The occurrence of glutathione in the foliage of horticultural plants, and its relation to the use of sulfur as a fungicide

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OCURRENCE OF GLUTATHIONE IN THE FOLIAGE
OF AGRICULTURAL PLANTS & ITS RELATION
TO THE USE OF SULPHUR AS A FUNGICIDE

LOVEJOY 1934
THE OCCURRENCE OF GLUTATHIONE IN THE FOLIAGE
OF HORTICULTURAL PLANTS AND ITS RELATION
TO THE USE OF SULFUR AS A FUNGICIDE

Richard Perry Lovejoy

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INTRODUCTION

Sulfur, either in the elemental form or as one of its easily decomposable compounds, is widely used as a toxic spray or dust for the control of fungous diseases of orchard and garden crops.

The exact nature of the fungicidal action of sulfur has been the object of much study and considerable controversy. However, Marsh and McCallan and Wilcoxson have independently studied the matter very carefully and presented conclusive evidence that the actual toxic effects of sulfur to fungi are exerted by hydrogen sulfide produced from elemental sulfur, or its compounds, by the reducing action of the spores of the fungi or of the leaves of the plants.

McCallan and Wilcoxson showed that the leaves of every one of the 26 species of higher plants which they tested, the spores of 16 species of fungi, and the sporophores of 8 species of mushrooms all evolved hydrogen sulfide when in contact with sulfur. They also made quantitative determinations of the hydrogen sulfide produced under certain conditions from definite areas of leaf surface of strawberry leaves and by the spores of 8 different fungi.

Following the work of Marsh, Barker tested the leaves of 61 species of higher plants for their ability to form
hydrogen sulfide when dusted with sulfur. He found that 15 of these gave positive results, while the remainder gave either inconclusive or negative results by his method of testing. He also tested 24 species of lower plants, including fungi, algae, and mosses, with positive results in 19 cases, while 5 species of fungi gave a negative test. However, he took his samples of leaves, fruits, vegetables, etc., in September and admits that at this season of the year the foliage is probably not representative of its condition during the early growing season when sulfur fungicides are used. In a footnote, Barker suggested that the active agent in this sulfur-reduction reaction in plants is glutathione.

Sluiter reported detailed studies of the production of hydrogen sulfide from mixtures of known concentrations of sulfur and glutathione obtained from various animal tissues.

Guthrie and Wilcoxson demonstrated the production of hydrogen sulfide from sulfur by the action of cysteine, glutathione, and extracts of potato tubers, gladiolus corms, lilac buds, yeast, and calf's liver (the latter two being the best known sources for glutathione).

In the meantime, Streeter had observed that injury to orchard foliage from summer spraying with lime-sulfur is directly proportional to humidity and temperature conditions in the orchard. These relationships are so similar to those
found by Sluiter and by McCallan and Wilcoxson to govern the reaction between glutathione and sulfur, as to suggest this explanation for the observed injury.

These facts, together with certain personal contacts of Dr. Roscoe W. Thatcher with some of the above mentioned investigations, indicated to him the desirability of a more detailed study of the occurrence of glutathione in horticultural plants and its possible relation to the use of sulfur sprays and dusts as a fungicide. At his suggestion, the writer decided to investigate this subject.
Occurrence and Properties of Glutathione

Pollacci, in 1962, appears to have been the first to observe the fact that many kinds of living tissue reduce sulfur to hydrogen sulfide at ordinary temperatures. He reported that grape leaves treated with sulfur evolved hydrogen sulfide, which he recognized by its odor and the blackening of lead acetate paper.

Selmi, as well as Cugini, likewise observed the formation of hydrogen sulfide when sulfur is applied to certain fungi.

In 1888, de Rey-Pailhade stated that an alcoholic extract of yeast, on being mixed with sulfur, rapidly evolved hydrogen sulfide at ordinary temperatures. He showed that the substance producing the hydrogen sulfide exists in the living cell, and noted that the action is most rapid at a temperature of from 25° to 40° C, and is inhibited by previously heating the solution to 70° C. This property was not confined to yeast cells, but the same reaction was obtained from a variety of animal and vegetable tissues. De Rey-Pailhade considered this reaction to be brought about by a specific enzyme, which he thought was a "hydride of protein", and
therefore assigned to it the name of "philothion". Throughout a long series of communications he maintained the view that the labile hydrogen thus shown to exist in living cells has important respiratory functions.

A review by Pozzi-Escot of the subject of reducing enzymes presents the status of philothion in 1902. This author reports that philothion readily undergoes oxidation, which destroys its power to reduce sulfur. He also observed that philothion was capable of reducing selenium, tellurium, arsenic, and phosphorus, forming the hydrides of these elements.

There is a reaction characteristic of compounds containing the -SH group, namely, the production of a violet color on treatment with sodium nitroprusside and ammonia. Several investigations had shown that a substance giving this test was widely distributed in plant and animal tissue. Neffter in 1909, however, first gave definite form to the view that the presence of the -SH group, whatever its associations, owing to its labile hydrogen, may be, in part at least, responsible for the reducing properties of protoplasm, and also, perhaps indirectly, for oxidations in the cell.

Based upon known analogies, he made the suggestion that during the autoxidation of the sulfhydryl groups peroxide of hydrogen may be formed and the peroxide oxygen then transferred, with or without the influence of a peroxidase, to other substances in the cell. For then, if -SH groups can be supposed to act continuously as promoters of cell oxidations, their own spon-
taneous oxidation to -S-S- groups must be reversible. In this connection, Heffter called attention to the fact that cystine can be reduced to cysteine by sodium sulfite, and suggested that in the cell some substance might, like the sulfite, act as an "acceptor" for the oxygen of the water molecule, leaving the hydrogen of this to reduce the disulfide group once more to sulfhydryl groups.

If an attempt to determine the chemical nature and physiological significance of the cell constituents and to see if he could experimentally support Heffter's theoretical views, Hopkins made an exhaustive fractionation of tissue extracts. He succeeded in isolating, from yeast, muscle and mammalian liver, a substance called "glutathione". This substance gave the nitroprusside reaction and therefore contained the -SH group. It seemed to be a dipeptide of cysteine and glutamic acid and exhibited the properties of de Rey-Pailhade's "philothion". Besides containing the -SH group, it is readily oxidized under suitable conditions by atmospheric oxygen, and exhibits the property of reducing sulfur to hydrogen sulfide at ordinary temperatures.

Subsequently Stewart and Tunnicliffe synthesized the dipeptide, cysteine-glutamic acid, which was thought to be identical in constitution and properties with the reduced form of glutathione. When this was oxidized to cystine-glutamic acid it appeared to be identical with the oxidized form of glutathione. This seemed to confirm the idea that
glutathione, in its reduced form was the dipeptide cysteine-glutamic acid, having the formula,

\[
\text{SH.CH}_2\text{.CH.NH.CO.CH}_2\text{.CH.NH}_2\text{.COOH}
\]

However, it was pointed out by Hunter and Eagles\textsuperscript{15} that various preparations of glutathione which they obtained did not analyze in agreement with the formula ascribed to it; that their preparations contained more than the theoretical amount of nitrogen and less sulfur, and therefore that glutathione could not be a dipeptide.

In a reinvestigation of the whole problem, Hopkins\textsuperscript{16} confirmed the work of Hunter and Eagles and reached the conclusion that glutathione is a tripeptide, which on hydrolysis yields cysteine, glutamic acid and glycine.

At about the same time, Kendall, McKenzie and Mason,\textsuperscript{17} working independently, arrived at the same conclusions.

Additional work, reported by Kendall and his associates\textsuperscript{18,19} somewhat later, indicated that in glutathione, the glutamic acid is attached to cysteine and that the latter is in turn attached to the amino group of glycine, as represented by the following formula,

\[
\text{COOH.CH.CH}_2\text{.CO} \quad \text{NH.CH.CO} \quad \text{NH.CH}_2\text{.COOH}
\]

\[
\text{NH}_2 \quad \text{CH}_2\text{.SH}
\]

The crystals of glutathione, prepared by several dif-
ferent methods, occur in orthorhombic prisms, or plates, bounded by many narrow faces.

Mason,\textsuperscript{20} in 1931, presented evidence to show that by aeration at pH 7.6, glutathione is quantitatively converted into the oxidized form without loss of nitrogen or sulfur. When dehydrated with alcohol, it contains approximately two molecules of alcohol for each molecule of the oxidized glutathione.

Voegtlin, Johnson, and Rosenthal\textsuperscript{21} studied the oxidation catalysis of glutathione. Their conclusions may be summarized as follows: "The oxidation of crystalline glutathione, in phosphate buffer solution of physiological pH, is accelerated by the relatively small amounts of the following substances: salts of copper, palladium, gold, and cobalt; also by selenates, selenites, and tellurites. Copper is by far the most effective catalyst. Under the same experimental conditions, the following substances do not influence the rate of oxidation of glutathione: salts of iron, manganese, nickel, tin, lead, cerium, mercury, platinum, chromium, osmic acid, and arsenite (in relatively low concentration). Salts of silver, zinc, cadmium, bismuth, and antimony, in suitable concentrations, decrease the rate of oxidation of glutathione. The so-called autoxidation of crystalline glutathione is due to the presence of minute traces of copper in this material."

The authors have furthermore shown that the addition of
small amounts of pure cysteine plus iron salts to glutathione solutions has no influence on the rate of oxidation of glutathione. This indicates that the oxidation of this substance is not dependent on the presence of a catalytically active iron-cysteine complex, but rather that the oxidation of glutathione is catalyzed by copper.

Probable Physiological Function of Glutathione in Plant and Animal Tissue

The following discussion is somewhat apart from the specific purpose of the investigations reported in this thesis, but is presented here to show the important role which glutathione undoubtedly plays in plant and animal life.

General Theories of Physiological Oxidations

To understand the function of glutathione in physiological oxidation, it will be necessary to review the more prominent theories concerning such reactions and to see what part glutathione plays in them.

According to Wieland, the oxygen of water is utilized for all biological oxidations. Atmospheric oxygen only serves to fix the hydrogen of the water. In general, Wieland sup-
poses that oxidations are always accompanied by simultaneous reductions, for always the hydrogen and not the molecular oxygen is said to be activated. The latter is said to remain entirely passive and to form no unsaturated molecules. Hence, according to Wieland, the oxidation is due solely to the formation of active atomic hydrogen. If the active hydrogen of water is tied up to a hydrogen acceptor, the residual unsaturated oxygen of the water can unite with various substances. It is believed that the action takes place somewhat as represented by the following equations:

(1) \[ \text{CO} + \text{H}_2\text{O} \rightarrow \text{HCOOH} \rightarrow \text{CO}_2 + 2\text{H} \]

(2) \[ \text{M (methylene blue)} + 2\text{H} \rightarrow \text{MH}_2\text{ (methylene white)} \]

(hydrogen acceptor)

In opposition to Wieland's theory is that of Bach\(^2\) that molecular oxygen reacts with some constituent of the protoplasm (oxygenase) to form peroxide. The latter is broken down by an enzyme (peroxidase) yielding active oxygen which is capable of oxidizing the metabolites in the tissues.

\[
\text{R} + \text{O}_2 \rightarrow \text{R} + 2\text{O}_2
\]

(autooxidizable substance atmospheric oxygen organic peroxide)

The enzyme (peroxidase) catalyzes the decomposition of the organic peroxide,
According to Hawk and Bergen\textsuperscript{24} not only may oxidations be brought about by activation of either oxygen or hydrogen, but both processes may occur simultaneously. Under anaerobic conditions, the oxidation of substance (R) in the presence of methylene blue (M) may be represented as follows:

\[(1) \quad RH_2 \rightarrow R + 2H \quad \text{(2) } M + 2H \rightarrow MH_2\]

Under aerobic conditions,

\[(a) \quad RH_2 \rightarrow R + 2H \quad \text{Oxidation by dehydrogenase}\]
\[(b) \quad 2H + O_2 \rightarrow H_2O_2 \quad \text{Peroxide formation}\]
\[(c) \quad H_2O_2 \rightarrow H_2O + O \quad \text{Peroxidase action}\]
\[(d) \quad R_1 + O \rightarrow R_1O \quad \text{Oxidation of second substance}\]
\[(e) \quad H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{Catalase producing molecular oxygen}\]

Warburg\textsuperscript{25} considers that oxidations in the living cell are due to a respiratory enzyme, a hemin derivative, the action of which is related to the presence of iron in the molecule.

Possibly the rôle of glutathione in oxidative processes may be explained by simultaneous activation of hydrogen and...
In his first paper, Hopkins pointed out certain relations in the behavior of fresh tissues toward methylene blue and glutathione. Fresh tissues reduce methylene blue. This dye is likewise reduced by the reduced form of glutathione. From these observations, he concluded that the reduction potential of tissues is greater than that of glutathione, which in turn has a greater reduction potential than methylene blue. These relations are not always fixed, but, according to Hopkins' first experiments, are closely dependent on the hydrogen-ion concentration of the medium. The explanation lies in the fact that the -S-S- group of the tripeptide acts first as a hydrogen acceptor. The hydrogen taken up is then transferred to the methylene blue.

In physiological oxidations, we must therefore consider the relation of glutathione to oxygen, the most available of hydrogen acceptors. The changes which glutathione undergoes in oxidation may be represented as follows:

\[
\begin{align*}
G - SH & \quad HS - G \\
+ O_2 & \\
G - S & \quad S - G + H_2O_2 \\
+ H_2 & \downarrow \\
G - SH & \quad HS - G
\end{align*}
\]

*In these equations and subsequently in this thesis the expression GSH is used to represent reduced glutathione and GS-SG to represent its oxidized form, as is common in much of the literature on this subject.
Those equations represent glutathione in the position of an intermediary, removing hydrogen from the tissues, or more properly metabolites, and passing it on to the oxygen. The hydrogen peroxide is formed as a by-product and is perhaps prevented from accumulating by catalase, which according to this scheme, assumes a definite function.

Harrison\textsuperscript{26} observed that the \(-\text{SH}\) group of glutathione is oxidized by molecular oxygen only in the presence of minute traces of iron.

Meldrum and Dixon\textsuperscript{27} undertook experiments which showed that pure glutathione is not appreciably autoxidizable even in the presence of iron. They came to the conclusion that the effect of muscle powder in preventing the oxidation of glutathione is due to the fact that the tissue removes from the solution some impurity other than iron which catalyzes the oxidation. They conclude that the impurity in crystallized glutathione which forms active complexes with iron is free cysteine and it appears that the amino group must be left free in order that active complexes may be formed. They claim that the cysteine impurity does not act by the alternate oxidation and reduction of its sulfur group, but merely by converting the iron into a catalytically active complex. It is the sulfur of glutathione which undergoes alternate oxidation and reduction in the presence of tissue, the ferro-cysteine catalyzing the reaction between glutathione and oxygen. The two most probable explanations of the oxidation of
glutathione in the tissues according to the above authors are: (1) Autoxidation catalyzed by hematin. (2) Oxidation by hydrogen peroxide either directly or indirectly.

In 1922, Hopkins and Dixon\(^2\) presented evidence to show that a tissue that has been washed until it no longer respires, will, upon the addition of glutathione, again take up oxygen and yield carbon dioxide. Muscle may be extracted with boiling water, washed with alcohol, dried in vacuo, and powdered and yet in the presence of glutathione exhibit a capacity for taking up oxygen. This property is not exhibited, however, in the absence of glutathione. It is apparent, therefore, that the thermostable, water-and-alcohol-insoluble tissue residue, must contain something which acts in conjunction with glutathione and which ordinarily has a higher reduction or lower oxidation potential than glutathione.

Meyerhof\(^2\) showed that dead yeast cells (acetone-washed yeast) lost the property of respiration after being washed with water. By means of qualitative reactions, he determined that the washings contained something which had free -SH groups. He discovered that he could restore the respiratory properties of the dead yeast by the addition of substances containing the sulfhydryl group. The relation of the thermostable, water-insoluble residue of tissues to oxidation in the presence of glutathione was shown by Meyerhof to be due to the lecithin fraction and more particularly to
the unsaturated fatty acids of the lecithin molecule. According to Meyerhof, glutathione may function as an oxygen carrier transferring oxygen to oxidizable constituents in the tissues, such as fatty acids, amino acids, etc.

Hopkins has also shown that glutathione promotes oxidation of fats and proteins but that oxidation of carbohydrates is not catalyzed by glutathione. The effect of hydrogen ions is an important factor in this process. Hopkins states that in neutral or alkaline systems, during the oxidation of the -SH group fatty acids are simultaneously oxidized in such a way that equipartition of oxygen occurs. The oxidation of a protein seems to depend upon its possessing an -SH group. Protein is oxidized under the influence of glutathione in a neutral or faintly alkaline solution but not in an acid medium. Hopkins states that proteins that do not contain the -SH group (e.g., gelatin) show no tendency to reduce oxidized glutathione. During oxidation of proteins the -SH group of the protein is oxidized. The amount of oxygen taken up amounts to about ten times the oxygen equivalent of the sulfhydryl present. After the -SH group has disappeared the oxidized protein can be reduced, by contact (as a solid phase) with cysteine to thioglycollic acid and glutathione. After this reduction the protein exhibits free -SH groups and is able to take up ten times the amount of oxygen with which it would be expected to combine on the basis of the content of -SH.
Meldrum claims that glutathione is kept mainly reduced in the cell by several reducing factors. Fixed -SH groups in muscle form one such factor; but he claims that dehydrogenase systems play no part in this process. He concludes that the part played by glutathione in the life of the cell is still obscure in the extreme, but that possibly it is connected with protein metabolism rather than cell oxidations.

Mason finds that when glutathione is kept in aqueous solution at 37°-62° C it undergoes cleavage into pyrrolidonecarboxylic acid and cysteinylglycine. With Sullivan's test for cysteine, cysteinylglycine gives a purplish red color and if present in small amounts it cannot be distinguished from the color due to a very small quantity of cysteine. He suggests that Meldrum and Dixon attribute to cysteine a catalytic activity in auto-oxidation of glutathione which is probably due to cysteinylglycine.

Examples of Physiological Role of Glutathione

The following are some examples in which glutathione plays a physiological role.

(1) Blood from cancerous persons contains less glutathione than does that of healthy persons (Willheim and Stern).

(2) Oxidized glutathione was found to be absent from
all normal body tissues except blood (Bierich and Rosen-
bohm$^{33}$).

(3) Fever, experimentally induced in man, leads to
increase of iodine reducing titre, probably due to increased
 glutathione (Waelsch and Weinberger$^{34}$).

(4) The toxicity of iodo-acetic acid to the oxidation
of glucose by brain tissue is decreased or inhibited by glu-
tathione (Quastel$^{35}$).

(5) The livers of dogs which had been receiving two
grams daily of magnesium sulfate contained larger amounts of
reduced glutathione than did livers of other dogs (Wolff and
Manjean$^{36}$).

(6) Intravenous injections of insulin increased the
 glutathione content of blood (Zunz$^{37}$).

(7) Sodium hyposulfite increased the glutathione content
of the liver in the normal animal and arrested its fall in
experimental obstruction of the bile duct (Binet and Arna-
udel$^{38}$).

(8) The glutathione content of the thyroid, adrenals,
kidneys, brain spleen, lungs, heart, muscles submaxillary
glands, pancreas, liver, testes, and veins is greater in
normal rats and mice than in those infected with experimental
sarcoma and carcinoma. During the growth of the tissue the
glutathione decreased, particularly in the thyroid (Medve-
dev$^{39}$).

(9) Glutathione was found to increase during the growth
of tumors on plants (Binet and Magrou\textsuperscript{40}).

(10) Animal and plant proteases may be activated by glutathione (Grassman, et al\textsuperscript{41}).

(11) Hydration of dried pea seeds caused the formation of glutathione (Vivario and LeClous\textsuperscript{42}).

(12) Treating potato tubers with ethylene chlorohydrin was found to break the dormancy of the tubers and the glutathione content was found to increase. Crystalline glutathione has actually been isolated from tubers treated this way (Guthrie\textsuperscript{43}).

According to Barker,\textsuperscript{44} in leafy tissue such as those with which we are most concerned in this investigation, the glutathione is probably extracellular and occurs on the under surfaces. The same author also finds no glutathione in the veins of the leaves. The exact function of glutathione in this type of tissue, however, remains in doubt, as it does in animal tissue.

The Relation of Glutathione to Liberation of Hydrogen Sulfide from Sprays and Dusts

An ideal fungicide is one that is highly toxic to the parasitic fungus and not injurious to the tissue of the host plant.

Extensive orchard and garden tests have shown that sul-
fur, applied in the elemental form as a dust, or resulting from the decomposition of polysulfides (such as "lime-sulfur", "livers of sulfur", etc.) applied as a spray or dust, is very toxic to many fungous spores or growing mycelia. Experience has shown that it is also very safe to use on dormant plants, as a winter or early season fungicide, but that injury to foliage is often caused if the spray or dust is applied in the summer to growing foliage, unless the concentrations used are kept very much lower than those safely used in dormant spraying.

There have been several series of studies of the fungicidal action of sulfur, of which two have definite applications to the problem which is the subject of this thesis. These are reported in the series of papers entitled "The Fungicidal Action of Sulfur", by Wilcoxson and his associates at Boyce Thompson Institute for Plant Research at Yonkers, New York; and the series under the same title by Barker and his associates of the Long Ashton Agricultural and Horticultural Research Station at Bristol, England.

A brief summary of the facts developed by Wilcoxson and his associates which are pertinent to this investigation reads as follows: "Leaves and spores of all species of plants tested produce hydrogen sulfide when in contact with sulfur. The production of hydrogen sulfide by spores varies according to the species and is directly proportional to the number of spores. The optimum temperature is 35° C, and in-
hibition takes place at 55°C; but the production occurs over a relatively wide pH range. Actual contact between spores and sulfur is not necessary, as action will take place through a collodion membrane, the hydrogen sulfide being produced on the spore side of the membrane and not on the sulfur side. The action also takes place across air space. The action of a spore on sulfur is primarily a reducing one. Hydrogen sulfide is extremely toxic to fungous spores. The order of sensitivity of spores of different species to sulfur and to hydrogen sulfide is identical and the sensitive spores produce more than one toxic unit of hydrogen sulfide. Finally, compounds such as glutathione which contain the -SH group readily react with sulfur, producing hydrogen sulfide at ordinary temperature and such compounds have been shown to exist in fungous spores." In conclusion the hypothesis is presented that the fungicidal action of sulfur depends upon the diffusion of sulfur vapor from sulfur particles to the spores and its reduction within the spores to form toxic hydrogen sulfide.

This excellent and conclusive evidence of the toxic action of sulfur on spores of fungous diseases of plants is well supplemented by the studies of Barker on the liberation of hydrogen sulfide from sulfur by the leaves of plants, upon which fungi grow as parasites. He found that the youngest leaves give most intense production of hydrogen sulfide from sulfur; with increasing age of leaves the reaction becomes
less and old leaves fail to give a positive test by this method. He concludes that leaves under active growing conditions react with sulfur by means of a reducing substance which is produced by the metabolism of the cells of the leafy tissue. At the time he prepared his report, he was not aware of the existence of glutathione; but, in a footnote appended after his paper was submitted for publication, he indicates his belief that the substance which he postulates to be present in the plant tissue is undoubtedly glutathione, as recently isolated and identified by Hopkins. Barker further found that the sulfur-reducing substance in leaves is extracellular and that its reaction with sulfur takes place on the under (stomatal) side of the leaves and not on the upper surface. He also studied the reducing action of the living plant tissues on various other chemicals than sulfur and found that in every case the reducing effect was confined to the under side of the leaves.

In connection with his studies of the mechanism of the sulfur-reducing action, Barker applied his method of testing to the leaves of 61 species of plants, chiefly fruits and vegetables, but including ornamentals. This method consists of clamping filter paper moistened with basic lead acetate to the surface of the tissue which had previously been dusted with flowers of sulfur. He records that with fifteen of these, the lead acetate paper was "colored black or brown"; with 9, it was "coloured faintly brown, but sulfide forma-
tion doubtful"; and with 37, it was "uncoloured". He com-
ments on the fact that his samples were taken in September
and that different results might have been obtained if the
samples had been taken earlier in the growing season. More-
over, McCallan and Wilcoxson found positive results from
every one of the 26 species of plants which they examined
by their methods of sulfur-reduction tests.

Hence, it seemed probable that the same reaction which
makes sulfur toxic to spores of parasitic plant diseases
may take place also with the foliage of the host plants and
may be responsible for injury to the latter if environmental
conditions are suitable for such injury to take place.

For this reason, it seemed to be desirable to study
further the possible presence of glutathione in horticultural
plants and its relation to the use of sulfur or sulfur com-
pounds as a fungicide on such plants.
EXPERIMENTAL WORK

Preparation of Glutathione from Yeast

It was deemed advisable to obtain a pure sample of crystallized glutathione for the purpose of making standards for sulfur generation and for check purposes on the various stages of the research. It was decided to obtain this from yeast.

Review of Methods

Several procedures for obtaining glutathione from this source have been described. Hopkins\(^6\) isolated the tripeptide from yeast. His method is as follows: "The yeast is boiled for five minutes with water containing 0.1 per cent acetic acid. The mixture is then filtered and a saturated solution of neutral lead acetate added, followed by a 10 per cent solution of mercuric sulfate in 5 per cent sulfuric acid. After filtering, the precipitate is decomposed with hydrogen sulfide, which takes about 48 hours. The excess of hydrogen sulfide is removed by a current of air, followed by hydrogen, and sufficient sulfuric acid added to make the solution approximately 0.5N. The solution is then heated
to 45°-50° C, and an aqueous suspension of cuprous oxide cautiously added. The cuprous oxide must be added in small quantities at a time and an excess avoided, as a relatively small excess may cause resolution of quite a large proportion of the precipitate. The precipitate is separated by centrifuging and washed by the use of the centrifuge, once with 0.5N sulfuric acid, and then with successive quantities of distilled water (previously well boiled and allowed to cool in a closed flask) until absolutely free from sulfate. The moist precipitate is suspended in four or five times its bulk of distilled water and decomposed with hydrogen sulfide. The filtrate from the copper sulfide is freed from hydrogen sulfide by a stream of hydrogen and evaporated in vacuo to a volume of 6-8 cc. for each kilo of yeast extracted. It is then placed in a crystallizing dish, mixed with half its volume of alcohol, and the dish allowed to stand in a thoroughly evacuated desiccator containing fresh sulfuric acid. Hopkins states that the precipitation with cuprous oxide removes only about 70 per cent of the peptide from the solution.

Kendall, McKenzie and Mason found that toluene or benzene liberated glutathione from the yeast cell at room temperature, and found by titration with iodine that more glutathione was present than from similar solutions which were heated. The rest of their process was not as simplified as Hopkins's method and the yield was not so high.
Pirie\textsuperscript{46,47} used a mixture of ether, alcohol and sulfuric acid for the extraction, and omitted the precipitation with lead acetate and mercuric sulfate. He added cuprous oxide directly to the filtrate from the ether-alcohol-and-sulfuric acid mixture. He allowed the glutathione to crystallize from water solutions.

Kozlowski\textsuperscript{48} used a mixture of acetone and water for the extraction, and to the clear supernatant liquid, after centrifuging, added neutral lead acetate. The precipitate formed was decomposed with 3 per cent sulfuric acid and after the filtrate had been made 0.5M with sulfuric acid, cuprous oxide was added as in Hopkin's method. After removal of the hydrogen sulfide with hydrogen, he added barium hydroxide to remove all traces of sulfates. This solution instead of being taken down in vacuo to a small volume, he immediately dropped into ten volumes of ice cold alcohol-ether mixture, from which the glutathione separates readily.

**Preliminary Tests of Methods**

As a preliminary test of the different possible procedures it was decided to take a small quantity of yeast and to divide it into two equal parts.

Pirie's method was followed with one portion and the yeast mixture was centrifuged. The liquid was not very clear and attempts were made to clarify it by filtering through a
funnel covered with Kieselguhr. This filtered very slowly and after a while filtration practically stopped. The filtrate was still cloudy. Using one-fourth of this extract the copper salt was precipitated with cuprous oxide. A slightly discolored white floculent precipitate settled out. A test for the -SH group was made by two different methods and the typical purple color appeared in both cases.

With another portion of the same solution, the same procedure was followed except that a solution of silver sulfate was used to precipitate the glutathione. A floculent white precipitate was obtained. It was noticed that when the hydrochloric acid was added a precipitate of silver chloride appeared, but later went into solution when the various reagents were added. It was thought that possibly a silver complex was formed which interfered with the reaction. A purple color was obtained but disappeared almost instantly. Another water suspension was made of the supposed glutathione-silver salt and hydrochloric acid added and the silver chloride filtered off. Both methods of the nitro-prusside test were then applied to this solution and a good test for the -SH group obtained by either method.

The second half-pound portion of yeast was treated with acetone and water, according to Kozlowski's method. A clear solution resulted from the centrifuged yeast mixture. One-fourth portions of this extract were taken and the same procedure followed as with the ether extract. The copper and
silver salt were obtained in both cases, and the final test for the -SH group.

**Attempted Isolation of Glutathione from Yeast**

As a result of these preliminary tests, it was decided to follow the procedure of Kozlowski for the isolation of a quantity of glutathione from yeast, omitting the precipitation with neutral lead acetate and removal of sulfate with barium hydroxide. Five pounds of yeast were taken and the copper salt of glutathione precipitated. The procedure of Hopkins was followed from this point. (Note: About four days elapsed before work could be done on the original extract and during this delay it had become turbid. All of these solutions were kept in the ice box at around 2°-3° C but evidently some fermentation had set in.)

No appreciable amount of glutathione was obtained in this case. It seems that there were three possible reasons why this process failed. (1) The copper glutathione may have been too impure and contaminated with other substances. The precipitate by this method was a floculent, grayish-white precipitate, rather than a fine white crystalline salt with a silky sheen. (2) The decomposition of the copper glutathionate with hydrogen sulfide may not have been complete; possibly because the gas was bubbled through the solution in an open beaker. (3) Due to the possible fermentation of
the acetone extract a considerable portion of the glutathione may have been oxidized or decomposed.

**Final Isolation of Glutathione**

Five pounds of yeast were taken and these sources of error avoided as much as possible. 1260 cc. of acetone and 750 cc. of water were used and the mixture allowed to stand for half an hour. This mixture was centrifuged. The yeast residue was washed with 470 cc. of acetone and 250 cc. water and the mixture centrifuged as before. The first extract was 2340 cc. (Extract No. 1) and the washings 825 cc. (Extract No. 2). By titration with 0.001M iodine solution, using starch as an internal indicator, a titration equivalent to 4.68 grams of glutathione was obtained from the first extract. A titration equivalent to 0.8 gram of glutathione was obtained on the second extract.

To the combination of the clear extracts No. 1 and No. 2 there was added 500 cc. of 15 per cent neutral lead acetate. The precipitate was centrifuged off and washed with 50 per cent acetone and finally decomposed with 3 per cent sulfuric acid. The lead sulfate was centrifuged off and an aliquot of the clear solution titrated with N potassium hydroxide to determine the acidity. The solution was then diluted to 0.5M. A titration equivalent to 3.4 grams of glutathione was obtained at this point.
The cuprous glutathionate was precipitated with cuprous oxide. This procedure was carried on more rapidly than before and no chance for fermentation was allowed. This time the characteristic, crystalline, white precipitate, with a silky sheen, as described by Hopkins, appeared.

The copper salt of glutathione was washed and suspended in water and this time the hydrogen sulfide was passed into the solution in a closed flask under pressure, and allowed to stand over night. The copper sulfide precipitate was washed and hydrogen was bubbled through the solution until all the hydrogen sulfide had been removed.

This solution was then evaporated in a vacuum desiccator at room temperature. (Note: The room temperature was considerably higher than usual and the solution went to dryness before morning.) The dry residue was dissolved in a little water distilled in glass and poured into an ether-alcohol mixture. A fine crystalline, white precipitate settled out. The yield of glutathione was somewhat less than was expected, probably because of going to dryness before adding the alcohol-ether mixture.

Yield of Glutathione Obtained

The yield of glutathione after it had been dried in a vacuum to a constant weight was 0.6144 gram.

Approximately one-seventh of the total solution, after
deleading, was taken and the copper salt of glutathione which was obtained from this was washed until free from sulfate with water and then alcohol. This salt was dried to constant weight in a vacuum. The weight of this copper glutathione was 0.4417 gram.

The total yields were: 0.6144 gram of pure glutathione + 0.4417 gram of copper salt.

Properties of Glutathione Obtained from Yeast

This first crystalline compound was studied under the microscope and compared with photographs of glutathione crystals and also compared with a sample of glutathione which had been purchased from the Eastman Kodak Company. Form, shape, and appearance of the two sets of crystals were identical. A minute amount of each lot of crystals was dissolved in water and the nitroprusside test applied. The intensity of the purple color was the same for both samples. The generation of hydrogen sulfide test (which will be described later) was applied to both sets of crystals and the color on the lead acetate paper was the same in both cases. Samples of both preparations were titrated with 0.001N iodine. The commercial preparation was found to be 97.3 per cent pure and our preparation was 96.3 per cent. The melting points of both samples of glutathione were determined. Each was found to melt sharply at 135°-136° C.
Hence, it was concluded that the two samples were identical and could be used for further experimental studies.

Determination of Glutathione in Tissues of Horticultural Plants

Determination of Applicability of Methods to Desiccated and Live Tissues

It was at first planned to study the progressive change of glutathione content in tissues of horticultural plants from the beginning of the formation of the leaves, through the various stages of growth, till the period when the leaves dropped off the tree in the fall. When work on this problem was first started methods for testing the live tissues had not been determined upon. In order to preserve the earlier samples for future study, it was decided to take the tissues to as nearly dryness as possible in the electric refrigerator at a temperature of 2°-3° C. Samples of buds and bark, and later of leaves and bark were treated in this way. After most of the moisture had evaporated, the samples were ground and then dried again in the refrigerator to constant weight. These powdered samples were then put into stoppered glass bottles and set aside for later use. The average percentage of moisture left in the samples was 6 to 10 per cent. It was hoped, by this treatment at a low temperature, to prevent fer-
mentation and that the glutathione which was in the tissues would not be decomposed or oxidized. It was thought that the first samples of buds and bark would contain little or no glutathione; but that the glutathione content would progressively increase up to a certain point and then drop again. Later, the three methods which are mentioned in the next section were applied to these samples and negative results obtained in all cases. Since it was uncertain whether the samples originally contained no glutathione, or had lost it in the drying process, it was decided to abandon attempts at a study of the progressive steps in the development of glutathione in tissues of horticultural plants and rather to develop more accurate methods of measuring the glutathione content of these tissues. It was also decided to make a survey of various tissues by measuring the glutathione content of the tissues when in full foliage.

So far as is known to the writer, no attempt has been made to make systematic determinations of the glutathione content in this type of plant tissue. Some measurements have been made of hydrogen sulfide generation and its toxic effect upon plant tissues, but this hydrogen sulfide generation has not been measured in terms of glutathione content. The only other plant tissue, other than yeast cells, from which glutathione has definitely been isolated is from potato tubers by Guthrie and Wilcoxson⁵.
Methods for Determination of Glutathione in Plant Tissues

The determination of the glutathione content of tissues has been the subject of much study since Hopkins succeeded in isolating glutathione in 1921. Most of the attempts to get a satisfactory method have been made upon animal tissues or with blood. It appeared to be uncertain whether the methods used for the determination of glutathione in such tissues are applicable to horticultural plant tissues, and a series of preliminary studies of methods was undertaken.

The most prominent methods for determining the glutathione content of animal tissues and in blood are of three different types; namely, iodine titration, nitroprusside tests and the hydrogen sulfide generation methods.

(1) Titration Methods

The titration method which has been most commonly used is that of Tunnicliffe^50 or some modification of it. His method is as follows: A known weight of the tissue is ground in a mortar with sand and 10 per cent trichloroacetic acid. He found the use of trichloroacetic acid to have the following advantages: (1) It yields a clear protein-free filtrate; (2) it does not interfere with the subsequent iodine titra-
tion; (3) oxidation of the -SH groups during the extraction is prevented by the acidity of the solution; (4) glutathione is not precipitated by the trichloroacetic acid. The clear extracts so obtained are titrated with standard iodine solution, using sodium nitroprusside as an external indicator.

Perlzweig and Delrue\textsuperscript{51} modified this method by adding to the trichloroacetic acid extract 2 cc. of 5 per cent solution of potassium iodide and using starch solution as an internal indicator, adding an excess of iodine solution and titrating back with an equivalent thiosulfate solution.

Gavilescu\textsuperscript{52} found that more satisfactory results were obtained by adding an excess of potassium iodide and titrating the solutions near the neutral point.

Woodward and Fry\textsuperscript{53} used thiosalicylic acid instead of trichloroacetic acid as a protein precipitant in the case of blood filtrates and claim it is more satisfactory. They also claim that back titration with sodium thiosulfate is apt to give higher results, due to the presence in the filtrates of substances with varying speeds of iodine reaction. The authors recommend using thiosalicylic acid for extraction, adding extra potassium iodide, and using starch as an internal indicator, titrate to the blue end point with potassium iodate.

The other titration methods which have been proposed may be mentioned briefly.

Fleming\textsuperscript{54} uses a solution of neutral red as a standard to compare the color developed by -SH groups using sodium
nitroprusside and sodium chloride in an alkaline medium.

Schelling\textsuperscript{55} uses sodium azide (NaN\textsubscript{3}) to test for free -SH ions and finds this to be a very sensitive test. Aliphatic sulfhydryl compounds, of the type of cysteine, give this test, whereas cyclic compounds, such as thiophenol and thioresol, fail to give a positive reaction. His method is as follows: To the solution to be tested add about 0.1 gram of sodium azide and the same amount of finely powdered potassium iodide. Shake well and from a burette drop 1-2 cc. of 0.1M iodine solution into the mixture. In the presence of free sulfhydryl groups an immediate formation of nitrogen gas takes place.

Method Used

For the purpose of this research, it was decided, for most purposes, to use 0.001M iodine solution for titration, using freshly filtered starch as an internal indicator, adding an excess of iodine and back-titrating with sodium thiosulfate. Experiments were also carried out with direct titration, using 0.001M potassium iodate solution; but the iodate titration, while satisfactory for the total titrations, would not work on the lead acetate fraction.

Stock solutions of 0.1M were made in all cases and standardized frequently. These stock solutions were found to change very little and the 0.001M solutions were made up
fresh daily by diluting the stock solutions.

Special chemically pure sand was used for grinding up the tissue for total titrations and 10 per cent trichloroacetic acid was used for extraction except in the case of a few experiments with thiosalicylic acid, to ascertain whether the latter was satisfactory for our purposes. No appreciable blanks were obtained either from the trichloroacetic acid, thiosalicylic acid, or from the sand.

In using the iodine solutions, it was necessary to add extra potassium iodide and this was added in the form of a 25 per cent solution. If the solutions were diluted too much before titration it was found that the values were too high. This was compensated for by adding extra potassium iodide; but it was found to be better in all cases to keep the volume as small as possible.

The calculations used to convert the titrations into glutathione are as follows:

\[
\text{cc. } 0.001\text{M} \text{I}_2 \text{ or } \text{KI} \text{a} \quad \frac{x}{2.26} \text{ (theoretical titer for } 1 \text{ mg. GSH)}
\]

Effect of Trichloroacetic Acid upon the Iodine Titration of Glutathione

Experiments were undertaken to see if adding pure glutathione solution to trichloroacetic acid had any appreciable effect upon the end point. Ten mgs. of glutathione were made
up to 100 cc. with water distilled in glass so that a 10 cc. aliquot equals 1.0 mg. of glutathione. Two and one-half cc. of 25 per cent potassium iodide solution was added before each titration, using 10 cc. of glutathione solution and titrating past the blue end point with 0.001N iodine solution and back titrating till the blue color just disappeared. When potassium iodate was used instead of the iodine solution, the solution was titrated to the first appearance of the permanent blue color.

Table I

Effect of Trichloroacetic Acid upon Iodine and Iodate Titrations of Known Amounts of Glutathione

<table>
<thead>
<tr>
<th>Mg. GSH</th>
<th>cc. CCl₃COOH</th>
<th>cc. 0.001N I sol.</th>
<th>cc. 0.001N KIO₃ sol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>3.3</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>3.1</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>---</td>
<td>3.3</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>---</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The conclusion from these results is that the presence of varying amounts of trichloroacetic acid does not interfere with the accuracy of the iodine titration of glutathione.
(2) Nitroprusside Tests

Two different methods for applying the nitroprusside test were used. (1) A few cc. of saturated ammonium sulfate solution is added to the solution which is being tested, followed by 6 drops of 2 per cent sodium nitroprusside and 2 cc. of concentrated ammonium hydroxide. (2) A modification suggested by Fleming was tried. This is supposed to be a more sensitive test but did not show much difference in results. To the solution which is being tested, 0.5 cc. of 1 per cent sodium nitroprusside is added, followed by a few crystals of sodium chloride and then three drops of concentrated ammonium hydroxide.

Attempts were made to apply the nitroprusside test method to the plant extracts. These were unsuccessful, though definite -SH tests were obtained in experiments to be described later, when the copper salt was precipitated. The failure to obtain a nitroprusside test with plant tissues was possibly due to the obscuring of the purple end color, by the color of the tissue extracts, or to secondary reactions taking place. A brown precipitate appeared in nearly all cases when the solution was made ammoniacal.
(3) Hydrogen Sulfide Generation Method

It has long been known that sulfhydryl compounds react with sulfur to form hydrogen sulfide and that this reaction is shown by many tissues.

Guthrie and Wilcoxon\textsuperscript{5} maintain that the reduction of sulfur is more specific for sulfhydryl compounds than the reduction of iodine. The reaction for this reduction is as follows:

\[ \text{EGSH} + S \rightarrow \text{G - S - S - G} + \text{H}_2\text{S} \]

The authors state that they did not find a non-sulfhydryl compound likely to be present in tissues that reacts with sulfur to form hydrogen sulfide. In order to determine the general applicability of this reaction, they made a study of the yield of hydrogen sulfide from solutions of cysteine and glutathione, and also from extracts of various tissues. The yield of hydrogen sulfide from tissue extracts to which known amounts of glutathione had been added were investigated. The sulfur reduction method was also tested by applying it to the iodine reducing substances of the potato after they had been fractionated with lead acetate. The authors used the method of Almy\textsuperscript{56} which depends on the absorption of the hydrogen sulfide in zinc acetate solution and its conversion
to methylene blue by the addition of para-amino-dimethyl- 
amaline and ferric chloride. The best results were obtained 
when the hydrogen sulfide was aerated rapidly with nitrogen 
from the reaction mixture into zinc acetate. The methylene 
blue had to be developed in the receiving tube, since the 
zinc sulfide formed could not be washed quantitatively from 
the receiver. They killed the tissue by dropping it into 
boiling water prior to the extraction. This prevents the 
destruction of the glutathione by oxidation, especially in 
the presence of oxidase. Most satisfactory results were 
obtained when a saturated solution of sulfur in absolute 
alcohol was used and the pH kept at 6.8 by use of a phos-
phate buffer of that pH. The average per cent recovery from 
pure glutathione solutions was 96.7 per cent. In order to 
investigate the usefulness of the sulfur reduction method 
and to compare it with the iodine reduction method, they 
applied it to extracts of various tissues. The iodine titra-
tion was applied according to the procedure of Perlzweig and 
Delrue.50 The following tissues were used: potato tubers, 
potato leaves, gladiolus corms, lilac buds, yeast, and calf's 
liver. In some cases a known amount of glutathione was added 
to the extracts after the evolution of hydrogen sulfide by 
the sulfur reduction method had been completed, and the re-
covety from these extracts determined by adding more sulfur 
and aerating into fresh zinc acetate. The percentage recovery 
determined in this way shows that the recovery of added glu-
Glutathione as shown by the hydrogen sulfide recovered is substantially complete only in the case of yeast, although complete recovery can be obtained with pure glutathione solutions. In the case of the potato leaves, which is a type of tissue such as we are studying, recovery is only 51 per cent. Even after correcting the values obtained by the sulfur method for percentage recovery, they are much lower than the iodine method, except in the case of yeast. The authors believed that the large discrepancies in the case of the other tissues are due to the presence of non-sulfhydryl iodine-reducing substances. Evidence of the simultaneous presence of sulphydryl and non-sulphydryl iodine-reducing compounds in the potato will be mentioned later. Guthrie and Wilcoxson acknowledge that the lead acetate paper method as used by McCallan and Wilcoxson is more sensitive for measuring small quantities of hydrogen sulfide, but believe it to be less accurate for larger quantities.

Method Used and the Making of Standards

After careful consideration of the above reports and some experimental tests, it was decided to use the lead acetate paper method in this study of plant tissues. The determining factors in this decision were: (1) The low percentage of recovery when glutathione was added to plant extracts. (2) Some preliminary experiments which showed that when the leafy tis-
Sue was dropped into boiling water and an extract made of the killed tissues, the hydrogen sulfide was considerably less than when the dry sulfur was dusted directly upon the leaves.

It was decided to use a paper made from Whatman's No. 1 filter paper dipped into a basic lead acetate solution made up as described in the Official Methods of the Association of Agricultural Chemists. The shape of the test paper used was a triangle with a base of 2 cm. width and sides with a length of 4 cm., with a stem of 0.4 cm. width and attached to the base of the triangle, and 4 cm. in height. The stem of this paper was inserted into a stopper with one hole and a solid glass rod inserted through the hole to hold the paper in place. This stopper with paper attached was inserted into the neck of a 100 cc. Pyrex flat-bottom extraction flask. A flat pan was used for a water bath and the temperature of the bath was maintained at 35° C for a number of hours and later raised somewhat to be sure to drive off all of the hydrogen sulfide.

It was decided to see if we could obtain a set of satisfactory standards from known amounts of pure glutathione. Solutions of glutathione dissolved in water distilled in glass were mixed with a sulfur paste and the hydrogen sulfide generated in the apparatus just described. This method was not successful, as very little generation of hydrogen sulfide was obtained. Good results were obtained, however, by
modifying the procedure in accordance with the observations of Guthrie. He found that the pH of a glutathione solution has a marked effect upon hydrogen sulfide generation and that the most satisfactory generation could be obtained by using a saturated solution of sulfur in absolute alcohol instead of using a sulfur paste with water. This solution was prepared by dissolving an excess of sulfur in hot absolute alcohol, allowing to cool and filtering. The phosphate buffer having a pH of 6.8 was made by mixing equal volumes of \( \frac{1}{15}\) Na\(_2\)HPO\(_4\)·12H\(_2\)O and \( \frac{1}{15}\) KH\(_2\)PO\(_4\). The test paper was moistened with basic lead acetate already inserted into the stopper and as soon as 3 cc. of alcoholic sulfur had been added, the stopper was inserted.

Following this improved procedure, a series of standards were made using pure glutathione; 25 milligrams weighed accurately and made up to 50 cc. with 6.8 phosphate buffer solution and taking the necessary aliquots to make a set of standards ranging from 0.1 mg. to 3.0 mg. of glutathione. A very satisfactory set of standards was obtained by this method, and standards made from glutathione prepared in this laboratory and the commercial product were identical.

**Tests on Various Types of Sulfurs with Regard to the Availability of the Different Types**

Tests were next made with the different kinds of sulfur which were available, to see if these would themselves yield
hydrogen sulfide in blank tests. The sulfurs were mixed with water to make a paste and tested for hydrogen sulfide generation. The results are shown in Table II.

It was desirable to know also the type of sulfur which gave a blank test that would give maximum generation on the type of leafy tissue which we were studying. Samples of fresh strawberry leaves of approximately the same surface area were dusted with the dry sulfur and the sulfur and water paste was also rubbed on the leaves, both upper and lower surfaces being so treated. The results of these tests are shown in Table II.

It appeared from these tests that the Liberty dusting sulfur, dusted upon the dry surface of the leaves, gave the best results as this appeared to have the finest size particles of all the samples tested, gave maximum hydrogen sulfide generation, and no blank. Accordingly, this particular sample of sulfur was used in all subsequent work.

Incidental Studies of the Glutathione Content of Various Horticultural Plant Tissues as Determined by the Hydrogen Sulfide Generation Method

In as much as the preliminary studies were completed before the various horticultural plants which it was proposed to study reached their full foliage stage, there was opportun-
Table II

Hydrogen Sulfide Generation Obtained from Different Types of Sulfur and the Generation from These Sulfurs When They were Dusted on and When Rubbed on the Strawberry Leaves

<table>
<thead>
<tr>
<th>Type of sulfur</th>
<th>Generation from blank</th>
<th>Generation from strawberry leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowers of sulfur.................................</td>
<td>---</td>
<td>xxxx*</td>
</tr>
<tr>
<td>Flowers of sulfur made into paste form................</td>
<td>Nil</td>
<td>x</td>
</tr>
<tr>
<td>Fine dry sulfur.................................</td>
<td>---</td>
<td>xxxx</td>
</tr>
<tr>
<td>Fine dry sulfur made into paste form.................</td>
<td>Nil</td>
<td>x</td>
</tr>
<tr>
<td>Liberty dusting sulfur...........................</td>
<td>---</td>
<td>xxxx</td>
</tr>
<tr>
<td>Liberty dusting sulfur made into paste form..........</td>
<td>Nil</td>
<td>x</td>
</tr>
<tr>
<td>Flotation sulfur.................................</td>
<td>---</td>
<td>xxx</td>
</tr>
<tr>
<td>Flotation sulfur made into paste form..............</td>
<td>Nil</td>
<td>x</td>
</tr>
<tr>
<td>Colloidal sulfur made into paste form...............</td>
<td>Nil</td>
<td>x</td>
</tr>
<tr>
<td>Colloidal sulfur paste...........................</td>
<td>Nil</td>
<td>xx</td>
</tr>
<tr>
<td>Liberty mix made into paste form...................</td>
<td>x</td>
<td>---</td>
</tr>
<tr>
<td>Lime sulfur made into paste form...................</td>
<td>xxxx</td>
<td>---</td>
</tr>
</tbody>
</table>

*xxxx = strong; xx = medium; and x = weak generation.
ity for the following more or less incidental, but interesting, preliminary experiments.

Samples of strawberry leaves taken after the berries had ripened showed a decrease of hydrogen sulfide generation compared with a sample taken in early June before the berries were ripe.

Samples of leaves from nearly all the fruit-bearing trees showed a decrease of hydrogen sulfide generation, measured in terms of glutathione, as the fruit appeared on the tree and began to mature. The glutathione content of the leaves of the cherry tree began to increase after the fruit had ripened and had been picked. These results are shown in Table III.

Hydrogen sulfide generation has been obtained from the fruit of the grape and it is possible that glutathione exists in all fruits and that during the process of growth of the fruits, glutathione is transported from the leaves to the fruit. McCallan and Wilcoxson showed, from an experiment upon a potted strawberry plant, that the supply of glutathione is being continually replenished in growing plants. It seems probable that the glutathione which has been lost from the leaves and passed through the sap to the fruit, is regenerated in the leaves and after the demand of the fruit for the glutathione has stopped, the glutathione which is being continually replenished, remains in the leaves.
<table>
<thead>
<tr>
<th>Type of leaf</th>
<th>Amount of $\text{H}_2\text{S}$ generation expressed in mg. of GSH/100 sq. cm. of surface area</th>
<th>Amount of $\text{H}_2\text{S}$ generation expressed in mg. of GSH/gm. of leaves</th>
<th>Date sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peach</td>
<td>$1.0 \pm 0.71$</td>
<td>$1.07$</td>
<td>6-13-33</td>
</tr>
<tr>
<td></td>
<td>$0.71$</td>
<td>$0.75$</td>
<td>7-4-33</td>
</tr>
<tr>
<td></td>
<td>$----$</td>
<td>$----$</td>
<td>8-3-33</td>
</tr>
<tr>
<td>Apple</td>
<td>$0.82$</td>
<td>$0.96$</td>
<td>6-13-33</td>
</tr>
<tr>
<td></td>
<td>$0.15$</td>
<td>$0.20$</td>
<td>7-7-33</td>
</tr>
<tr>
<td></td>
<td>$----$</td>
<td>$0.25$</td>
<td>7-27-33</td>
</tr>
<tr>
<td>Pear</td>
<td>$0.65$</td>
<td>$0.87$</td>
<td>6-13-33</td>
</tr>
<tr>
<td></td>
<td>$0.72$</td>
<td>$0.92$</td>
<td>7-7-33</td>
</tr>
<tr>
<td></td>
<td>$----$</td>
<td>$0.40$</td>
<td>7-25-33</td>
</tr>
<tr>
<td>Grape</td>
<td>$0.84$</td>
<td>$0.99$</td>
<td>6-14-33</td>
</tr>
<tr>
<td></td>
<td>$0.16$</td>
<td>$0.20$</td>
<td>7-11-33</td>
</tr>
<tr>
<td></td>
<td>$----$</td>
<td>$0.25$</td>
<td>7-28-33</td>
</tr>
<tr>
<td>Cherry</td>
<td>$0.20$</td>
<td>$0.22$</td>
<td>6-13-33</td>
</tr>
<tr>
<td></td>
<td>$0.09$</td>
<td>$0.10$</td>
<td>7-1-33</td>
</tr>
<tr>
<td></td>
<td>$----$</td>
<td>$0.20$</td>
<td>7-28-33</td>
</tr>
</tbody>
</table>
Glutathione Content of the Bark of Fruit-Bearing Trees

Samples of fresh bark from the pear, apple, and cherry trees were weighed and dusted with sulfur as before and the generation of hydrogen sulfide measured. These results are expressed in Table IV.

It must be remembered, however, that in this tissue we do not know whether the glutathione is extracellular or not. Therefore, the glutathione content of these tissues as found by hydrogen sulfide generation may be too low.

Determinations of Glutathione in Leaves of Fruits and Vegetables

Development of Method for Obtaining Comparative Results Between Iodine Titrations and Hydrogen Sulfide Generation

It became apparent at an early stage of the investigation that the iodine titration obtained with a trichloroacetic acid extract of the leaves, was yielding much higher results, when expressed in terms of glutathione, than were obtained from hydrogen sulfide generation tests.

An early sample of strawberry leaf required 19.80 cc. of 0.001M iodine solution per gram, which in terms of glutathione would be approximately 6 mg. This amount was much higher than the results obtained from early generation tests. Similar results with peach, cherry, and grape leaves are shown in Table V.
### Table IV

Glutathione Content of the Bark of Fruit Trees

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Weight of sample in grams</th>
<th>Mg. of GSH by H₂S generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pear bark</td>
<td>4.4504</td>
<td>0.50</td>
</tr>
<tr>
<td>Cherry bark</td>
<td>4.2873</td>
<td>2.00</td>
</tr>
<tr>
<td>Apple bark</td>
<td>4.3300</td>
<td>0.75</td>
</tr>
</tbody>
</table>
## Table V
Comparison of Results of Glutathione Determinations by Iodine Titrations and Hydrogen Sulfide Generation

<table>
<thead>
<tr>
<th>Type of leaf</th>
<th>Glutathione found expressed in mg. per gram:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By iodine titration</td>
</tr>
<tr>
<td>Peach</td>
<td>9</td>
</tr>
<tr>
<td>Cherry</td>
<td>4</td>
</tr>
<tr>
<td>Grape</td>
<td>7</td>
</tr>
</tbody>
</table>
These results agreed with those obtained by Guthrie and Wilcoxson who report that, in most horticultural plant tissues, there are present non-sulfhydryl iodine-reducing substances. Ascorbic acid, a non-sulfhydryl compound that reacts with iodine in acid solution, has been isolated from oranges, cabbage, and other tissues. It is probable that such sulfhydryl iodine-reducing compounds are present in many tissues. The same authors showed that it is possible to separate the iodine-reducing substances of the potato tuber into two fractions, one of which gives no nitroprusside test, thus showing definitely the presence of non-sulfhydryl iodine-reducing substances and indicating that the iodine method as used hitherto does not give a true measure of the sulfhydryl content of extracts of potato tissue.

The same fact was found to be true in this work, definite positive nitroprusside tests being obtained on the lead fraction and none upon the rest of the solution.

The following preliminary experiment showed that all of the glutathione is extracellular in this type of tissue: The leaves were weighed and dropped into boiling water and this extract taken for lead acetate fractionation. The material remaining after the extraction was ground up with distilled water and sand and filtered. This extract gave no precipitate with neutral lead acetate, indicating that all the glutathione had been removed by the boiling water. Hence, a hot water extract of the whole leaves was used for the iodine
titrations in all further experiments.

**Method for Fractionation of Iodine-Reducing Substances**

A method for fractionation of the iodine-reducing substances in this extract was used somewhat similar to that used by Guthrie and Wilcoxson,\(^5\) but differing in the following important particulars.

To the leaf extract, there was added enough saturated lead acetate solution to throw out all of the materials which would precipitate with this reagent. This precipitate was centrifuged and washed once with lead acetate solution. It was then delead by adding 10 per cent sulfuric acid and centrifuging out the lead sulfate. This precipitate was washed with water, and the supernatant liquid and washings made up to a known volume. In the later experiments this volume was maintained at 50 cc. An aliquot of this solution was pipetted into a beaker and taken just past the neutral point with 20 per cent sodium hydroxide solution, using methyl red as an indicator. It was then brought back to as nearly the neutral point as possible with a weak solution of trichloroacetic acid. Table VI shows the relationship between the glutathione content obtained in this way compared with that from hydrogen sulfide generation tests.

These results show that the first lead acetate fraction, as obtained by this method, still contains non-sulfhydryl
### Table VI
Comparison of Results of Glutathione Determinations with Iodine Titrations on First Fractionation of Extracts of Tissues

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Generation of $H_2S$ expressed as mg. of GSH per gm.</th>
<th>Iodine titration expressed as mg. of GSH per gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberry leaves</td>
<td>1.0</td>
<td>1.45</td>
</tr>
<tr>
<td>Peach leaves</td>
<td>1.0</td>
<td>2.47</td>
</tr>
<tr>
<td>Raspberry leaves</td>
<td>1.0</td>
<td>6.19</td>
</tr>
<tr>
<td>Pear leaves</td>
<td>1.0</td>
<td>2.18</td>
</tr>
<tr>
<td>Apple leaves</td>
<td>0.1 - 0.25</td>
<td>1.17</td>
</tr>
<tr>
<td>Cherry leaves</td>
<td>0.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>
iodine-reducing substances, as the iodine titration invariably yields higher results than the hydrogen sulfide generation test. This is probably due to the presence in these tissues of ascorbic acid, which reduces iodine but contains no −SH group. This, and possibly other substances having similar properties, have been shown by several investigators to be commonly present in plant tissues.

It is known that ascorbic acid is soluble in water when precipitated neutral lead acetate solution is added but is precipitated by the basic lead acetate. It seemed possible that this fact afforded a method of separating glutathione from ascorbic acid. One of the methods of precipitating ascorbic acid is by adding a hot saturated solution of neutral lead acetate and then making the solution ammoniacal.

It seemed reasonable to suppose that if, in our extracts, the solution was neutral or faintly on the alkaline side, some of the ascorbic acid would precipitate as the lead salt. Some of these extracts from the leaves were tested and found generally to be neutral or faintly alkaline. Hence, it was decided to make all these extracts just slightly acid with acetic acid and then add a hot saturated solution of neutral lead acetate. By doing this and following all precautions in titration, which will be mentioned later, satisfactory agreement between the iodine
titrations from the lead acetate fraction expressed in terms of glutathione content and the hydrogen sulfide generation expressed in the same terms, could be shown. In a few cases only, satisfactory agreement could not be obtained, due to unknown interfering substances.

Final Studies of the Glutathione Content of the Leaves of 39 Different Species of Plants

Obtaining and Selection of Samples

In making the final studies of the glutathione content of the leaves of 39 different species of plants it was found to be important to obtain as fresh samples as possible for this work. Some of the samples had to be obtained at a considerable distance from the laboratory and as the tests were to be made as soon after sampling as possible, it was found to be advisable not to collect more than three samples daily and usually only two samples were taken. All steps of the experiments were done on the same day that the samples were collected. Actively growing leaves were used wherever possible, and leaves were taken which had a weight per square centimeter surface area as nearly alike as possible. It was of course realized that samples of equal weight do not always have the same surface area; that the samples of the same type
of plant grown under different conditions might show variation in glutathione content; and that samples of the same plant obtained at different times would also show a variation.

In the earlier work, an attempt was made to correlate the glutathione content of the leaves with a definite surface area, but this was found to be not possible, probably because of the unequal distribution of the glutathione in the web-tissue, veins and mid-ribs of different areas of the leaves. Hence, in all final work, a definite weight instead of a definite area of the leafy tissue was used.

**Type of Determinations**

In the final studies three determinations were made on each sample, namely: (1) Hydrogen sulfide generation tests; (2) total iodine titration; (3) iodine titration of the first lead acetate fraction.

1. **Hydrogen Sulfide Generation**

The method previously described was used for the determination of hydrogen sulfide generation from the leafy tissues. Generation from the extract of the killed tissue was found not to be satisfactory and in all subsequent work the live leaf tissues were dusted with sulfur, on both sides.
A number of samples of different sizes were taken for the hydrogen sulfide generation tests. This was partly because it was not known in advance whether this generation would be high or low and partly to show whether this generation increases proportionately with the weight or surface area.

(2) Total Iodine Titration

In preliminary experiments with the tissues, extractions were made with both thiosalicylic acid and trichloroacetic acid, but as there appeared to be no advantage in using thiosalicylic acid, trichloroacetic acid was used for extraction for all of the titrations of total iodine-reducing substances. Water extractions were also tried, but there seemed to be no advantage in this method.

A weighed amount of the tissue (usually 1 gm.) was ground in a mortar quartz sand and 10 cc. of 10 per cent trichloroacetic acid. This mixture was diluted somewhat with distilled water and filtered through cloth which was suspended in a platinum cone, the latter placed in a glass funnel which was connected with the suction pump. After filtration the tissue was washed with 10 cc. of trichloroacetic acid and distilled water. The combined extract was titrated with 0.001N iodine solution, 2.5 cc. of 25 per cent potassium iodide solution being added before titration. Freshly filtered starch was
used as an internal indicator, an excess of iodine added, and the solution back titrated with 0.001M sodium thiosulfate solution.

(2) Iodine Titration upon First Lead Acetate Fraction

Take a weighed amount of the tissue (usually 7 gm.) and boil it gently for ten minutes. This extract is decanted off, the tissue washed with distilled water and the washings added to the extract. Add enough dilute acetic acid to make the extract slightly acid, and add 20 cc. of saturated lead acetate solution. Separate the precipitate by centrifuging and wash with lead acetate diluted 1-4. Delead the precipitate by adding 10-15 cc. of 10 per cent sulfuric acid and centrifuging out the lead sulfate. Wash this precipitate with water and make the supernatant liquid and washings to 50 cc. Pipette 10 cc. of this solution into a beaker and take just past the neutral point with 20 per cent sodium hydroxide solution, using methyl red as an indicator. Bring back to as nearly the neutral point as possible with a weak solution of trichloroacetic acid, making the solution on the acid side rather than on the alkaline. Cool the solution, add 2.5 cc. of 25 per cent potassium iodide solution, add some freshly filtered starch solution and titrate past the blue end point with 0.001M iodine solution and back
titrate with 0.001N sodium thiosulfate solution.

**Precautions to be Followed**

It was found by experience that the solution should be on the acid side rather than on the alkaline, but the nearer neutral the solution was maintained, the better the results. Sometimes, due to interfering substances in the extracts, the neutral end point color was so obscured that it was very difficult to determine. The best results were obtained when no extra water was added to these solutions before titration. Experiments showed that, if the solutions were diluted too much, high results were obtained. In some cases, however, the neutral end point was so difficult to determine that it was necessary to dilute the solutions. This end point was the most important limiting factor in these titrations. If the solution had to be diluted, however, an extra amount of potassium iodide would somewhat compensate for this dilution.

The results obtained with these three different methods are shown in Table VII.
### Table VII

**Total Iodine Titrations, Iodine Titrations of the First Lead Acetate Fraction, and Hydrogen Sulfide Generation Tests**

<table>
<thead>
<tr>
<th>Order</th>
<th>Kind of plant</th>
<th>Total Iodine titration (cc. 0.001M iodine per gram)</th>
<th>Glutathione (mg. per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Iodine titration</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Fraction 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tree fruits:**

<table>
<thead>
<tr>
<th>Rosaceae</th>
<th>Apple</th>
<th>51.62</th>
<th>2.02</th>
<th>0.62</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pear</td>
<td>13.80</td>
<td>1.71</td>
<td>0.53</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Peach (1)</td>
<td>20.82</td>
<td>2.52</td>
<td>0.77</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Cherry</td>
<td>21.00</td>
<td>0.96</td>
<td>0.28</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Plum (1)</td>
<td>41.95</td>
<td>2.50</td>
<td>0.76</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Plum (another variety)</td>
<td>47.05</td>
<td>1.99</td>
<td>0.62</td>
<td>trace</td>
</tr>
</tbody>
</table>

**Small fruits:**

<table>
<thead>
<tr>
<th>Rosaceae</th>
<th>Raspberry (1)</th>
<th>1.40</th>
<th></th>
<th></th>
<th>0.38</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blackberry (1) (El Dorado)</td>
<td>31.90</td>
<td>0.75</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Blackberry (Snyder)</td>
<td>35.25</td>
<td>2.20</td>
<td>0.63</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Strawberry</td>
<td>23.25</td>
<td>*</td>
<td>*</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Blueberry</td>
<td>18.55</td>
<td>2.40</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>40.70</td>
<td>*</td>
<td>*</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>Black Currant</td>
<td>50.10</td>
<td>*</td>
<td>*</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Gooseberry</td>
<td>25.25</td>
<td>2.72</td>
<td>0.84</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Elderberry</td>
<td>23.10</td>
<td>*</td>
<td>*</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Grape</td>
<td>20.70</td>
<td>0.30</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Vegetables:**

<table>
<thead>
<tr>
<th>Gramineae</th>
<th>Sweet Corn</th>
<th>5.05</th>
<th>0.93</th>
<th>0.28</th>
<th>0.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liliaceae</td>
<td>Onions</td>
<td>1.70</td>
<td>0.54</td>
<td>0.16</td>
<td>0.25</td>
</tr>
<tr>
<td>Polygonaceae</td>
<td>Rhubarb</td>
<td>13.10</td>
<td>1.32</td>
<td>0.41</td>
<td>0.40</td>
</tr>
<tr>
<td>Chenopodiaceae</td>
<td>Beet (1)</td>
<td>10.36</td>
<td>2.34</td>
<td>0.72</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Swiss Chard (1)</td>
<td>12.05</td>
<td>2.16</td>
<td>0.66</td>
<td>0.50</td>
</tr>
<tr>
<td>Cruciferae</td>
<td>Cabbage (1)</td>
<td>5.90</td>
<td>1.12</td>
<td>0.65</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Cauliflower (1)</td>
<td>10.50</td>
<td>1.07</td>
<td>0.33</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Horse Radish (1)</td>
<td>35.60</td>
<td>1.36</td>
<td>0.42</td>
<td>0.25</td>
</tr>
</tbody>
</table>

(Continued)
Table VII (Conc.)

Total Iodine Titrations, Iodine Titrations of the First Lead Acetate Fraction, and Hydrogen Sulfide Generation Tests

<table>
<thead>
<tr>
<th>Order</th>
<th>Kind of plant</th>
<th>Iodine titration (cc. 0.001N iodine per gram)</th>
<th>Glutathione (mg. per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Fraction 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vegetables (Con.):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leguminosae</td>
<td>Beans</td>
<td>16.21</td>
<td>2.14</td>
</tr>
<tr>
<td>Malvaceae</td>
<td>Okra</td>
<td>3.80</td>
<td>1.15</td>
</tr>
<tr>
<td>Martyniaceae</td>
<td>Martynia</td>
<td>22.20</td>
<td>2.07</td>
</tr>
<tr>
<td>Aizoaceae</td>
<td>New Zealand Spinach (1)</td>
<td>4.97</td>
<td>1.71</td>
</tr>
<tr>
<td>Umbelliferae</td>
<td>Carrot</td>
<td>17.80</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Celery</td>
<td>-----</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Parsley</td>
<td>42.10</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>Parsnip (1)</td>
<td>23.05</td>
<td>0.82</td>
</tr>
<tr>
<td>Convolvulaceae</td>
<td>Sweet Potato</td>
<td>24.19</td>
<td>0.214</td>
</tr>
<tr>
<td>Solonaceae</td>
<td>Tomato</td>
<td>9.40</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>Pepper (1)</td>
<td>20.86</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>Egg Plant</td>
<td>7.75</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Irish Potato</td>
<td>5.70</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Tobacco</td>
<td>9.34</td>
<td>1.56</td>
</tr>
<tr>
<td>Cucurbitaceae</td>
<td>Winter Squash</td>
<td>5.55</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Winter Squash (different location)</td>
<td>-----</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Cucumber</td>
<td>3.63</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>4.90</td>
<td>1.21</td>
</tr>
</tbody>
</table>

*Not determinable, due to unknown interfering substances.
+Not determined.
(1)Midrib removed.
(2)Midrib included.
Discussion of Results

All the samples used in this work were taken in the middle of the summer and young actively growing tissues were used in all cases.

These results show that glutathione is almost universally present in leaves of horticultural plants, at least during midsummer growth; and that hydrogen sulfide is produced when those leaves are brought into contact with sulfur.

With a few exceptions the results obtained by the two different methods of determinations from the leafy tissues of vegetables were more in agreement than those from fruit plants. In most cases, the total iodine titrations were lower in this class, indicating that they contain much less non-sulphydryl iodine-reducing material. In the case of the parsley, squash, and sweet potato plants the results were much higher by the hydrogen sulfide reduction method than by iodine titration. As a general rule, the iodine titrations were slightly higher than the corresponding hydrogen sulfide generations. Apparently the only explanation for these three exceptions is that either the conditions of the tissue were not favorable for the precipitation of the lead salt or that during the extraction the glutathione was either oxidized or hydrolyzed. In the case of a celery plant which had been dusted with a copper containing fungicide, a titration but
no hydrogen sulfide was obtained; whereas, from the untreated sample we obtained comparable generation and titrations, in agreement with those obtained from titration upon the dusted sample. It seems possible that some substance which is in the spray or dust inhibits the generation of hydrogen sulfide or that the hydrogen sulfide which is generated reacts with the copper in the fungicide to form copper sulfide. Also lower hydrogen sulfide generation has been noticed from samples of sprayed grape and plum leaves.

Check titrations were made without difficulty with vegetable tissues, but in the case of leaves from fruit-bearing trees and shrubs the proper end point was difficult to obtain in some cases.

Isolation of Glutathione from Leaves of Horticultural Plants

Preliminary experiments were undertaken to ascertain the possibility of actually isolating glutathione from the type of plant tissues studied, as a further confirmation of its presence in these tissues. There was insufficient time to continue the isolation of glutathione to the final crystalline form, however.

Preliminary extractions were made, using strawberry and cabbage leaves. The leaves were extracted as outlined above
for the fractionation experiments, with the exception that the leaves were ground in a special grinder and then dropped into boiling water, boiled for 10 minutes and filtered. The filtered extract was treated with lead acetate in a slightly acid solution, the precipitate deleadcd, the solution made .5N in sulfuric acid, and cuprous oxide added in the same way as with the yeast extract from which our supply of glutathione had been obtained.

In the case of the extract from the strawberry leaves the characteristic white crystalline salt with a silky sheen precipitated out, though it was more or less contaminated with metallic copper. This precipitate was separated by the use of the centrifuge, suspended in water and tested for the -SH group with sodium nitroprusside and a strong positive test obtained. Hence, it had all of the characteristics of the copper-glutathione salt as precipitated from yeast extract.

No appreciable amount of copper salt was secured from the cabbage leaf extract, though a strong -SH test and iodine titration was obtained. Extractions from cabbage leaves using ether, alcohol and water, and acetone and water, were made. On these, strong -SH tests were obtained in both cases, but no appreciable quantities of copper salt. In the case of the cabbage leaves, so much sulfuric acid had to be added to thoroughly decompose the lead salt that to reduce the solution to 0.5N it had to be diluted to such a large volume that
undoubtedly the concentration of the glutathione per cc. was so small that the peptide would not precipitate at this concentration.

General Discussion

One of the severest handicaps in investigating the presence, action, and physiological function of glutathione in plant tissues is that so little is known of the other constituents of these tissues. Nearly all studies upon glutathione have been carried on with animal tissues and blood, and the methods used and conclusions reached are not necessarily applicable to plant tissues.

Many difficulties and unexplainable results which were encountered during this work would be easier of solution if complete analyses could be obtained of these horticultural plant tissues.

The problems of how glutathione is synthesized in plant tissues, why it varies in amount during different parts of the season, what its function is in the metabolism of the plant, and whether its decrease is a question of hydrolysis, oxidation, or some other process, still remain obscure.

A method of estimating the oxidized glutathione in plant tissues would help to solve the last mentioned of these problems.
We are still in doubt with regard to the loss of glutathione from dried tissues. Here again it is possibly a question of either oxidation or hydrolysis.

We are of the opinion that glutathione can be isolated from most of the leafy tissues and are confident that if time had been available to start with larger quantities of material we could have isolated pure glutathione from the strawberry leaf. The copper salt of glutathione was obtained from this plant and if sufficiently large quantities were used the isolation of glutathione should be accomplished.
SUMMARY

(1) The existing knowledge concerning the presence of glutathione in plant tissues and its relation to the generation of hydrogen sulfide when these tissues are brought into contact with sulfur has been reviewed, and the possible relation of this phenomenon to the use of sulfur (or its compounds) as a fungicide on horticultural plants discussed.

(2) A review of the possible relations of glutathione to the physiological functions of plant and animal tissues has also been presented.

(3) A quantity of pure glutathione was isolated from yeast and used as a basis for study of methods for its determination and for the preparation of standards for later work.

(4) Several proposed methods for the quantitative estimation of glutathione in animal tissues were tested to determine their applicability for use with plant tissues.

(5) An iodine-titration method, modified to suit the conditions of work with plant tissues, and a hydrogen-sulfide-generation method similarly modified, were found to give results which were generally in satisfactory agreement, when applied to various samples of leaves from horticultural plants.

(6) These two methods were applied to samples of leaves
from 39 different kinds of fruits and vegetables, and the results tabulated.

(7) It was found that glutathione was present in determinable, although varying, amounts in every one of these tissues; from which it is concluded that glutathione is probably universally present in the actively-growing foliage tissue of horticultural plants.

(8) It was shown that, in every case, the presence of this glutathione in the tissues results in the liberation of hydrogen sulfide when sulfur is applied to the surface of the leaves.

(9) Some evidence was obtained to show that the proportions of glutathione which is present in such tissues varies with their stage of development and with the fruit-production process, in the case of fruit-bearing plants.

(10) Some indications were obtained that glutathione is also present, probably in much smaller amounts than in the foliage, in the bark and buds of the plants which were examined; but the season of the year at which the work was done prevented the obtaining of conclusive evidence on this point.

(11) The work of other investigators has shown that sulfur, when in contact with the spores of fungous diseases, liberates hydrogen sulfide, which is toxic to the spores and acts as a fungicide to destroy them. The work here re-
ported shows that a similar reaction undoubtedly takes place with the glutathione which is present in the leaves when sulfur is applied to the foliage of orchard or garden crops for fungicidal purposes. It is possible that, under proper conditions of temperature and humidity, injury may be caused to the foliage by the hydrogen sulfide thus liberated. Some evidence of such injury exists, and the result of these studies, indicating as they do that glutathione is present in such tissue in widely varying amounts in different species of plants and at different seasons of the year, suggest this as a possible explanation of such injuries.

(12) While it was not within the range of the studies here recorded, it is apparent that a further study of the possible relation of glutathione content of foliage to injury to such foliage from applications of sulfur fungicides would be a fruitful field of investigation.
LITERATURE CITED

(1) Marsh, R. W.

(2) McCallan, S. E. A., and Wilcoxson, Frank.

(3) Barker, B. T. P.

(4) Sluiter, Emma.

(5) Guthrie, John D., and Wilcoxson, Frank.

(6) Streeter, Leon R.
Unpublished data communicated verbally before his death in 1932.

(7) Polacci, Egidio.

(8) Selmi, F.

(9) Cugini, G.
(10) De Rey-Pailhade, J.
Sur un Corps d'origine Organique Hydrogénant le Soufre à Froid.
Cited by McCallan and Wilcoxson (2). p. 3.

(11) Pozzi-Escot, M-Emm.
The Reducing Enzymes.

(12) Heffter, A.
Die reduzierende Bestandtiele der Zellen.
Medizin-Naturwissenschaft Archiv., 1, 81-108 (1903).

(13) Hopkins, Sir Frederick Gowland.
On an Autoxidizable Constituent of the Cell.

(14) Stewart, Corbet Page, and Tunnicliffe, Hubert Erlin.
Glutathione. Synthesis.

(15) Hunter, George, and Eagles, Blythe Alfred.
J. Biol. Chem., 72, 147-166 (1927).

(16) Hopkins, Sir Frederick Gowland.
On Glutathione: a Reinvestigation.

(17) Kendall, Edward C., McKenzie, Bernard F., and Mason, Harold L.

(18) Kendall, Edward C., McKenzie, Bernard F., and Mason, Harold L.
A Study of Glutathione. III. The Structure of Glutathione.

(19) Kendall, Edward C., Mason, Harold L., and McKenzie, Bernard F.
A Study of Glutathione. IV. Determination of the Structure of Glutathione.

(20) Mason, Harold L.
A Study of Glutathione. V. The Spontaneous Cleavage of Glutathione in Aqueous Solution.


The Glutathione Content of the Blood of Cancer Patients.
Biochem. Z., 260, 130-136 (1933).
Abs. in C. A., 27, 3522 (1933).

(33) Bierich, R., and Rosenbohm, A.
The Glutathione Content of Tissues.
Abs. in C. A., 27, 2161 (1933).

(34) Waelsch, H., and Weinberger, E.
Blood-glutathione in Fever.
Abs. in C. A., 25, 529 (1933).

(35) Quastel, J. H.
Glutathione, Iodoacetic Acid and Glucose Metabolism.
Abs. in C. A., 27, 2477 (1933).

(36) Wolff, R., and Manjean, S.
Reduced Glutathione Content of Guinea-Pig Tissues.
Abs. in C. A., 27, 529 (1933).

(37) Zunz, Edgard.
The Effect of Insulin on the Blood Content of Reduced Glutathione.
Abs. in C. A., 26, 2506 (1932).

(38) Binet, Leon, and Arnaudel, A.
Quantitative Variations of Reduced Glutathione in the Liver under Diverse Experimental Conditions.
Abs. in C. A., 26, 2225 (1932).

(39) Medvedev, N. B.
Glutathione in Organs in Presence of Experimental Tumors.
Abs. in British Chem. Abst., 1932, 35.

(40) Binet, Leon, and Magrou, J.
The Increase of Glutathione in Tumors of Plants.
Compt. Rend., 192, 1415-1416 (1931).
Abs. in C. A., 25, 4300 (1931).

(41) Grassman, W., Schoenebeck, O. V., and Eibeler, H.
Plant Proteases (XVI). Activation of Animal and
Plant Proteases by Glutathione.
Abs. in C. A., 25, 1870 (1931).

(42) Vivario, R., and Lecloux, J.
Formation of Glutathione during Growth.
Abs. in C. A., 25, 3688 (1931).

(43) Guthrie, John D.
The Isolation of Glutathione from Potato Tubers
Treated with Ethylene Chlorhydrin.

(44) Barker, B. T. P., et al.
Investigations of the Fungicidal Action of Sulfur.
1929, 57-75 (1919).
II. Ibid., 1927, 72-80 (1927).
1922, 130-143 (1929).

(45) Wilcoxson, Frank, et al.
The Fungicidal Action of Sulfur.
I. Phytopathology, 20, 391-471 (1930).
III. Ibid., 2, 509-523 (1931).
IV. Ibid., 4, 415-424 (1932).

(46) Pirie, Norman Wingate.
The Preparation of Glutathione from Yeast and Liver.

(47) Pirie, Norman Wingate.
Cuprous Glutathionate.

(48) Kozlowski, Anton.
Kupferverbindung des oxydierten Glutathion der Hefe.
Biochem. Z., 241, 403-408 (1931).

(49) Tunnicliffe, Hubert Erlin.
Glutathione. Relation between the Tissues on the
Oxidized Dipeptide.

(50) Perlzweig, Wm. A., and Delrue, Georges.
The Use of the Starch-Iodine End-Point in Tunnicliffe's
(51) Gavilescu, Nicolae.
The Iodine Method for the Determination of Glutathione.

(52) Woodward, Gladys E., and Fry, Edith G.
The Determination of Blood Glutathione.

(53) Fleming, R.
A Modification of the Sodium Nitroprusside Reaction for Hydrogen Sulfide in Low Concentrations.
Abs. in C. A., 25, 337A (1921).

(54) Schelling, Victor.
A Study of Blood Glutathione.

(55) Fleming, R.
Determination of Glutathione by a Colorimetric Method.

(56) Almy, L. H.
A Method for the Estimation of Hydrogen Sulfide in Proteinaceous Food Products.


(58) Guthrie, John D.
Personal communication (1933).
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Approved by:

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Graduate Committee

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