HILGARDIA

A Journal of Agricultural Science Published by the California Agricultural Experiment Station

VOLUME 9 JANUARY, 1935 NUMBER 2

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UNIVERSITY OF CALIFORNIA • BERKELEY, CALIFORNIA

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H. S. REED AND J. DUFRÉNOY

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H. S. REED[3] AND J. DUFRÉNOY[4]

INTRODUCTION

THE TERM "MOTTLE-LEAF" designates a functional disease of certain species and varieties of the genus *Citrus*. The most evident symptom of this disease is the absence of chlorophyll from certain areas between the veins of the leaf and, in more advanced stages, dwarfing of the leaves. The exact cause, or causes, of mottle-leaf have not been determined, but the evidence at hand supports the belief that the disease is independent of parasitic microörganisms. Climatic conditions often influence the occurrence and intensity of the disease, but it cannot be said that they have been shown to be the determining factor.

Soil conditions have been found to be more directly related to the occurrence of mottle-leaf. The application of nitrates (Vaile[(20)]) or of excessive amounts of urea (Haas[(7)]) may produce mottling of orange-tree foliage. The ratio of calcium to potassium also has certain pertinent relations to the disease (Kelley and Cummins,[(10)] Reed and Haas[(17)]), yet applications of calcium to the soil are by no means a corrective. Frequent applications of organic manures have been found to be one of the most satisfactory means in California for holding in check this disease, or for ameliorating the condition of trees badly affected with mottle-leaf.

The use of iron and zinc salts for the control of functional diseases of fruit trees has received much attention in California in recent years. Peach trees affected with little-leaf (Chandler, Hoagland, and Hibbard[(5)]) and orange trees affected with mottle-leaf (Johnston[(9)]) have shown striking benefits from applications of zinc sulfate singly or in combination with iron sulfate.

Mazé[(12)] pointed out a significant relation between zinc and sulfur metabolism in maize. He found that roots which were grown in a solution deficient in zinc were soon coated with an ocherous deposit. When traces

[1] Received for publication July 26, 1934.

[2] Paper No. 299, University of California Citrus Experiment Station and Graduate School of Tropical Agriculture, Riverside, California.

[3] Professor of Plant Physiology in the Citrus Experiment Station and Graduate School of Tropical Agriculture and Plant Physiologist in the Experiment Station.

[4] International Fellow, Rockefeller Foundation.

of zinc were present, so that the plants could survive for a while, although suffering from inadequate supply of zinc, sulfur compounds were formed as a response. The presence of sulfides in the ash of the roots of the plants which received suboptimum amounts of zinc indicates that in them the process of sulfur metabolism was impaired. When a maize plant was transferred to a zinc-free solution, it died within a few days after the first symptoms of zinc starvation, and therefore had no time to react by the formation of sulfur compounds.

From these results Mazé concluded that zinc has a specific effect: in low concentrations it acts as a "food" for the cell, and its physiological action seems to be linked with sulfur translocation. However, zinc, when present in soluble form, is definitely toxic even at a very high dilution; for example, zinc nitrate at a concentration of 25 parts per million inhibited the growth of maize roots and killed the plants within one month, although under these conditions the amount of zinc was not sufficient to meet the zinc requirements of the plants.

Mazé[12] assumes that in the presence of calcium carbonate, zinc is precipitated as an insoluble salt from which necessary quantities are dissolved out by root excretions and absorbed as they are needed by the plant without any undue accumulation.

The importance of small quantities of zinc for the healthy growth of citrus has been shown by Haas[7] (pp. 488 and 489): "Where the cultures of citrus were maintained for periods of several years the addition of zinc was necessary to maintain health." And: " . . . . no other heavy metal in small concentrations has thus far been found to bring about the same beneficial response from the root system over a period of months or years."

On the other hand, concentrations of zinc higher than 5 p.p.m. may inhibit growth of citrus seedlings, as Haas[7] has shown.

Bertrand and Benzon[2] and Bertrand and Andreitcheva[1] found small quantities of zinc in the juicy part of citrus fruits (tangerines and oranges), but found larger amounts in chlorophyll-bearing organs. Their findings in relation to green and etiolated leaves are of particular interest in relation to the study of mottle-leaf. They showed that the green leaves of various salad plants were richer in zinc than etiolated leaves. It appears, therefore, that there may be a relation between zinc and chlorophyll production. Zinc may be assumed to play a rôle in chlorophyll production, or at least in some process in which chlorophyll is involved.

MATERIAL AND METHODS

The present article will be concerned mainly with the effects of zinc and iron salts on the cellular physiology of orange leaves, with reference to their beneficial effects on the mottle-leaf disease. The Division of Orchard Management of the Citrus Experiment Station has been studying the effects of applications of iron and zinc to the soil and has also sprayed certain badly mottled trees with a solution of zinc sulfate. Both of the treatments mentioned were followed by disappearance of the symptoms of mottle-leaf. Through the kindness of that division we had the opportunity of collecting material for cytological study.

The material designed for cytological study was fixed in Nemec's or in Meves' solutions. When prepared for micro-incineration, it was fixed in a mixture of equal parts of formalin and 95 per cent alcohol to avoid the introduction of chromates. The most favorable staining results were usually obtained by the use of hot acid fuchsin in aniline water, counterstained with toluidine blue and aurantia.

CYTOLOGY OF LEAVES

From an early stage the cells of mottled leaves are different from those of healthy leaves. The dissimilarity is indicative of deep-seated changes in the physiology of the affected leaves. The palisade cells of affected leaves are broad and often divided transversely, with the resulting formation of rhomboidal rather than columnar cells. When one examines tangential sections of leaves he finds that these large hypoplastic cells occur in rather definite groups with smaller cells of normal size intervening (Reed and Dufrénoy[15]).

The nuclei of these hypoplastic cells are elongated (fig. 3*B*) and have no nucleoli. Their appearance suggests a transformation from a sol to a gel condition.

The thin film of cytoplasm along the cell wall usually can be seen only by the use of the highest magnifications.

The strongest evidence of profound alterations in the physiology of the mottle-leaf cells is shown by their contents. The palisade and adjoining layers of cells are (as might be expected from the leaf color) very deficient in chloroplasts. The palisade cells generally have a polarized appearance due to the aggregation of cytoplasm and plastids at one end of the cell. Both plastids and mitochondria are in close contact with numerous small vacuoles of cytoplasm which show a honeycomb structure typical of hypoplastic cells. The chloroplasts of mottle-leaf cells generally contain thin, elongated starch grains which only partially fill the cavities in the plasts in which they lie. Probably the rates of hydrolysis of starch in such cells and translocation of sugars are greater than the rate of formation. A similar condition has also been observed in the cells of leaves affected with virus disease or in malnutrition. The stromata of the plastids are often rich in fat globules.

STARCH ACCUMULATION AND PHLOEM NECROSIS

The remarkable difference in the translocation of starch in green and mottled orange leaves has been mentioned in a previous paper (Reed and Dufrénoy[15]). Plastids showing fatty degeneration at one end frequently contain thin starch grains toward the other end. The fact that mottled leaves retain some starch after being kept in darkness for several weeks points to inhibition of starch utilization and starch translocation. This unhydrolyzed starch may indicate that amylase has not been produced, or that some by-product of the reaction has retarded its activity. The latter possibility seems to be in harmony with the necrotic condition of the phloem in the leaf veins.

The contents of the vacuoles of hypoplastic cells are rich in the class of phenolic material which is stained yellow by potassium bichromate or blackened by osmic acid. The former precipitates the phenolic substances either in the form of floccular precipitates or in the form of spherical granules, according to the degree of dispersion which existed in the vacuolar solution. One of the most important indexes of impaired physiological function is to be found in the occurrence of spheres of phytosterol or lecithin in the vacuoles. They probably represent material which the cell was unable to burn or to transform.

The fact that the emulsion of lecithin in water is capable of holding in solution or suspension a variety of substances which are otherwise insoluble, as, for instance, sterol, may explain how sterol, which is itself insoluble in water and can only be contained in the vacuolar watery solution in the form of colloidal indiffusible emulsion, might concentrate in the spherical inclusions here discussed. There are a number of ways in which the sterol might be conceived to alter the level of the autocatalyst of growth; for example, by modifying the action of lecithin or by exerting an effect of its own upon nuclear synthesis.

The lecithin-sterol inclusions in mottled leaves may be linked with the premature storage of starch and calcium in the leaf primordia in contrast to the deficiency of the same elements in adult leaves on mottled trees.

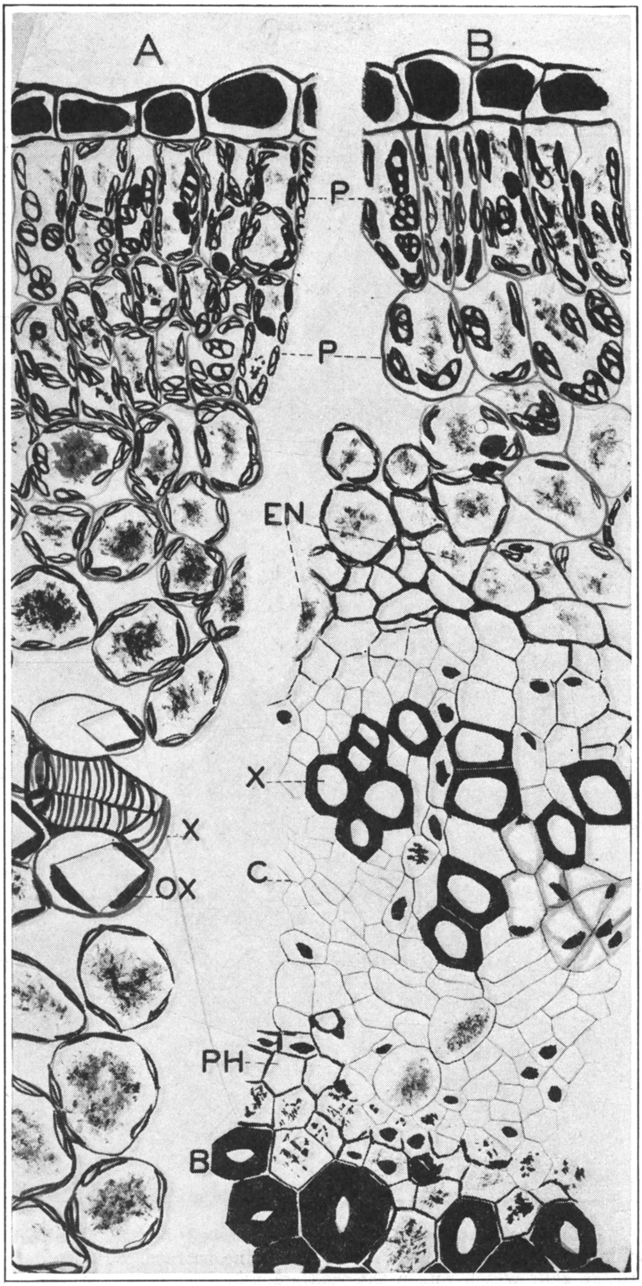


Fig. 1.—Sections of leaves from orange trees which showed beneficial effects of applications of 20 pounds of zinc sulfate per tree: *A* and *B* collected 15 and 17 months, respectively, after the applications were made. *P*, Palisade cells, many of which manifest the form which is characteristic of mottle-leaf palisade. However, they can have an abundance of healthy chloroplasts uniformly distributed in the cells. *B*, Bast fibers; *C*, cambium initials; *EN*, endodermis; *OX*, calcium oxalate; *PH*, phloem; *X*, xylem.

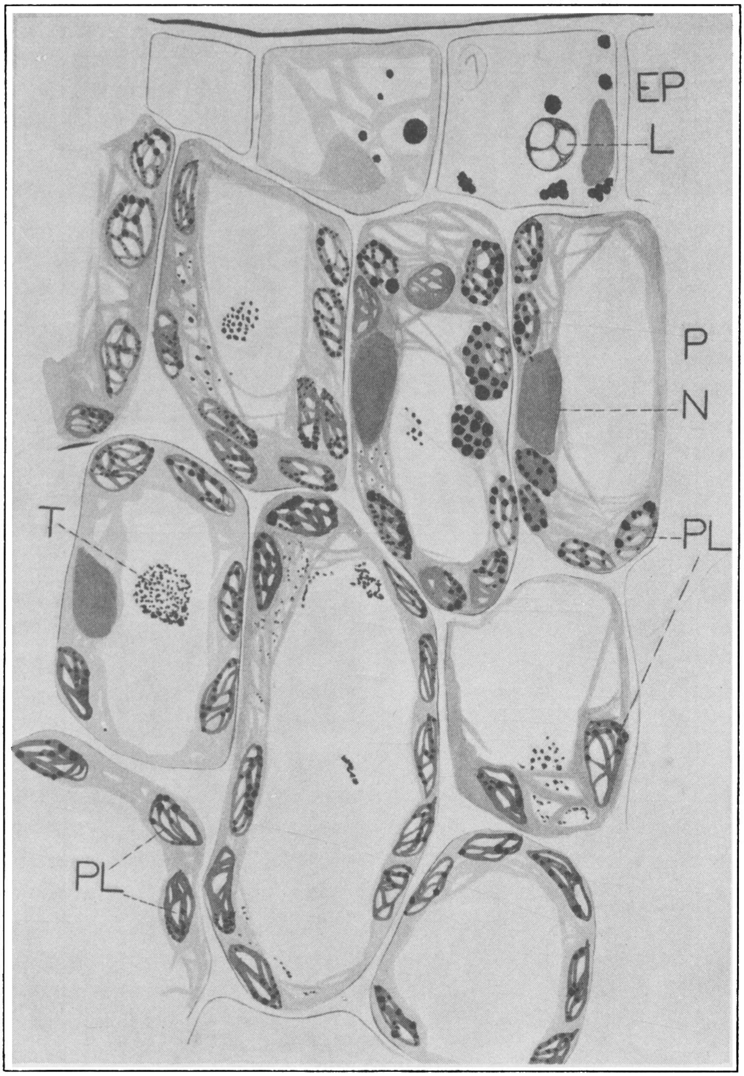


Fig. 2.—Section of orange leaf which showed beneficial effects of spraying with a 2.65 per cent solution of zinc sulfate. *EP*, Epidermis; *P*, palisade cells which retain the isodiametric dimensions characteristic of mottled leaves, although their contents show recovery from the hypoplastic condition; *T*, tannic substances; *PL*, plastids; *L*, lecithin.

THE EFFECTS OF ZINC ON THE CYTOLOGY OF LEAVES

Soil Treatments.—When one examines the cells of leaves from trees which were benefited by the application of zinc sulfate, one finds a different condition. The leaves show none of the irregular yellow areas and, naturally, the leaf cells contain healthy chloroplasts. The cell contents (fig. 1) are normally distributed in contrast to the characteristic clumping which prevails in the cells of mottled leaves.

The chloroplasts are regularly arranged in the peripheral regions of the cell, their stromata stain bright red with acid fuchsin, and they contain numerous starch grains. In the case of material killed with Meves' solution, tiny, dark oil drops show conspicuously in the bright-red background of the stromata (fig. 2).

Phenolic material, though present, is not abundant. The vacuolar inclusions composed of lecithin or phytosterol (which were present in the cells of mottled leaves) may be found in the cells of leaves from zinc-treated trees, but they are scarce.

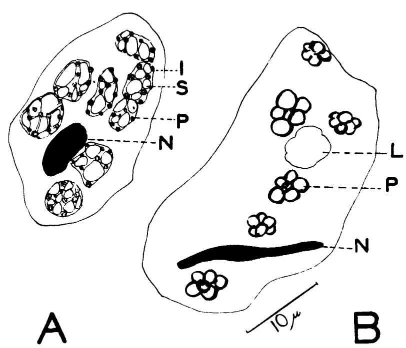


Fig. 3.—Cells from the mesophyll: *A*, normal condition in which chloroplasts, *P*, are regularly distributed, each of which contains numerous starch grains, *S*, and a few small oil droplets, *I*; *N*, nucleus. *B*, Hypoplastic condition from badly mottled leaf in which chloroplasts, *P*, contain many large oil droplets; *L*, lecithin; *N*, nucleus.

Many of the palisade cells of green leaves from zinc-treated trees are broad, resembling those of mottled leaves, and indicating that the tree may not have recovered fully (fig. 2). This condition is not surprising when the tremendous disorganization of cellular contents in badly mottled leaves is recalled. The succeeding cycles of leaves may show a closer approach to the normal organization of these complex cells. The appearance of leaf cells undergoing the changes incident to recovery are shown in figure 1. The trees from which the specimens were taken had received 20 pounds of zinc sulfate March 12, 1932. The drawings represent the cellular conditions 15 and 17 months later.

In mottled leaves, the chloroplasts contain many oil globules in the spring and early summer, as previously mentioned (Reed and Dufrénoy[16]). With the advance of the growing season, the number and size of the oil globules increase, eventually reaching a stage where the few small starch grains in the plastids may be masked by the surrounding large oil drops (fig. 3).

In contrast to that condition, the plastids of green leaves produced on trees in the Citrus Experiment Station grove which had been benefited by the previous application of zinc sulfate contained starch and a minimum of oil globules. Their stromata stained brightly, as did those in unaffected leaves, and showed normal starch grains, with very few small oil globules intervening.

Until now, we have made no specific study of the fibrovascular elements of the mottled or of the healthy orange leaf, although, as frequently noted, the tissue in the vicinity of the veins is the last to turn yellow when the leaf is affected with this disease. The cytological features of the fibrovascular bundles are described in the following paragraphs.

The xylem elements in affected leaves are interspersed with wood parenchyma, whose cells contain large vacuoles within which there is a large amount of phenolic material readily demonstrated with blue dyes or when the material is fixed with potassium bichromate or osmic acid (plates 1 and 2). The xylem elements in green leaves from orange trees which had been treated with zinc are more compactly grouped and may contain narrow medullary rays consisting of a row of cells whose cytoplasm is richly dotted with mitochondria, and whose vacuoles contain little phenolic material (plate 1). We have observed that these mitochondria in the xylem and phloem parenchyma frequently develop into plastids containing pigment, as shown in figure 5.

The phloem elements, even in healthy plants, often have a transitory existence, since they are superseded by, or crushed between, newly formed elements which differentiate from a cambial layer. A section of a vein from a green leaf from a tree which was treated with zinc shows that the phloem is mainly composed of living elements, each containing uniformly distributed mitochondria in the cytoplasm surrounding vacuoles wherein little, if any, phenolic material is demonstrable (plate 1).

The phloem elements in mottled leaves, on the contrary, show the following evidences of necrosis, even from an early stage: (1) overstaining of the nuclei and adjoining cytoplasm; (2) collapse of the cytoplasm accompanied by an aggregation of material at one end of the cell (polarization), presence of phenolic material in the vacuoles of many cells (plate 2) readily demonstrated when stained with blue dyes; (3) swelling of the pectic material in the middle lamella. The evidence of plasmolysis and of cytoplasmic disintegration may be seen in many cells, while those adjoining them retain a healthy appearance with abundant mitochondria in their cytoplasm.

The vascular bundles in petioles and leaves are surrounded by the pericycle layer (plates 1 and 2). In the vicinity of the xylem elements, the pericycle consists of a row of cells containing large vacuoles in which phenolic compounds are abundant. The contents of these vacuoles,

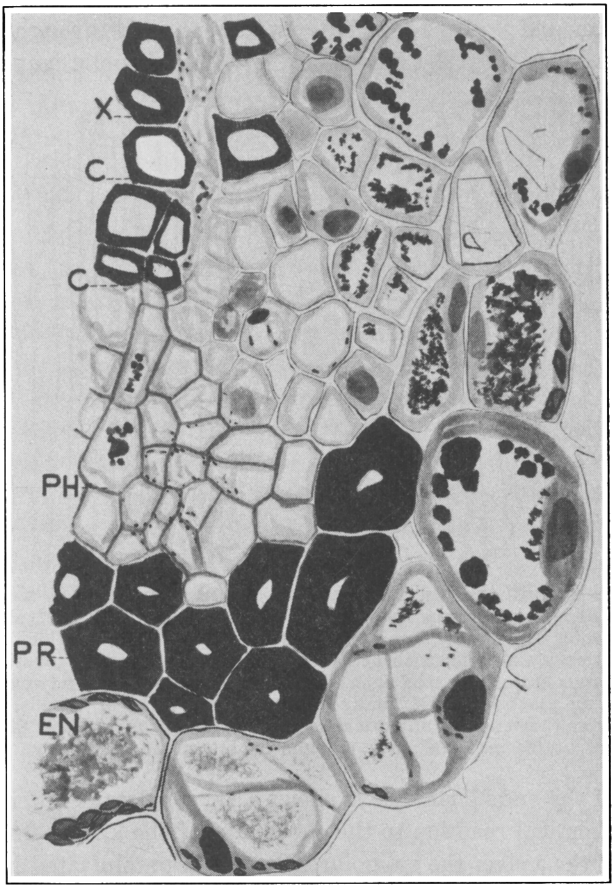


Fig. 4.—Section of a small vascular bundle of an orange leaf from a tree which had been treated 17 months earlier with zinc sulfate. Leaves had a healthy green color. *EN*, Endodermis; *PR*, pericycle; *X*, xylem; *PH*, phloem; *C*, cambium.

in green as well as in affected leaves, are readily precipitated when penetrated by vital dyes or by killing fluids containing chromates. The phenolic materials are precipitated in the form of small aggregates. The pericycle in contact with the phloem in green leaves forms a compact strand of fibers (plate 1). The pericycle fibers in mottled leaves show the same tendency to disperse which we have already noted in the xylem (plate 2).

The endodermal layer (plates 1 and 2) consists of cells which alternate with those of the pericycle, forming a sheath (the so-called "starch sheath") around the fibrovascular bundle. In the orange leaf we have found, however, relatively few amyloplasts in endodermal cells, and they are crowded closely to the outer wall of the cell through the expansion of the large central vacuole (fig. 4). The phenolic material in the

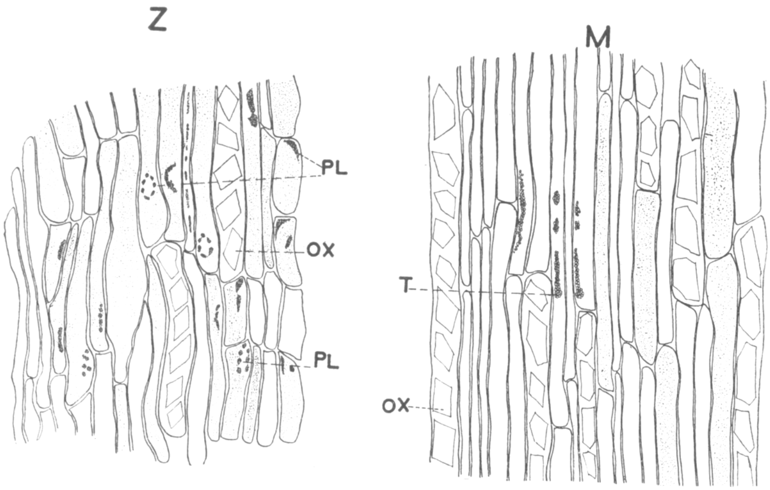


Fig. 5.—Longitudinal section through vascular bundles of zinc-treated (Z) and of mottled orange leaves (M). (Vital staining with neutral red.) In Z, each cell or vessel contains a vacuole (shown by shading) in which the vacuolar solution stains pink with neutral red. Green or yellowish plastids (PL) are numerous around the nucleus which, being unstained, is merely outlined by surrounding plastids. In M, many of the phloem cells, or vessels, evidence necrosis, for they contain no stainable vacuolar solution, but show (T) brown tannic flocculated material; OX, calcium oxalate crystals.

vacuoles of the endodermal cells shows a very constant difference in its physical-chemical reaction to the chromates of the killing fluids. In the vicinity of the xylem the vacuolar material is precipitated in the form of globules which stain densely with acid fuchsin. In the vicinity of the phloem the phenolic material seems to have more stability, or perhaps to form more stable complexes in the vacuolar solution; for, when precipitated, it appears as light, fluffy masses filling most of the space within the vacuole.

Calcium oxalate crystals (fig. 4 and plates 1 and 2) occupy the vacuolar cavities of many of the endodermal cells. A longitudinal section shows the abundance and distribution of the crystals (fig. 5). Penzig apparently saw and figured calcium oxalate in the endodermis (Penzig[13], Tav. IV, fig. 1) but made no comments on it. Rufz de Lavison[18] was one of the first investigators to suggest that the endodermis is the layer which controls the exchange of ions between the vascular system and the surrounding parenchymatous cells. The calcium oxalate crystals in the endodermal cells may result, therefore, from the meeting at that point of the incoming calcium ions with the oxalic acid synthesized in the plant cells.

Spray Treatments.—Additional material for the study of the effects of zinc was obtained from another series of trees which the Division of Orchard Management had sprayed 7 months previously with a solution containing 2.65 per cent commercial zinc sulfate. (The sample used contained 0.001 gram arsenic per pound of $ZnSO_4$, according to analyses kindly furnished by B. M. Laurance. In the concentrations used, the effect of the arsenic is negligible.) The spray was even more effective than the soil treatments previously mentioned. The foliage, which was badly mottled previous to the spray treatment, became green, and the trees, which had made but little growth for several years, produced many new, vigorous shoots. The small, depauperate leaves which were formerly yellow developed new plastids from the inactive mitochondria and showed a healthy green color, although there was no increase in their size. The palisade cells (fig. 2) showed cytological evidence of normal physiological functions. The palisade and adjoining layers of cells contained rather less phenolic material than similar cells from trees to which zinc had been applied through the roots. The older leaves showed a few instances in which the spray material killed cells in the epidermis and palisade layers. Immediately beneath these spots there was a layer of wound tissue. The underlying palisade cells showed evidence of great activity, although the broad shape of the cells was a witness to their former hypoplastic condition.

EFFECTS OF IRON ON THE CYTOLOGY OF LEAVES

Iron salts are known to be essential for green plants, and it is therefore pertinent to inquire whether the lack of green color in mottled orange leaves might be related to a lack of iron. The question has been raised frequently in the past few years on account of the prevalence of chlorosis, mottle-leaf, little-leaf, and other functional disorders of fruit trees. In some instances, there have been marked improvements in the condition of the trees after the application of commercial iron salts to the soil; in other instances no effects were found.

We have studied this question in leaves of orange and pomelo collected from healthy and mottled trees. Significant differences between green and affected leaves with respect to their iron content, however, could not be demonstrated. Sections of orange leaves, both severely affected and unaffected, were examined by the use of MacCallum's method. The sections were treated first with alcohol containing 3 per cent nitric acid to "unmask" organic iron. After careful washing with neutral alcohol, the slides were placed in the 0.5 per cent hematoxylin for 6 to 18 hours. Then the sections were washed with a mixture of alcohol and ether to remove the uncombined yellow-brown hematoxylin. The inorganic iron forms with hematoxylin, a blue-black substance which can be identified with the microscope. Iron was evident in the remains of plastids and nuclei of affected as well as of healthy leaf tissue. The analysis of the ash of healthy and of mottled orange leaves[10] likewise showed no significant differences in their iron content.

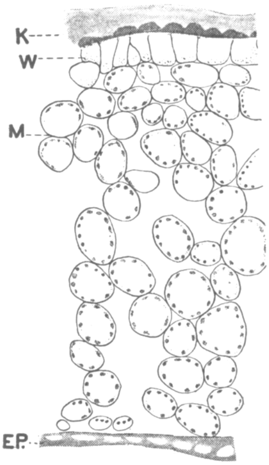


Fig. 6.—Transverse section of orange leaf which had been sprayed with a 2 per cent solution of iron sulfate. *K*, Remains of epidermal and palisade tissues; *W*, wound phellogen; *M*, mesophyll; *EP*, epidermis. (Drawn from section of living leaf, mounted in a 10 per cent cane sugar solution.)

Some material of unusual interest was obtained from an orange tree which had definitely responded to iron sulfate applied as a spray to the foliage. The trees had been heavily sprayed in January with a 2 per cent solution of chemically pure iron sulfate. Many leaves suffered scorch in the vicinity of the midrib where the residual salt was deposited after evaporation of the solution. The leaves showed, however, marked beneficial results from the application of the solution. They formed wound phellogen beneath the necrotic areas and developed a normal green color where previously they had been mottled. The underlying cells subsequently grew and produced a peculiar warping of the leaf. Figure 6 shows the condition of the cells in one of these leaves.

The overlying epidermis and palisade cells had been killed by the iron sulfate, leaving a layer of purplish-brown material beneath which there was a layer of wound tissue (*W*). The mesophyll cells (*M*) showed striking evidence of growth, with a resulting increase both in numbers and in size. The microscopical features thus afforded further evidence of the favorable effect of iron salts on growth. When sections were stained by Mawas' method, it was possible to demonstrate the presence of iron in the nuclei of a large number of cells, especially in those of the wound phellogen. The presence of tannic material in the necrotic layer (*K*) was revealed by its reaction with the iron.

We have obtained rather more precise information about the distribution of iron in the leaf by means of the micro-incineration technic. After having been incinerated (as described in the following section), the slides bearing the mineralized sections were flooded with a 0.5 per cent solution of hematoxylin, washed with water, dehydrated with alcohol, and mounted in balsam. One could see that most of the iron in the palisade cells had been present in the plastids. Confirmatory results were obtained when incinerated sections were treated with a freshly prepared solution containing 0.75 per cent potassium ferrocyanide and 0.25 per cent HCl.

CYTOLOGICAL ANALYSIS BY MEANS OF MICRO-INCINERATION AND MICROCHEMISTRY

Extremely interesting results have been obtained by a method of incineration adapted to the problem under investigation. Policard[14] suggested micro-incineration more than ten years ago as a means for accurate localization of the mineral constituents of the tissues. The success of the method lies in the fact that the incinerated sections (spodograms) preserve in an extraordinary way the topography of the tissues and also of the cells.

We used a mixture of equal parts of 95 per cent alcohol and formalin for killing fluid in order to avoid the introduction of metallic ions which might obscure the true mineral constitution of the tissues. The material was sliced during immersion in the killing fluid to insure good penetration and to avoid premortal translocation of the minerals in the cells. The killed tissue was dehydrated in alcohol, cleared in n-butyl alcohol, embedded in paraffin, and sectioned 6 or 8 microns thick. The paraffin ribbons were fixed to microscope slides with albumen, as for staining with dyes.

The sections were incinerated in an electric furnace where the temperature was gradually raised to 500° C within 3 hours and maintained below 600° C for 2 or 3 hours longer. The furnace was not opened after switching off the current until the slides were cool. Atmospheric dust, as Policard[14] pointed out, is a source of error. We kept the slides under glass covers as much as possible, even in the electric furnace during incineration.

The incinerated sections preserved histological and cytological details of the tissues and adhered to the glass so well that they could be mounted in balsam or treated with various reagents without destroying the patterns. We found it better, in most cases, to study the spodograms in a dry condition without balsam. The area was outlined with four strokes of a wax pencil; this made a ridge thick enough to support the cover glass and prevent it from coming in contact with the ash. The cover glass was sealed with a marginal seal of hot paraffin.

Since the ash adhered to the slides so well, it was possible to identify zinc and iron and to determine their distribution in the tissues. Comparisons were made with control sections stained by the usual methods.

We have had the best success in identifying zinc *in situ* in the tissues by the use of the Bradley[3] test developed for the identification of zinc in shellfish. It ultilizes sodium nitroprusside in concentrated solution

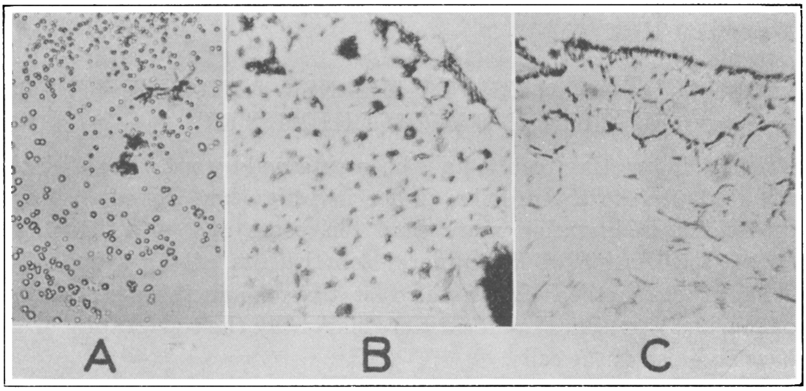


Fig. 7.—*A*, Photomicrograph of zinc nitroprusside formed by adding a drop of concentrated sodium nitroprusside solution to a drop of 1:100,000 solution of zinc chloride. *B*, Photomicrograph of ash of a section of a leaf from a tree to which zinc sulfate had been applied 20 months previously. *C*, Photomicrograph of the ash of a section of a leaf from a tree which had received no zinc sulfate.

which forms, with zinc, $Zn \cdot NO \cdot Fe(CN)_5 \cdot H_2O$, zinc nitroprusside, a salt of low solubility which is precipitated *in situ* on the slide as faintly brownish grains or disks catenulated into botryoidal masses. On standing, these grains may unite to form imperfect cubes, octohedrons, or dodecahedrons, as shown in figure 7*A*, which is a control photomicrograph of a precipitate formed by adding a drop of concentrated sodium nitroprusside solution to a drop of 1:100,000 solution of zinc chloride on a slide, decanting the excess liquid, and focusing on the crystals which had been formed (Chamot and Mason[4]). The crystals of zinc nitroprusside are isotropic. A photomicrograph of crystals obtained by treating the ash of an orange leaf from a tree to which zinc sulfate had been applied 20 months previously shows similar botryoidal masses (fig. 7*B*). A photomicrograph of the ash of a section of a leaf from a tree which had not received zinc sulfate yielded no crystals; the ash merely outlined the pattern of the cell wall (fig. 7*C*).

Manganese forms a nitroprusside which is indistinguishable from that of zinc, and we recognize that some of the crystals found were not those of zinc. However, the intimate relation between the application of zinc to the soil and the presence of nitroprusside crystals in the ash of incinerated leaf sections makes it improbable that any appreciable amount of the crystalline material observed was manganese nitroprusside.

The presence of zinc in the leaves of treated orange trees has, moreover, been definitely established by analyses of their ash by its highly specific reaction with potassium mercuric thiocyanate. Four samples

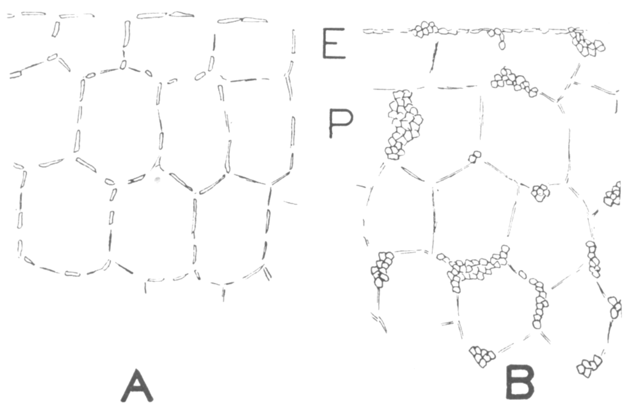


Fig. 8.—Incinerated sections of palisade layers of orange leaves: *A*, from tree which had not received zinc sulfate, shows no crystals of zinc nitroprusside; *B*, from tree having benefited from a soil application of zinc sulfate 20 months previously, shows abundance of crystals of zinc nitroprusside around palisade cells. *E*, Epidermis; *P*, palisade cells.

of orange leaves were collected, dried, and incinerated in crucibles. The samples were:

1. Control: leaves from mottle-leaf trees to which no zinc had been applied.

2. Healthy green leaves which had developed on trees subsequent to the application of zinc sulfate as a spray.

3. Old leaves from same tree as No. 2, marked with small necrotic spots resulting from the zinc sulfate spray applied 7 months previously.

4. Mature leaves from trees sprayed 10 days previously with zinc sulfate plus lime and still carrying the spray residue.

The samples of ash were extracted with dilute sulfuric acid, washed, and filtered. The clear filtrates were then examined for zinc by a modification of the method given by Hammond.[8] A drop of 0.1 per cent $CuSO_4$ solution and a few drops of a dilute solution of freshly prepared potassium mercuric thiocyanate were added to the filtrate. The solution was boiled and cooled.

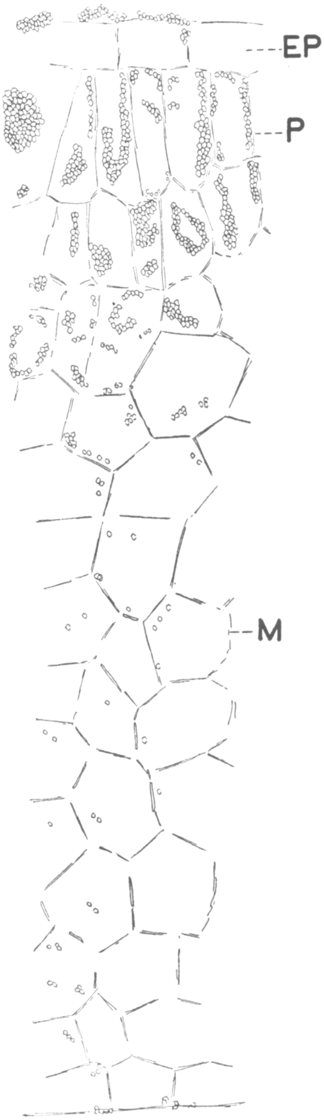


Fig. 9.—Ash of a transverse section of a dwarfed orange leaf which turned green after having been sprayed with zinc sulfate. The treatment with sodium nitroprusside revealed the presence of zinc as crystals of zinc nitroprusside almost exclusively in the palisade cells. *EP*, Epidermis; *P*, palisade cells; *M*, mesophyll.

By this method, violet crystals indicate the presence of zinc. If the amount was small, the violet crystals were not seen readily; therefore a drop of the sediment in the bottom of the test-tube was removed with a pipette and examined under the microscope. The crystals could then be seen readily. The following results were obtained from the samples analyzed:

1. Control: no violet crystals
2. Violet crystals present, but not numerous
3. Violet crystals numerous
4. Violet crystals present in abundance

This is in harmony with the results of the histo-chemical analyses with sodium nitroprusside and confirms the conclusion that the crystals obtained were formed principally by zinc.

The beneficial effects to citrus of an application of zinc sulfate to the soil are therefore concomitant with an absorption of zinc whose distribution in the leaf cells is evident after incineration.

Figure 8 shows camera-lucida drawings of two incinerated sections of orange leaves. Both drawings show the broad, isodiametric palisade cells characteristic of mottled orange leaves. No crystals of zinc nitroprusside occur in 8*A*, the control section, but they are evident in *B*, the ash of the leaf from a tree which received an application of zinc sulfate to the soil 20 months previously. The crystals occur in this material chiefly at the periphery of the palisade cells.

Especially illuminating results were obtained from the study of material from trees which the Division of Orchard Management had sprayed 6 months previously with a 2.65 per cent solution of zinc sulfate and which had had an extremely beneficial effect in ameliorating mottle-leaf and promoting growth. Figure 9 shows how the zinc was distributed in the cross section of a leaf. Crystals were found in large numbers in the palisade and intermediate cells, but were scarce in the mesophyll and epidermis cells. It should be stated that the leaves under dis-

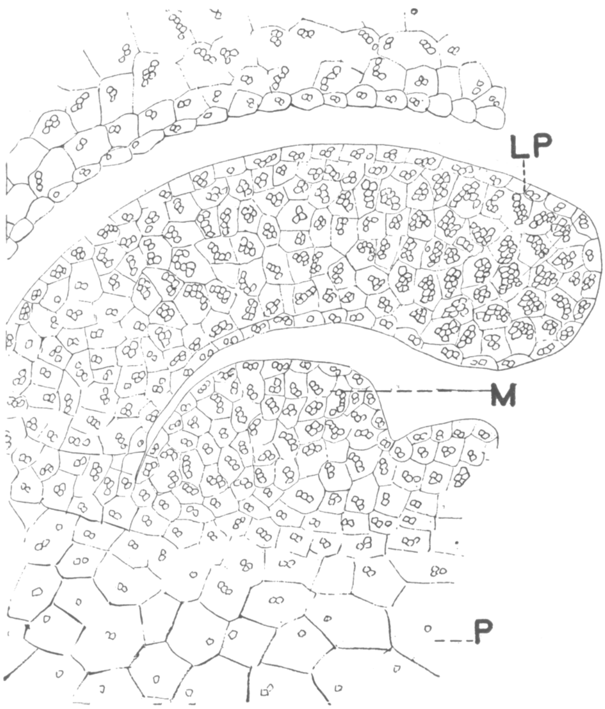


Fig. 10.—Incinerated section of an orange bud from a shoot developed since the tree was sprayed with zinc sulfate. The drawing shows the accumulation of zinc in the leaf primordium and apical bud. *LP,* Leaf primordium; *M,* meristem of bud; *P,* parenchyma.

cussion were formed after the trees had been sprayed. The distribution of the zinc was due, therefore, to physiological processes and not to the deposition of spray material on the foliage. The crystals of zinc nitroprusside were more numerous in the lumina of the cells than in the case previously noted. Mention has already been made of the close connection between chlorophyll-bearing cells and zinc, which has been clearly demonstrated by Bertrand and Andreitcheva.(1)

There is a striking accumulation of zinc in the meristematic tissues of buds of trees to which zinc sulfate was applied, either as a solution on the foliage or to the soil in which the trees grew. Figure 10 shows part of an incinerated section of a bud from a shoot which developed after the tree had been sprayed with zinc sulfate solution. The incinerated section had been treated with a solution of sodium nitroprusside, as previously described. The crystals of zinc nitroprusside were most numerous in the apical portion of leaf primordia and in the cone of meristem, in contrast to their numbers in the more mature parenchyma cells. The walls of the embryonic cells do not seem to be highly mineralized, and as a consequence were not conspicuous in the spodograms.

Buds collected from shoots of comparable age on unsprayed trees in adjacent rows, incinerated and treated in similar manner, showed no zinc nitroprusside crystals.

DISTRIBUTION OF CALCIUM

The tissues of citrus leaves contain numerous crystals of calcium oxalate, which are generally large in comparison with size of the cells. It is well known that calcium oxalate crystals generally form in vacuoles which are very rich in material that stains deeply with basic dyes. The evidence thus far obtained indicates that this material is composed largely of pentosans. After incineration, these calcium oxalate crystals leave residues which are very conspicuous. A word about calcium seems appropriate at this point, though the significance of the element cannot be fully discussed.

The localization of calcium oxalate can be studied either on freehand sections of living material (fig. 5) or in the ash of micro-incinerated sections of material killed with a mixture of alcohol and formalin.

Calcium oxalate crystals in fresh material or crystals of calcium oxide resulting from their micro-incineration are brightly illuminated by oblique illumination on the dark field. Figure 11, drawn with the help of a $\frac{1}{7}$-inch oil-immersion objective fitted with the diaphragm for dark-field illumination, shows the comparative aspect of ash from the transverse sections of vascular tissues and adjoining parenchyma. In figure 11*M*, from a mottled leaf, there is little calcium in the palisade tissues, as contrasted with the abundance of large crystals in the subepidermal cells of figure 11*Z*, from the leaf of a tree which recovered from its mottled condition after the application of zinc to the soil.

The endodermis around the vascular bundles of the treated leaf is made evident by the abundance of calcium in almost all cells. The endodermis of the mottled leaf can barely be made out, except in the vicinity of the bast fibers, where a few crystals of calcium oxide are seen. The walls of the xylem and of the bast fibers, and even those of the cambium and phloem, are more heavily mineralized in the treated leaf (fig. 11*Z*), where the histological pattern is well preserved, than in the mottled leaf, where it is barely discernible. The long palisade cells of the treated leaf also contain more ash constituents than the broad and short palisade cells of the mottled leaf.

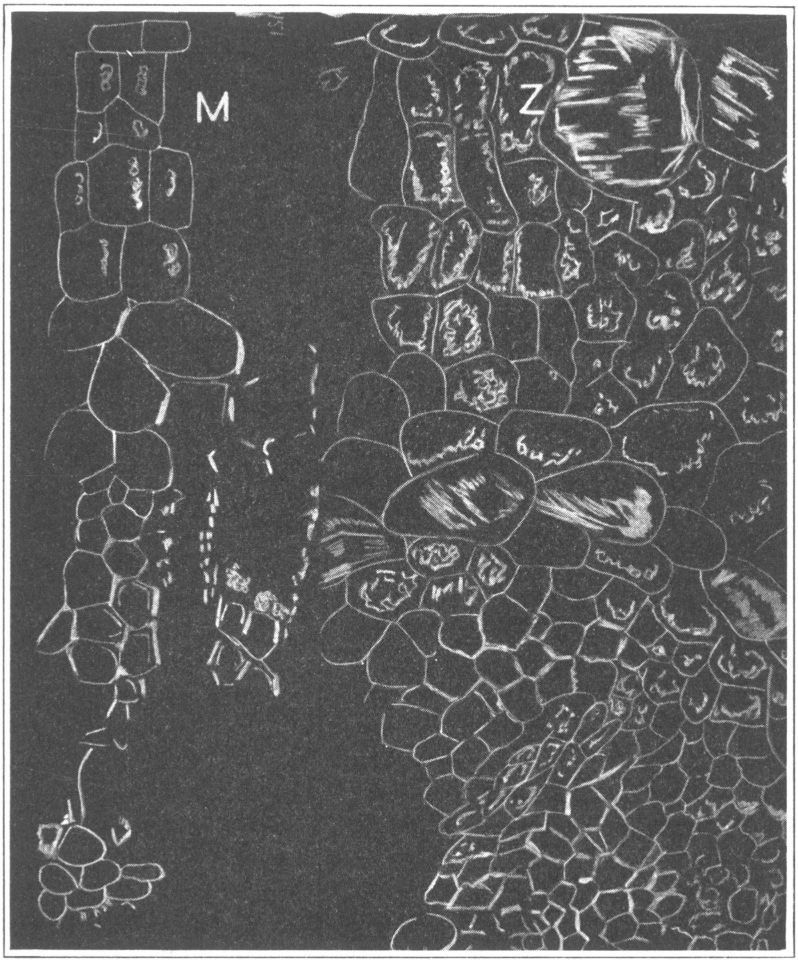


Fig. 11.—Incinerated sections of mottled (*M*) and zinc-treated (*Z*) orange leaves shown by dark-field illumination. Explanation in text.

The incinerated sections of buds also show conspicuous deposits of calcium in cell walls before any solutions are employed for microchemical tests. The walls of the embryonic cells leave a difficultly detectable residue, while those of the older postembryonic cells are clearly outlined by their mineralized residues.

It is known that meristematic cell walls consist of complexes of cellulose, pectin, and fatty acids. Their middle lamellae consist of a protein-pectin complex (Tupper-Carey and Priestley[19]). In the adult parenchyma, the composition of the cell wall is similar to that of the meristem, but the middle lamellae are composed of calcium pectate (Mangin[11]) and calcium soap. The process of micro-incineration clearly reveals the distribution of calcium salts in the tissues. The first evidence of calcium was detected in the primordia of leaves enfolding the apical bud. Calcium oxalate crystals were found there in great abundance in buds from mottled shoots, but were far less abundant in buds on shoots from zinc-treated trees. This apparent contradiction of an excess of calcium in buds of leaf primordia of mottled citrus and deficiency of calcium in adult leaves deserves further consideration. It will be recalled (Reed and Dufrénoy[16]) that starch is also accumulated early in leaf primordia of mottled citrus, while starch synthesis is inhibited in adult leaves. Further investigations will be needed to show the relations of zinc and calcium to organic acids in cellular metabolism.

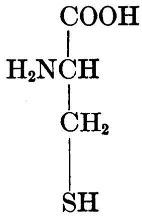
RELATION OF ZINC AND IRON TO CELL METABOLISM IN MOTTLED LEAVES

Cytological studies of citrus trees affected with mottle-leaf have led us to believe that the pathological symptoms are correlated with an accumulation of suboxidized metabolic substances which result from a low oxidation-reduction potential. The beneficial effects of zinc or iron salts upon mottled citrus leaves are so striking that it seems appropriate to discuss their relation to the sulfhydryl compounds in the cell, and to consider the system whereby the oxidation-reduction potential is determined only by the reduced form.

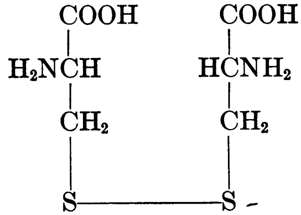
If the cell is to perform its life processes, it must maintain energy at a given level by oxidation; this level is defined by the oxidation-reduction potential. The ability of the cell to perform the oxidations through which energy is obtained for the maintenance of living processes is largely determined by the oxidation-reduction potential.

It seems evident, from the work of Hopkins and others, that certain sulfhydryl compounds, such as cystein, are present in all living cells and that they may control processes of oxidation and reduction. It is also known that salts of certain metals catalyze the partial oxidation of cystein to cystin. The oxidation of cystein to cystin seems to be a far more involved process than it was assumed to be when it was represented as the union of two molecules of cystein into one molecule of cystin with the concomitant removal of hydrogen:

Cystein



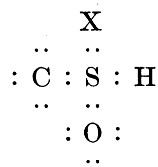
Cystin



The process probably cannot be adequately represented by:

$$2\ RSH \rightleftharpoons RSSR + HH$$

Since sulfur has two unoccupied electron pairs, it can be assumed that an atom of oxygen can be joined to the S atom, with resulting formation of a sulfoxide:



To obtain any appreciable amount of oxidation, it may be necessary for a molecule of sulfoxide to unite with one of sulfhydryl through the formation of complexes with metals which readily change their valences, thereby serving for H and O transfers.

Two or more molecules of amino acids may be linked with one of a metal such as Fe, Cu, Mn, or Zn. The catalytic effect of iron on the rate of oxidation of amino acid has been studied. It was found that there was a proportionality between the rate of oxidation and the percentage of iron present (above a certain minimum). The auto-oxidation activity of the amino acid seems to be related to this proportionality.

The demonstration of the specific effect of zinc in stabilizing the nitroprusside color reaction of glutathione by Giroud and Bulliard[6] confirms our belief that zinc had much to do with the activity of sulfhydryl compounds in regulating the oxidation-reduction potential within the cells of citrus.

This idea seems to be supported by the following observations:

1. The large accumulation of zinc in the meristematic cells of buds and in the palisade cells of leaves.

2. The resumption of activity in the cells of leaves after the trees had been sprayed with zinc sulfate solution, evinced by the normal nuclei, the fibrillar cytoplasm, and the development of normal chloroplasts.

3. The accelerated growth of new shoots on trees subsequent to the application of zinc and the accumulation of zinc in the tissues.

The effects of zinc applications suggest that some reaction has been initiated by which the proteins and carbohydrates of the cells have been utilized to supply energy to the cells, and we have evidence that this is a process of oxidation in which the sulfhydryl compounds play a controlling rôle.

The sterides characteristic of cells of mottled citrus leaves were described in a previous paper (Reed and Dufrénoy[16]). The name "sterinoplast," which had been coined by Guilliermond, was applied to the highly refringent spheres of phytosterol material, or lecithin, found in the epidermal or adjoining palisade layers of leaf cells. The size of the sterinoplasts made it evident that the fatty material in them was not emulsified; and their absence in cells of unmottled leaves lent support to the idea that they were suboxidized products of the metabolism of proteins and carbohydrates. The sterides were comparatively scarce in leaves to which zinc was applied. Apparently, the stabilizing of the sulfhydryl compounds promoted the oxidation of cell metabolites and thereby liberated energy for vital processes.

SUMMARY

The results of these investigations afford evidence that mottle-leaf of citrus is characterized by a shift in the oxidation-reduction equilibrium of the leaf cells. This relation is demonstrated by the results of certain chemical analyses such as those which show that nitrites exist in the expressed sap of mottled leaves, but not in that of green leaves. Indications of a reducing action in the palisade cells of mottled leaves are also shown by their power to reduce methylene blue and Nile blue A.

It is shown in the present investigation that profound changes in the cytological conditions are associated with the recovery of mottled trees after the application of zinc, either through the soil or in the form of a spray on the foliage. In the green leaves of new shoots whose growth had been promoted by zinc applications, neither calcium deficiency nor phloem necrosis is evident, while chloroplastids develop to fair size and form starch. The beneficial effects are especially striking when old, depauperate leaves are sprayed with a solution of zinc sulfate. Although their histological organization is not changed, there is evidence of marked cytological restoration.

The beneficial effects of iron salts on hypoplastic cells of mottled leaves are negligible. Cytological investigations showed that iron can be detected even in the degenerated plastids of hypoplastic cells.

The distribution of iron and zinc in the plant tissues was determined

by a combination of micro-incineration and micro-analysis. It was found that the ashes preserve the histological and cytological features of the tissues sufficiently to afford definite information concerning the distribution of these elements in leaves and buds.

The fact that zinc accumulates in buds which are to produce green tissues and in the palisade cells of green leaves indicates that it is intimately concerned with the oxidation-reduction potential of the cell.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to J. Enderud, who assisted in preparing stained material for the experiments discussed in this paper and in making the illustrations. Acknowledgment is also due Dr. K. V. Thimann, of the California Institute of Technology, who brought to the writers' attention Hammond's method of determining zinc by the use of potassium mercuric thiocyanate.

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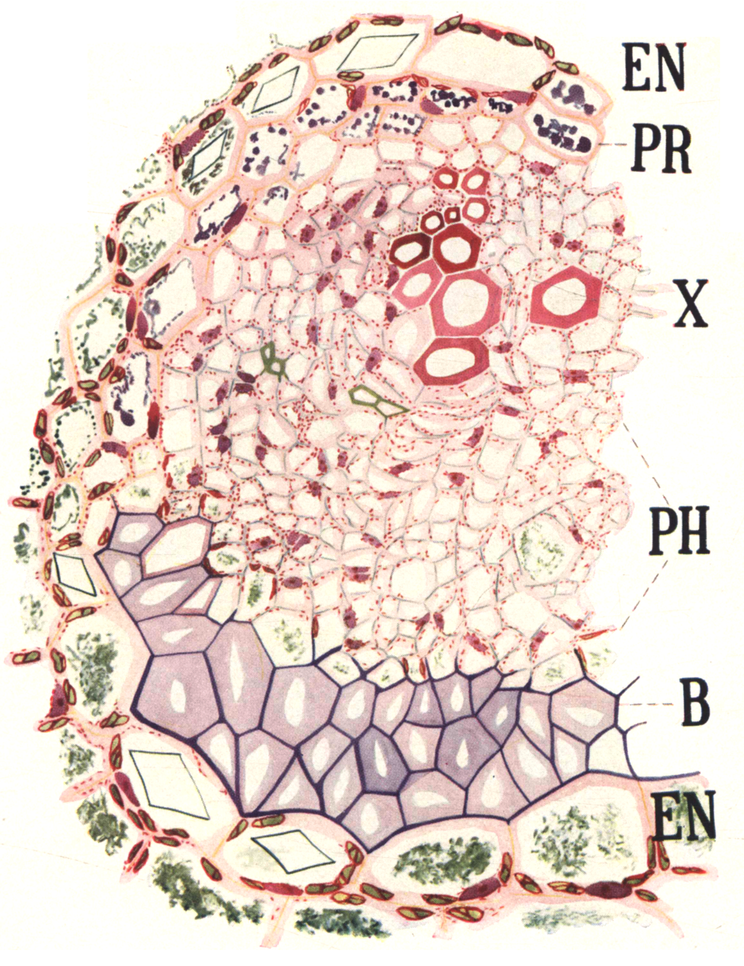
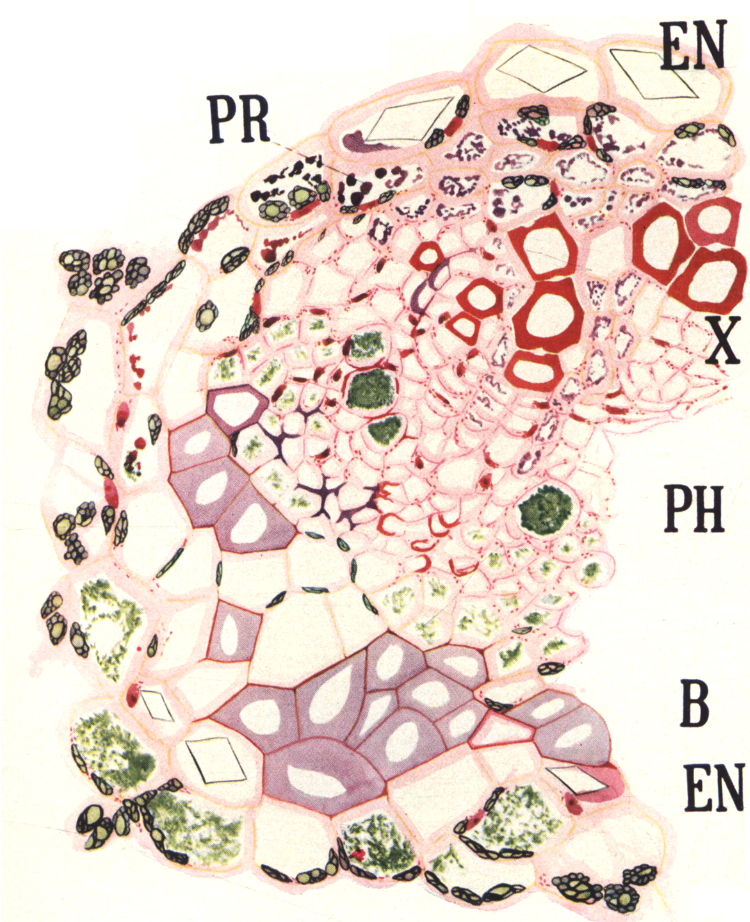


Section of a vascular bundle in a healthy orange leaf. *EN*, Endodermis; *PR*, pericycle; *X*, xylem; *PH*, phloem; *B*, bast.



Section of a vascular bundle in a mottled orange leaf for comparison with plate 1. *EN*, Endodermis; *PR*, pericycle; *X*, xylem; *PH*, phloem; *B*, bast.