1.09	75	30.2	19.6
Deep green peach leaves.....	1.47	75	36.9	26.7
Severely chlorotic apricot leaves.....	0.16	48	15.5	5.1
Moderately chlorotic apricot leaves	0.31	45	18.0	9.4
Light green apricot leaves.....	0.59	76	30.8	17.8
Deep green apricot leaves.....	0.90	62	37.7	27.2

no consistent relation exists between the total iron content and the chlorophyll content, while the relation between the amount of iron extracted with 1.0 N HCl and the chlorophyll content is very close. It is evident that the method of estimation of active iron as described in this paper is applicable to leaves of pear, apricot, and peach, and presumably also to other material.

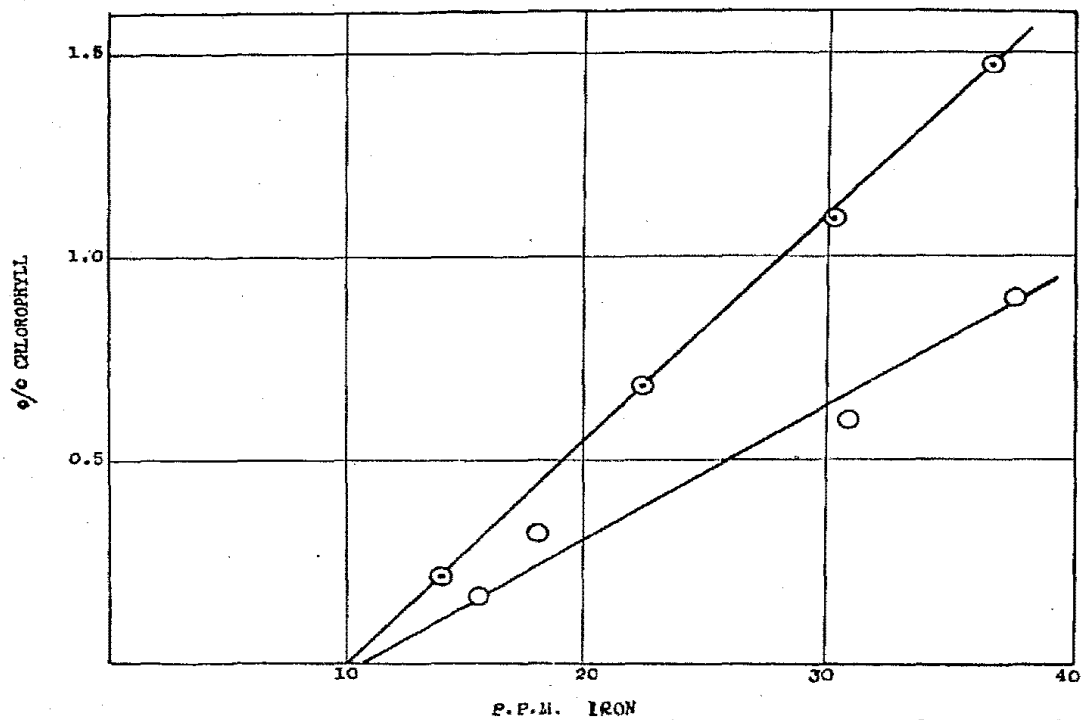

FIG. 5. Chlorophyll content and iron extracted from 1.0 N HCl from peach leaves (⊙) and apricot leaves (○).

V. Nature of active and inactive iron

When a solution of ammonium thiocyanate or potassium thiocyanate is added to 1.0 N HCl extract of pear leaves, the brown liquid turns red-brown. This indicates the presence of Fe^{+++} ions, or of an iron compound readily converted into Fe^{+++}. The 1.0 N HCl extract is deeply colored, however, owing to the presence of decomposition products of chlorophyll and of other compounds. For this reason the intensity of the red color of the iron thiocyanate cannot be determined directly on the extract. In order to separate the ionic iron from the rest of the extract, the following procedure was adopted.

The 1.0 N HCl extract of a leaf sample was made up to 175 cc. with 1.0 N HCl solution, and 25 cc. of 40 per cent. NH_4CNS were added to it. To this solution 50 cc. of ethyl acetate were added; the whole was shaken for a minute or two in a separatory funnel, the emulsion allowed to stand for 10–20 minutes, the aqueous phase drained, and the ethyl acetate collected

in a flask. The extraction with ethyl acetate was repeated five or six times until the ethyl acetate remained colorless. The ethyl acetate extracts were added together and evaporated slowly in pyrex beakers on a hot plate at a low heat. When the volumes of the liquid in the beakers were reduced to about 10–20 cc., the beakers were removed from the hot plates and allowed to cool, and concentrated nitric acid was added drop by drop, a few drops at a time with an interval of several minutes between each addition. After this process was repeated several times, about 5 cc. of concentrated nitric acid were added and the liquid in the beakers was evaporated at low heat until dry. The beakers were then put in an electric furnace and the residue ashed at low temperature (at a very dull red). The determination of the iron in the ash was carried on in the manner previously described.

TABLE V

IONIC AND IONIZABLE IRON IN 1.0 N HCl EXTRACTED FROM PEAR LEAVES

DESCRIPTION OF SAMPLE	DATE OF COLLECTING SAMPLE	HOURS OF EXTRACTION WITH 1.0 N HCl	TOTAL IRON IN 1.0 N HCl EXTRACT	IRON IN ETHYL ACETATE EXTRACT	IRON IN ACID RESIDUE	Fe_a
			IN P.P.M. OF DRY WEIGHT			
Severely chlorotic spur leaves	July 9	44	12.8	8.3	4.5	1.4
Light green spur leaves	"	47	24.7	21.2	3.5	7.2
Light green leaves from shoot terminals	"	7	15.2	12.7	2.5	8.3

The results of the ethyl acetate extractions are presented in table V. Practically all the iron in the HCl extract was removed by ethyl acetate. The small quantities found in the acid residue may have been due partly to traces of iron in the reagents used, and partly to some ethyl acetate which remained as a fine emulsion in the acid phase. The data in table V indicate that *practically all of the iron in the acid extract is present as ferric iron, or in a form which is readily converted into* Fe^{+++}. In this respect no difference exists between the active and the inactive iron in 1.0 N HCl.

This fact, however, does not disclose in what form the active and the inactive iron respectively are present in the living cell. So much, however, can be concluded: *these two forms of iron are present in the leaf cells in compounds which can be dissolved, or readily converted by 1.0 N HCl into ionic iron or ionizable iron.* It may be of interest to note that all the iron

compounds used successfully in this investigation for the cure of chlorosis were compounds which in aqueous solutions dissociate, at least partly, into ferric or ferrous ions. An attempt to treat chlorotic trees with potassium ferrocyanide, which in aqueous solution does not yield ionic iron in appreciable amounts, was not successful on account of the injurious effects of potassium ferrocyanide on the trees. No conclusion, therefore, can be drawn from this experiment regarding the ability of pear leaves to convert a non-ionic iron compound into active iron.

Since it has been found in several leaf samples that the amount of active iron greatly exceeds the amount of iron extracted with water, or the amount of iron contained in the "vacuolar sap," it is thus inferred that *the active iron is not present in these leaf samples in solution; at the most only part of it is soluble.*

Pear leaf tissue tested microchemically for iron with potassium ferrocyanide and potassium ferricyanide yielded negative results. Positive tests were obtained only with leaves which were taken from trees treated with iron. The positive reaction in these leaves was observed only in and near parts which showed injury effects due to an excess of iron. The microchemical tests thus carried out were not numerous, but the results obtained are in agreement with those of MILAD (8). This is not necessarily proof that no ionic iron readily soluble in hydrochloric acid exists in pear leaves. It has already been shown that the amount of active iron in pear leaves is not large, and it probably seldom exceeds 50–80 p.p.m. of the dry weight; often it is much less than that. Such an amount of iron when distributed in an excess of reagent may be diluted to the extent that it remains in solution notwithstanding the presence of large amounts of ferri- or ferrocyanide.

TABLE VI

VALUES OF E FOR VARIOUS LEAF SAMPLES

LEAVES COLLECTED FROM	PLANT	DATE OF COLLECTING SAMPLE	E
Spurs	Hardy pear	April 29	22.1
Spurs	"	May 13	25.1
Spurs	"	May 27	37.7
Spurs	"	June 16	38.3
Spurs	"	July 9	22.0
Base of shoots	"	July 9	22.7
Middle of shoots	"	July 9	33.2
Terminal end of shoots	"	July 9	44.3
Middle of shoots	Peach	July 20	33.4
Middle of shoots	Apricot	July 20	20.7

It is also possible that an appreciable fraction of the ionic iron is present as complex in the HCl extract, tied up with organic acids. The iron in these complexes may be readily ionizable, but the concentration of F^{++} or Fe^{+++} in the extract may be too small to yield a positive test with $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$.

The slope of the lines in figure 1 and figure 5 is given by the ratio $E = \frac{\text{chlorophyll content}}{\text{active iron}}$ per unit weight of leaves. This ratio is therefore a measure of the efficiency of the active iron in chlorophyll formation. In table VI the values of E are presented for ten series of samples. The values of E in this table are expressed in mols of chlorophyll[6] per gram atom of active iron. No consistent correlation is revealed between the age of the leaves and the value of E. The fact that the values of E are large and variable leads to the conclusion that it is *highly improbable that the active iron is combined with the chlorophyll in a stoichiometrical relation* if it is combined with it at all.

Iron is known to catalyze oxidation processes in living substances. The formation of chlorophyll in the living plants is, most likely, associated with an oxidation process, since several investigators claimed that the rate of chlorophyll formation in seedlings was greatly impeded by low oxygen pressure in the air surrounding the plants (for example, CORRENS **4**). The active iron presumably does not form a part of the chlorophyll molecule; it is likely, therefore, that its function consists in catalyzing an oxidation process or some oxidation processes connected with chlorophyll formation.

EMERSON (5) succeeded in growing *Chlorella* in sugar solutions deficient in iron. The algae in such solutions were devoid of, or deficient in, chlorophyll; but their rate of respiration was substantially the same as that of normally green *Chlorella*. The fact that the chlorotic algae had a normal respiratory rate suggests that they were not subnormal in regard to the amount of "respiratory ferment" they contained. In spite of this they were decidedly subnormal in their chlorophyll content. It seems, therefore, improbable that the active iron is identical with the respiratory iron ferment of WARBURG (**15**).

The nature and the localization in the cell of the inactive iron are also uncertain. The inactive iron extracted with 1.0 N HCl may differ from the active iron only in regard to its localization in the cell, *i.e.*, it may be present only in the interior of plastids or other protoplasmic bodies. On the other hand, it is equally plausible that the inactive iron compound may differ from the active in its chemical composition; nor is it certain that the

[6] One mol of chlorophyll was taken as equal to 897.4 which represents an average value for chlorophyll $(a+b)$, on the assumption that the ratio of chlorophyll *a* to chlorophyll *b* is 3.

inactive iron in the acid extract is derived from one compound only. This last statement may be applied also to the active iron.

The writer wishes to emphasize that while it is common to find chlorotic leaves which contain as much or more iron than green leaves of the same age, the inability to utilize iron for a normal development of chlorophyll is confined to leaves with a low content of iron during at least the first part of the growing season. This statement does not necessarily apply, of course, to yellow leaves in which the development of chlorophyll is abnormal, due to other causes than those which are responsible for lime-induced chlorosis.

This fact would indicate that the occurrence of chlorosis is not entirely independent of the amount of iron in the leaves. Indeed, a comparison of the iron content of leaves in the chlorotic orchard C with that of green leaves from orchard S, in a region free of chlorosis (fig. 6), shows that the

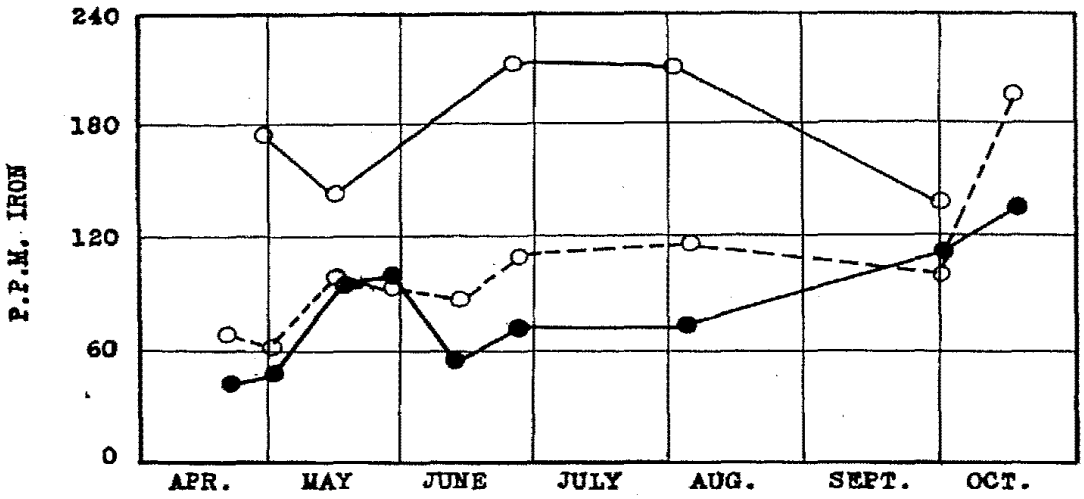

FIG. 6. Seasonal variation of iron content (on dry-weight basis) in Bartlett spur leaves of one-year-old wood: ——●——, chlorotic leaves, orchard C; – – –○– – –, green leaves, orchard C; ——○——, green leaves, orchard S.

iron in leaves from orchard S is on a higher level *throughout* the period of active growth. From experience the writer is inclined to conclude that the occurrence of chlorosis is highly improbable in pear leaves, the iron content of which remains above, say, 70–80 p.p.m. (on the dry-weight basis) during the first two or three months of their growth. An explanation for this fact is offered by the suggestion that a certain equilibrium exists between the active iron and the inactive iron in pear leaves. In leaves containing a small amount of iron, the balance between the two forms of iron may be shifted in such a way as to prevent the formation of an adequate amount of active iron for normal chlorophyll formation; while in leaves rich in iron, the active iron (although it may be only a small fraction of the total

iron) is present in sufficient amount for the normal development of chlorophyll.

Summary

1. Chlorotic pear leaves may contain as much or more iron than green leaves of the same age and taken from the same trees, regardless of whether the iron content is expressed on the fresh-weight or the dry-weight basis. The iron content, however, of green leaves from trees grown in districts free from chlorosis is higher than the iron content of either green or yellow leaves from chlorotic trees. Lime-induced chlorosis (dealt with in this paper) is confined to leaves in which the iron content is relatively low during the first two or three months of the growing season.

2. No correlation exists between the amount of iron extracted from pear leaves with water and with 0.5 N HCl and the chlorophyll content of leaves.

3. Only part of the iron in leaves, the *active iron,* is effective in chlorophyll formation.

4. A method is described for the estimation of the active iron in leaves, which is based on the assumption that the active iron, or its derivative, is contained in the 1.0 N HCl extract of dried leaves.

5. The chlorophyll content of leaves from chlorotic plants is proportional to the amount of active iron in the leaves.

6. The iron of the compound active in chlorophyll formation is present in the 1.0 N HCl extract as ionic iron or in a compound which readily yields ionic iron.

7. The values of the ratio $\frac{\text{chlorophyll}}{\text{active iron}}$ in leaves varies widely in different sets of leaves. It is inferred from this fact that the active iron is not present in leaves in a stoichiometrical combination with chlorophyll, if it is combined with it at all.

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