CHELATE COMPOUNDS AS SEED PROTECTANT FUNGICIDES AND THE EVOLUTION OF TOXIC GASES FROM CHELATE FUNGICIDES RELATED TO DITHIOCARBAMIC ACID

A THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN AGRICULTURE

by

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in the

DEPARTMENT OF AGRONOMY

WE ACCEPT THIS THESIS AS CONFORMING TO THE
STANDARD REQUIRED FROM CANDIDATES FOR THE
DEGREE OF MASTER OF SCIENCE IN AGRICULTURE

MEMBERS OF THE DEPARTMENT OF AGRONOMY

THE UNIVERSITY OF BRITISH COLUMBIA

APRIL, 1951.

ABSTRACT

An attempt was made to create effective fungicides by combining fungistatic chelates with mercury and other metals. Two chelates, cupferron and phenyl-thiodantoic acid, were found highly fungistatic, and the effectiveness of their mercury complexes as seed protectants was similar to that of the standard fungicide Arasan.

The mercury complex of cupferron was found relatively unstable, and mercury was easily displaced by metals forming more stable complexes. On the other hand, mercury could not be displaced from the phenyl-thiohydantoic acid complex, and fungistatically, this compound appeared to function as an undissociated molecule. Slopes of dosage-response curves of both chelates were decreased by the inclusion of mercury, and the efficiency of both compounds increased as respiration inhibitors. However, both chelates and their mercury complexes were more effective growth inhibitors than respiration inhibitors, and their fungistatic values could not be estimated by respiration methods.

Evolution of toxic gases from dithiocarbamate chelate fungicide during decomposition was measured in the Warburg

manometer. It was concluded that the dialkyl series of dithiocarbamates decomposed in the presence of slightly acid buffers to produce carbon disulphide and a dialkyl-amine. Dithane, on the other hand, decomposed with evolution of hydrogen sulphide and carbon disulphide, and, it was assumed, with a residue of ethylene thiourea.

ACKNOWLEDGMENTS

Grateful acknowledgments are due to Dr.W. Newton, of the Dominion Laboratory of Plant Pathology, Saanichton, for many helpful discussions and suggestions in connection with this work.

Grateful acknowledgments are due also to Dr.J.C.Woodward, Dominion Agricultural Chemist, for permission to submit this thesis based on work carried out at Saanichton, while the author held the position of Agricultural Research Officer in the Division of Chemistry of Science Service.

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INTRODUCTION

To any study involving the control of disease organisms by chemicals, Ehrlich's (25) concept of "dosis curativa" and "dosis tolerata", is of fundamental importance. In other words, these studies are concerned basically with the relationship between the dose toxic to the parasite, to the dose tolerated by the host.

In the early history of plant pathology, the inorganic compounds used for control of diseases were generally toxic to both the parasite and the host. Thus effective disease control was obtained only with some corresponding damage to the plant.

Following the introduction of the dithiocarbamate fungicides by Tisdale and Williams (52) in 1944, there appeared two very important features favoring organic over inorganic compounds for plant disease control. The first feature was that many organic compounds tend to be fungistatic, rather than fungicidal; that is, they inhibit cell growth, without at the same time, killing the cells. Secondly, the growth inhibiting action of organic compounds tends to be more specific than the action of inorganics. Both these features assist in selecting organic compounds which have a broader separation between the "dosis curativa" and the

"dosis tolerata".

The present paper consists of the following two phases of the problem of plant disease control by organic fungicides.

1 The production of active fungistatic agents from compounds related to the dithiocarbamates through common chelating properties.

2 A study of the conditions producing decomposition of dithiocarbamates, and the identification of the toxic gases given off from these compounds.

LITERATURE REVIEWS

Seed Protectant Fungicides

Due to the ubiquitous nature of soil-born plant pathogenic fungi in agricultural areas, McNew (38) has pointed out that it requires unfavourable weather conditions only to produce injury. Damping-off of seedlings by soil inhabiting fungi is considered by Leukel (29) to be the most serious problem encountered in obtaining good stands of vegetable crops, and one of the major types of damage of all crops. Although many fungi may be involved in the damping-off of seedlings, Heald (17) considers Pythium debaryanum to be of major importance. Of the other molds involved, Heald (17) includes Pythum ultimum, and members of the genus Phytophthora.

Seed treatment may be done for one of two purposes: to protect the seed from soil-born organisms, or to kill disease organisms that over-winter in, or on, the seed coat. Compounds of the first class are known as "seed protectants", and of the second, "seed disinfectants". Of these two types of compounds, seed protectants are the most universally required.

The control of damping-off by seed treatment has been discussed by Kadow and Anderson (28) and by Horsfall (24), while Taylor and Rupert (50) have given a review of the history of vegetable seed protectants. More recent developments of control

measures are given in a review by Leukel (29).

According to Martin (30), early methods of seed treatment consisted in soaking seeds in solutions of fungicides, principally copper sulphate and formaldehyde. Leukel (29) points out that following the discovery of copper carbonate dust as a bunticide, there was a gradual change to dust fungicides. This change was attributed to the ease of handling of dust fungicides made possible by the development of various home-made mixers and treaters. The introduction of red and yellow cuprous oxides by Horsfall (22) (19), and of zinc oxide (23) further stimulated interest in the use of dusts as seed protectants.

Organic seed protectants in the form of dusts date from the introduction of tetrachloro-para-benzo-quinone, or Spergon, by Cunningham and Sharwell (10) in 1940. Spergon was followed quickly by tetramethyl-thiuramdisulphide, or Arasan, and ferric dimethyl-dithiocarbamate, known as Fermate. These compounds received favorable comments from McNew (39), Taylor, Rupert and Leach (51), and many others.

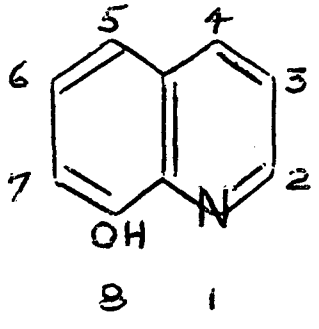
In the final analysis, the judgment of any fungicide is an economic one. McCallan (35) has discussed the favourable position held by seed protectants in this respect. Because of the costs of handling and the large amounts of material required, field sprays and dusts are limited to the high value per acre crops. On the other hand, seed protectants are being used profitably on the low

value per acre grain crops. The corollary is also true: that expensive materials in the form of organics or mercurials, regardless of effectiveness, can find economic use only in seed treatment.

Chelate Compounds and Chelate Fungicides

1 Characteristics of Chelating Agents

The first use of a chelate as a fungicide was in 1914, when 8-hydroxy-quinoline, or oxine, was tried by Hiltner (20) as a seed protectant. The same compound, under the proprietary name of Chinosol, had been placed on the market in Germany as a bacteriocide, twenty years earlier.



8-HYDROXY-QUINOLINE.

As can be seen from the accompanying formula, oxine consists of a toxic phenolic ring, attached to a non toxic pyridine ring (25). This compound appeared for many years to possess a number of toxicological reactions which were structurally anomolous. These reactions were:

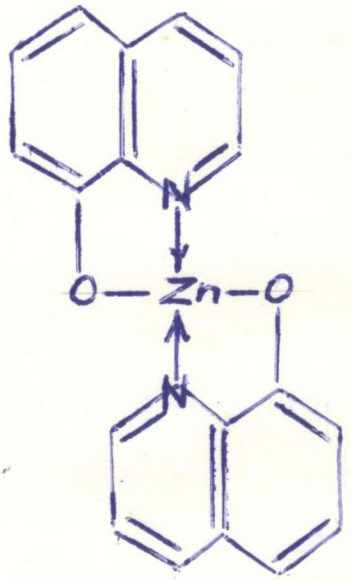
(a) The compound is fungistatic, not fungicidal, as would be expected by the presence of the toxic phenolic ring.

(b) Only when the hydroxyl group is in the eighth position is the compound fungistatic.

(c) The compound is more effective against Gram positive than against Gram negative organisms.

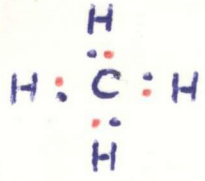
These ostensibly anomalous reactions were explained by Albert (1) and by Zentmyer (57), in 1944 when they co-ordinated anti-bacterial and anti-fungal activity of oxine with chelating ability.

The term "chelate", derived from "chela", the great claw of the crustations, was introduced by Morgan and Drew in 1920 (34). Chelate is a descriptive term for the caliper-like groups, which, functioning as two associated units, fasten onto a central metallic atom to form hetrocyclic rings. For example, oxine is able to bind zinc according to the accompanying formula. Here zinc is linked simultaneously to phenolic oxygen by normal valencies, and by nitrogen through co-ordinated covalent bonds.



ZINC COMPLEX.

The origin of co-ordinated covalent bonds may be briefly stated as follows: in the normal covalent bond of organic compounds, the single bond consists of a pair



COVALENT BONDS OF METHANE.

of shared electrons, as illustrated below for methane. Thus one electron comes from carbon, and the other from hydrogen. The shared electrons, constituting a single bond, are conventionally represented by a single line.

In some conditions, both electrons may be donated by a single atom. Referring to oxine, the valence of nitrogen in the pyridine ring is three, while nitrogen has a potential valence of five. Zinc on the other hand, has two valence electrons only, and these are already taken up by oxygen. Accordingly, both the electrons forming each single bond between nitrogen and zinc must be donated by the nitrogen atom. The direction of electrons donation is indicated conventionally by the use of an arrow.

As mentioned previously, fungistatic activity of oxine is limited to the anologue with the hydroxyl group in the eighth position. Chelating activity is similarly limited, since rings formed of more than five elements in this type of structure are unstable. Such instability is explained logically by the strain theory. The normal, (and hence the most stable) angle between valencies of the carbon atom directed at the verticies of a regular tetrahedron are 109°, while the angle between the sides of a regular pentagon are almost identical at 110°. The corresponding angles of a hexagon are, however, considerably larger at 120°. Elimination of strain in six membered chelate rings formed from oxine by zigzagging in two planes, such as Desha (11) describes for the benzene ring is apparently not possible. Thus such six membered rings formed from 7 hydroxy-quinoline are unstable,

and Albert (3) reports chelating ability as well as fungistatic ability is of a low order.

In the original oxine molecule, only two hydrophillic groups are present, - OH and -N=. Following complex formation, both these groups are masked, with the result that the complex is exceedingly insoluble in water. Accordingly, when oxine is added to a fungus culture, trace metals are precipitated and growth ceases, as Zentmyer (58) has shown, as a result of trace metal deficiency.

The example of metal-complex formation of oxine is in many respects typical of the action of chelates generally. Information on the action of other chelating agents is to be found in the monographs by Welcher (54) and Zoe and Sarver (59). Some additional features of chelate action pertinent to the following discussions, are given from Welcher.

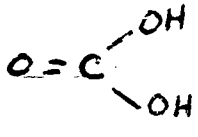
Oxine is an example of a chelate which forms nonionizeable complexes, referred to as inner complex salts. Inner complex salts react more as pure organics than as inorganic compounds, and the metal contained in such complexes cannot be detected by ordinary means, such as precipitation with hydrogen sulphide. These complexes, though usually very insoluble in water, are soluble in organic liquids.

Chelating agents generally combine with a considerable number of metals, and combine specifically only between certain narrow pH limits. The stability constants of different metal complexes of a chelating agent vary considerably. Mellor and Maley (32) suggested

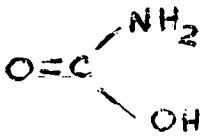
that the order of stability constants of metal-chelate complexes is probably similar regardless of chelating agent. Further evidence supporting this view is presented in the experimental portion of this thesis.

Most important of the chelates used as fungicides are the dithiocarbamates. In fact, McCallan (36) considers the dithiocarbamates the most effective organic fungicides so far discovered. The introduction of these compounds dates from 1934, when Tisdale and Williams (52) obtained a patent on their fungicidal use. The fungicidal action of these compounds has been studied in detail by Goldsworthy, Green and Smith, (16), and Dimond et al (12), while the present status of the dithiocarbamates as fungicides has been reviewed by Heuberger (18).

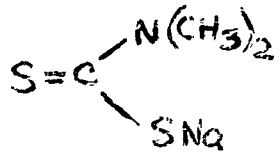
The dithiocarbamate fungicides are related to the chelate sodium dimethyl-dithiocarbamate, which in turn is related to carbonic acid as illustrated below.



CARBONIC ACID

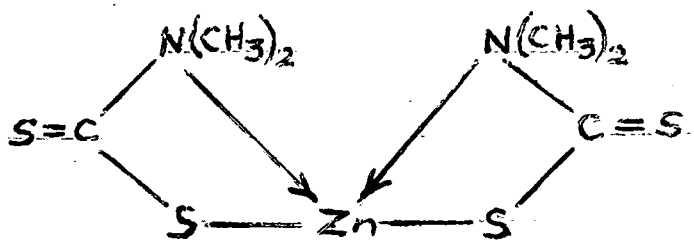


CARBAMIC ACID (UNSTABLE)



SODIUM DIMETHYL DITHIO-CARBAMATE

Sodium dimethyl-dithiocarbamate is a typical chelating agent, forming co-ordination compounds with metals of the following type:



DIMETHYL-DITHIOCARBAMATE ZINC COMPLEX.

As Horsfall (25) has shown, sodium dimethyl-dithiocarbamate is also a very powerful fungistatic agent. Nevertheless, the compound cannot be used directly as a protective fungicide because of high solubility. Insoluble derivatives have, as a result, been prepared by the following three methods:

1 By combining two molecules of the chelate through sulphur to produce tetramethyl-thiuram-disulphide, now known as Arasan.

2 By combining two molecules through the methyl groups to produce disodium-ethylene-bisdithiocarbamate, or Dithane.

3 By preparing insoluble chelate complexes from metal salts as previously illustrated. The best known of these, are the iron and zinc complexes, Fermate and Zerlate.

Reference to the formulae just given shows that while Dithane retains its chelating ability, Arasan does not. Thus, by

replacing sodium in Dithane, even more insoluble materials are produced. The best known of these compounds is the zinc complex Parzate. The chelating ability of Dithane is also of importance to the fungistatic action of this compound, as will be discussed later.

Very little has been reported on the value of other chelates as fungicides. Horsfall and Zentmyer (26) have shown however, that the chelates cupron, cupferron, and salacylaldoxime are active fungistatic agents. Horsfall (25) attempted to combine the toxicity of copper with the toxicity of salacylaldoxime in the form of the copper complex, but found the compound inactive. Copper xanthates have been tried as field protective fungicides, but were found wanting (25) (60). As will be explained later, copper was probably a particularly bad choice of metal in these cases. On the other hand, since this work was undertaken, the copper complex of cupferron, a compound examined in some detail later, has received favourable criticism as a protective fungicide, (27), and also as a preserver of fishing nets (27).

Mode of Toxic Action of Chelate Fungicides

Apart from oxine, little work has been done on the mode of toxic action of chelate fungicides. Oxine has gained particular attention since its action against bacteria is closely related to that of penicillin.

Gale (15) in describing this relationship, points out that amino acids are taken up by Gram positive bacteria in two ways,

(a) by simple diffusion,

(b) by an active cell process requiring energy.

It is this latter energy requiring transfer of amino acids which is inhibited by penicillin, and all cells able to synthesize their own amino acids are, as a result, resistant. Two trace metals, manganese and magnesium, play an essential part in this energy transfer. Oxine, by inactivating these two metals, is able to inhibit the absorption of amino acids.

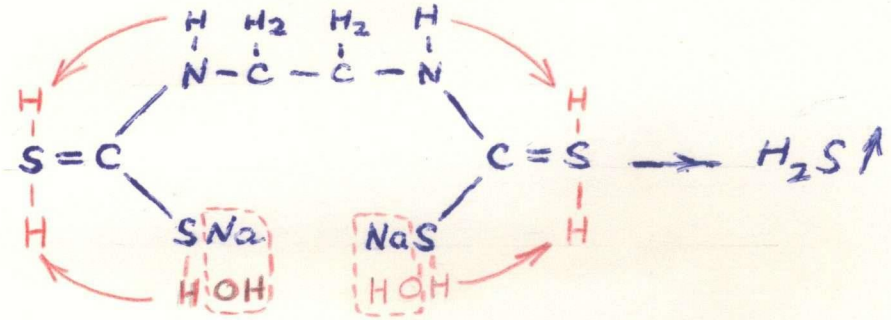
Inactivation by oxine of other trace metals, particularly copper and zinc, interferes with cell respiration and fermentation, but this requires a higher concentration of oxine than that required to inhibit amino acid absorption.

Horsfall (25) suggested that the fungistatic action of other chelates, namely quinone dioxine, salacylaldoxine and the dithiocarbamates may also be due to their action in precipitating trace metals.

A very significant fact concerning the action of chelates upon growth has been pointed out by Albert (2). In order for growth inhibition to take place, the trace metals on a bacterial surface must be inactivated. Apparently metals may be held by a biological surface in such a way that only chelates of appropriate architecture may have access to them. Albert (3) considered that this ability was limited to relatively few chelates, and that these were mainly related to oxine. Nevertheless, data cited by Horsfall (25) and results given in the experimental part of the present paper, indicate that a considerable number of chelates quite unrelated to oxine, are growth inhibitors.

Foote, et al (14) attempted to determine the inhibitory effect of Dithane and Arasan upon the enzyme carboxylase with the Warburg manometer. However, they reported inconclusive results, which they attributed to the insolubility of these compounds.

A further mode of toxic action of chelates which may function independently to that of metal precipitation, is the evolution of toxic gases. This type of action appears limited to those chelates containing sulphur. Dimmond and Horsfall (7) stated that the greater part of the toxicity of Dithane appeared to be due to the evolution of hydrogen sulphide, and suggested the following scheme to explain the evolution of this gas.



HYDROLYSIS OF DISODIUM ETHYLENE-BIS-DITHIO-CARBAMATE (DITHANE).

Hydrolysis first took place, followed by a double tautomerism between nitrogen and sulphur on the one hand, and between the two sulphur atoms on the other. Instability resulted from this dynamic state, and hydrogen sulphide was split off.

This explanation was apparently accepted until recently Rich and Horsfall (45) gave evidence that the gas given off by Dithane was a mixture. The second gas appeared to be even more toxic than

hydrogen sulphide. As a result, Rich and Horsfall concluded that no satisfactory theory has yet been offered to explain the fungicidal mechanism of Dithane. They express the hope, however, that a clue may be found by attempting to isolate and identify the toxic gases from this compound. As will be shown later in the experimental part of this work, the second toxic gas given off by Dithane, is carbon disulphide.

No reports have been made of the evolution of gases from other dithiocarbamate fungicides. However, it will be shown later, that probably all of these fungicides owe at least a part of their effectiveness to the evolution of toxic gases.

Laboratory Assay of Fungicides

Two principal methods for the laboratory assay of fungicides have been developed. The first of these is based upon the inhibition of spore germination, and has been adopted as a standard method by the Fungicide Committee of the American Phytopathological Society (4). The second is based upon the inhibition of mycelial growth, a method first proposed by Bateman (8), and developed later by Vincent (61).

The basic principal behind both these methods is the examination of the fungicide at different concentration levels. As the dose is increased, so is the inhibition increased. When the percentage inhibition is plotted against concentration, a dosage-response curve is produced.

Wilcoxon and McCallan (55) have shown that if such data

obtained from spore germination tests are plotted on logarithmic probability paper, a linear dosage-response curve results. Bateman (8) obtained similar linear dosage-response curves from mycelial inhibition data by plotting the logs. of concentration against logs. of percentage inhibition.

The great value of linear dosage-response curves in evaluating the fungistatic action of different compounds has been discussed by Dimond et all (13), and reviewed by Horsfall (25). It appears that different fungicides have different dosage-response curve slopes, and it is assumed that compounds with different slopes have different modes of action. Where dosage-response curves of two fungicides cross, a reversal of the order of effectiveness of toxic action results. Other factors being equal, effectiveness of toxic action of a fungicide with a flat slope will decrease more slowly with weathering, than that of a fungicide possessing a steep dosage-response curve.

Although more generally used, the spore germination method suffers the disadvantage that the test organism must germinate by germ tube. Thus, the mycelial inhibition method must be used in evaluating chemicals against damping-off species of Pythium or Phytophthora, since these organisms germinate by swarm spores.

EXPERIMENTAL

Part 1

The Preparation and Evaluation of Chelate Complexes as Seed Protectant Fungicides

An attempt was made to synthesize effective fungistatic agents by combining fungistatic chelate radicals with metallic ions. For this purpose, a number of chelates were first examined for fungistatic activity, and the most active of these selected for further study.

The method of measuring fungistatic activity was based on the procedure developed by Bateman (8) and Vincent (61). Briefly, this procedure consists in determining the mycelial growth rate of a fungus on agar plates containing sub-lethal doses of fungistatic agent.

Agar dilutions of the toxic agent are first prepared in Petri plates. When cool, these plates are inoculated with a fungus culture. The plate cultures are then incubated along with control cultures containing no toxic agent, and radial growth measurements made of the developing colonies at suitable time intervals.

Growth rates are determined by plotting the increase in

diameter of the colonies against incubation time, and drawing the best straight line through the points.

The percent of growth retardation of each concentration of toxic agent is then calculated by the following formula:

$$R = \frac{C - T}{C} \times 100\%.$$

Where R=% retardation, C= growth rate of control T= growth rate in presence of toxic agent.

The relationship between concentration and percent retardation is obtained by constructing dosage-response curves. These curves are obtained by plotting the retardation values against concentration on log. paper, and drawing the best straight line through the points.

Phytophthora erythroseptica Pethy. was the test organism used throughout this work. This mold was chosen since its suitable growth rate and colony features allowed accurate growth measurements, and because it is representative of organisms causing damping-off of seedlings in soils. Dosage-response curves produced with this organism were linear, providing measurements were commenced after colony diameter had reached 10 mm., and the total incubation period did not exceed five days.

Evidence was obtained that the position of the dosage-response curve may be affected by the nature of the agar medium as illustrated in Table I.

Table I

Inhibition of P. erythroseptica growing on corn meal and on oat meal agars by mercuric chloride

	% inhibition of growth per 100 ($x10^{-3}$) Gm. $Hg.-Cl_2$							Concentration at 50% inhibition ($x10^{-3}$) grams 100 ml.
	1.1	1.2	1.5	1.7	2.0	2.3	2.6	
Corn Meal	29	45	60	82				1.3
Oat Meal				25	35	47	54	2.4

The organism appeared even more susceptible to the action of mercuric chloride on a synthetic asparagine medium. This medium could not be used, however, since the mold grew as an irregular colony, and accurate growth measurements were impossible. In order to obtain comparable results, a large quantity of oat meal extract was prepared and used throughout the following experiments. This extract was prepared according to Rawlins' procedure (43) by heating 50 grams of oat meal in one liter of water at 70° for 1 hour.

Since the method of measuring growth in Petri plates necessitates a clear medium, the extract was filtered. Sufficient water was then added to produce a final nitrogen content determined by the Kjeldhal procedure of 0.5%, and the extract sterilized at 15 lbs. pressure for 20 min. The agar medium used in the following

experiments was prepared from this extract by adding 15% agar to suitable portions of extract, and re-sterilizing the medium.

Agar dilutions of the toxic agents were made by adding the toxic agent to hot, sterilized agar in Erlenmyer flasks. Fifteen ml. portions of agar were then dispensed into Petri plates, five plates per dilution. Seeding was accomplished by placing on the centre of each agar plate a 5.0 mm. disc of agar-mycelium cut from a seven day oat meal agar plate culture of P.erythroseptica.

The plates were incubated at 24°C, and colony diameters measured at approximately 24 hour intervals.

Inhibition values of eight chelates, and mercuric chloride are given in Table II. Concentrations producing fifty percent inhibition were estimated by interpolation of the dosage-response curves drawn from these data.

Data from Table II show that all eight chelates are active fungistatic agents. However, fungistatic values vary considerably, and only cupferron (ammonium-nitrosophenyl-hydroxylamine) and phenyl-thiohydantoic acid possess values of the same high order as that of mercuric chloride. Cupferron and phenyl-thiohydantoic acid were selected therefore as chelates with the potentialities for producing effective fungistatic complexes with metals.

Table II

Inhibition of Mycelial Growth of P.erythroseptica

by Chelating Agents and Mercuric Chloride

Compound	Percentage Inhibition: Gms. per liter ($x10^{-2}$)						Gms. per liter ($x10^{-1}$)					**I_{100}
	.08	1	2	3	6	8	1	2	3	6	8	
*Cupferron	30	50	98									.0002
Phenylthio-hydantoic acid		6	20	48	95							.005
Mercuric Chloride		10	35	73	100							.004
Salacylald-oxime					18	30	39	98				.02
Pyrogallol							85	90	94	100		.05
Benxoin-oxime							65	79	89	98		.05
Demethyl-glyoxime								28	40	52	80	.09
Gallo-cyanine								15	25	34	66	.11

*Cupferron concentration ($x10^{-1}$)

**Concentration in gm. per liter producing 100% growth inhibition.

Preparation of Metal Complexes of Cupferron and Phenyl-thiohydantoic acid

Cupferron complexes were prepared by adding solutions of metal salts to aqueous solutions of chelating agent. All metal salts were in the form of chlorides, except lead acetate. Sufficient acetic acid was added to the lead solution to prevent hydrolysis and precipitation of the hydroxide upon the dilution. To 150 ml. of a 1.0% solution of cupferron, 200 ml. of metal salt solution were added, containing five times the equivalent weight of metal required for total precipitation. All metals were in the divalent state, and it was assumed that the chelate radicals were bidentate. By precipitating the chelate radical completely, tests for the presence of chloride or lead in the wash water from the precipated complex was sufficient to establish purity. The precipitate was allowed to settle for one hour. Filtering was then done in a frittered glass filter, and the complex washed with hot distilled water. Washing was continued until no test for chloride could be obtained in the wash water with silver nitrate, or lead with hydrogen sulphide. All complexes were finally dried over sulphuric acid in a desiccator.

Preparation of metal complexes of phenyl-thiohydantoic acid were carried out in a similar fashion to those of cupferron. However, since this chelate is relatively insoluble in cold water, precipitation was done from hot solutions, and the complexes were washed with boiling water. In order to induce precipitation of

zinc, the pH of the zinc chloride was first adjusted to pH 5.5 with dilute ammonium hydroxide.

Evaluation of Metal Complexes by Greenhouse Experiments

An evaluation of the metal complexes as seed protectants was then made by a procedure based upon that of McCallan (37). In outline, this procedure consisted in determining the relative emergence resulting from seeds treated with metal complexes when planted in soil infected by the damping-off organism, Pythium ultimum.

Due to a limited amount of metal complexes available, radish seeds only, of Sparkler variety, were used in the first test. Seeds were treated with .0625% of seed weight of chemical by shaking in glass vials for fifteen minutes.

Infected soil was prepared by adding to freshly sterilized soil, three day cultures of Pythium ultimum on unhusked barley. An equivalent of 50 g. of infected barley was added per flat of soil. The flats were placed in the greenhouse, watered, and covered with wax paper for two days. After removing the paper, the treated seeds were planted in randomized ten-seed rows. Controls consisting of untreated seed, seed treated with a standard seed protectant fungicide Arasan, and seed planted in sterile soil were included. Final counts of emergence were made after ten days. This test was repeated twice, with essentially similar results. The percentage emergence obtained from seeds treated with the different complexes are given in Table III.

Table III

*Percentage Emergence of Chelate-complex Treated Seed

Metal	Percentage Emergence	
	Cupferron	Phenyl-thio hydantoic acid
Mercury	77.5	70.
Zinc	60.	52.5
Cadmium	58.8	
Lead	61.5	
Manganese	59.8	
Copper	60.	60.
Arasan	65.0	
Control	45.5	
Sterile soil	74.5	

Least significant difference 21%

*Mean emergence from nine replicates of 10 seed rows.

Although the percentage emergence of seeds obtained by all treatments is somewhat higher than that from the untreated control, only the results of the two mercury complexes are outstanding. The actions of the two mercury complexes were as a result, examined in more detail.

Using the two mercury-complexes with Arasan as a control, a second test of seed protectant values was made, using pea seed of the Lincoln variety.

Since McCallan (37) reports pale coloured pea seeds more susceptible to damping-off than green seeds, pale seeds were selected for use. Seeds were treated at the following three levels, expressed as percentage of seed weight:

.015% .0625% and 0.25%.

The seeds were planted as described for radish seed, and a final emergence count made after fourteen days. Dosage-response curves were drawn from the resulting data by the method of Wilcoxon and McCallan (55). Since resistance to disease is normally distributed among seeds, Wilcoxon and McCallan have shown that when the percentage emergence in the form of probits is plotted against logs. of concentration, a dosage-response curve in the form of a straight line results.

The zones of error of the ED50 (percentage dose for 50% emergence) were calculated by the graphical method of Wilcoxon and McCallan (55). The resulting dosage-response curves plotted on a logarithmic-probability scale are shown in Fig. 1.

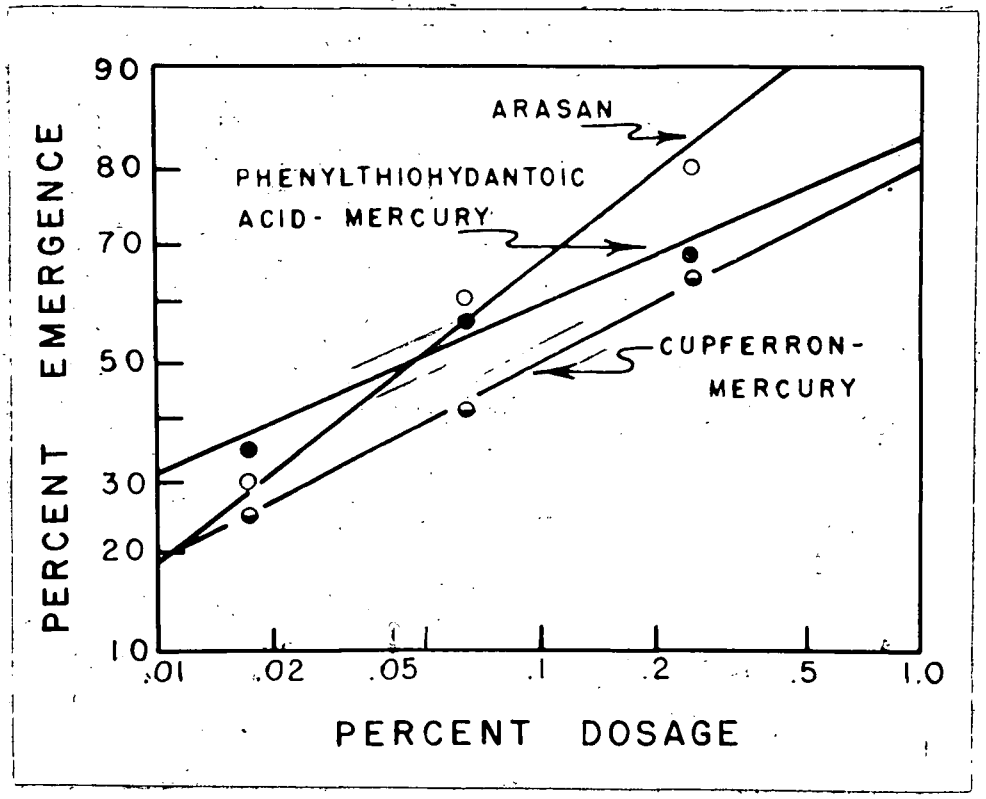


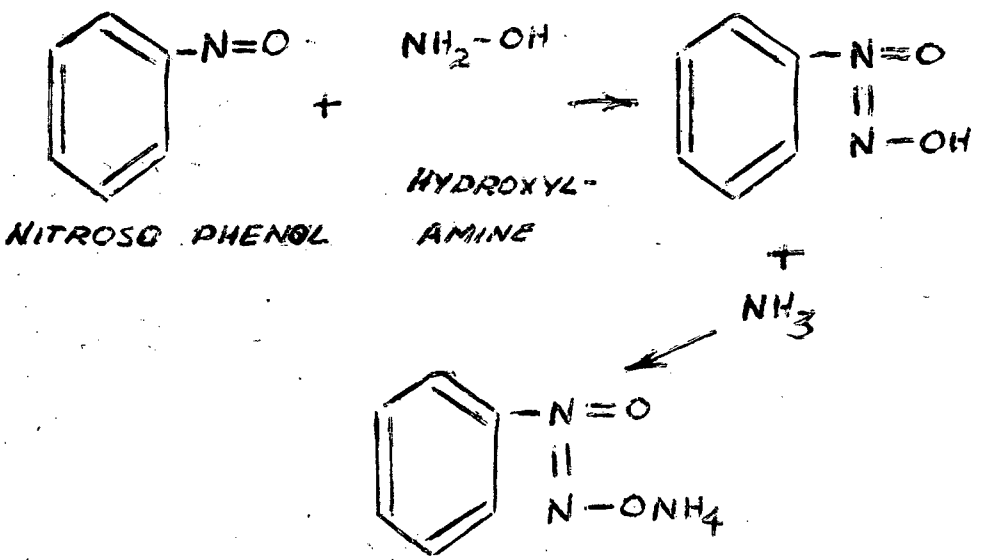
Fig.I. Dosage-response curves from pea seed emergence tests plotted on logarithmic-probability scale for mercury complexes and standard fungicide Arasan. Parallel lines indicate zone within which the ED50 may be expected to fall 19 times out of 20, under the same conditions. Points represent mean emergence of ten seed rows replicated six times.

Fig.I shows that the ED50 of Phenyl-thio-hydantoic acid complex is identical to that of Arasan. Since the curves cross, however, the order of fungicidal efficiency of the two compounds is reversed, Arasan being the more efficient above the point of intersection, and the phenyl-thiodantoic complex below. A similar crossing of the Arasan and cupferron complex curves takes place in the neighbourhood of 20% emergence.

Mode of Fungistatic Action of Mercury Complexes

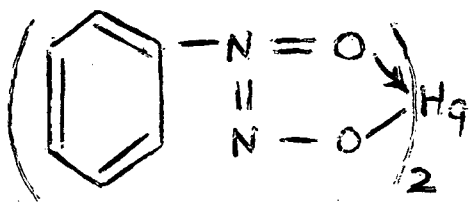
An examination of the mode of fungistatic action of the mercury chelate complexes first entailed a study of the composition of these compounds.

Cupferron (ammonium nitroso-phenol hydroxylamine) is related to nitroso-phenol as shown below:



AMMONIUM NITROSO-PHENOL-HYDROXYLAMINE (CUPFERRON)

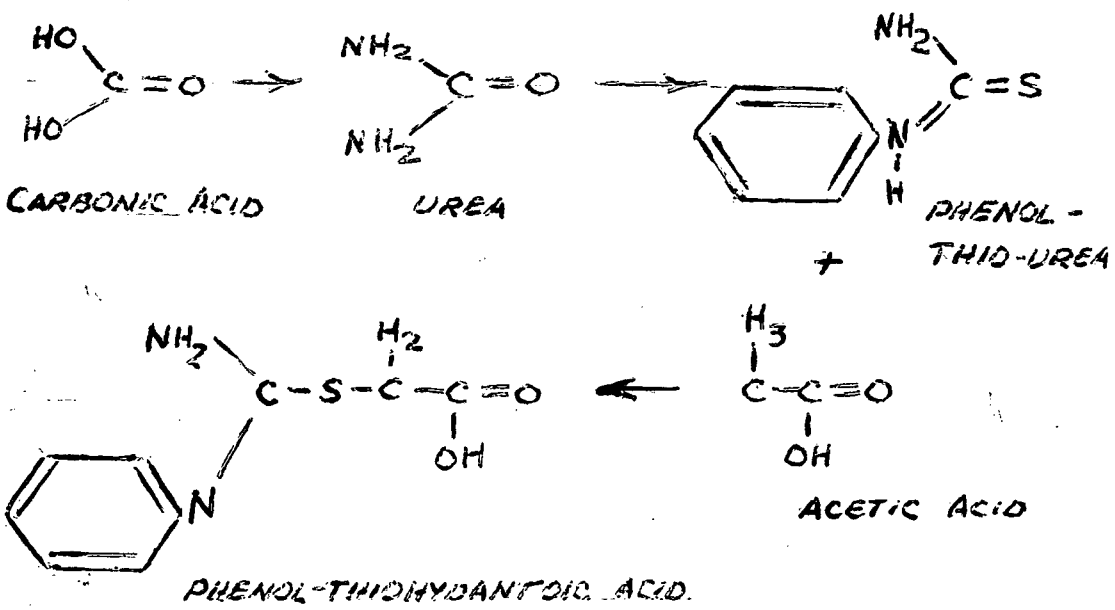
According to Smith (47), Cupferron forms co-ordination compounds with metals as illustrated for the mercuric complex:



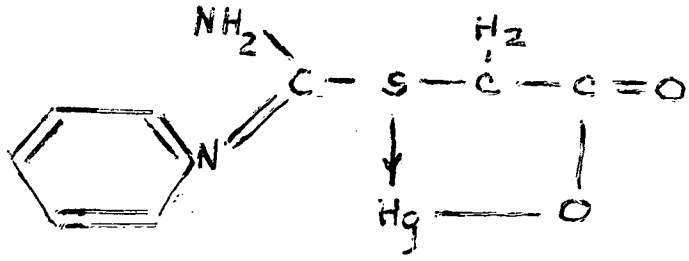
CUPFERRON MERCURY COMPLEX.

The theoretical mercury content of this compound is 42.3%. The actual mercury content of the prepared complex, as determined by a modification of Rupp's procedure (49), was 41.6%. The content of available mercury in ionized form present in a saturated solution of the complex as determined with para-dimethyl-benzal-rhodanine reagent (48) was 4.5 p.p.m.

Phenol-thiohydantoic acid, or phenol-thiourea-acetic acid, is related to carbonic acid in the following manner:



According to Willard & Hall (56), phenyl-thiohydantoic acid forms co-ordination complexes with divalent metal to give the empirical formula of the mercury compound as $(C_9 H_9 O_2 - N_2 S) Hg$. The theoretical mercury content of this compound is 32.4%. However, analysis of the prepared phenyl thiohydantoic acid mercury complex gave a mercury content of 48.10%. This result agrees closely to the theoretical mercury content of 48.97% of the mercurous complex, as in the form illustrated.



MERCUROUS COMPLEX.

It appears, therefore, that although mercuric chloride was used in preparing the above complex, under the conditions of preparation, mercury is reduced to the mercurous form. This reduction is probably due to the action of products resulting from slight decomposition of the chelate in hot solutions.

Tests for the presence of available mercury in saturated solutions of mercurous-phenylthiohydantoic complex showed that less than one ppm. of ionized mercury was present.

From the above results, the importance of available mercury

to the fungistatic action of the two complexes was estimated. This was done by comparing the available mercury content of the complexes with the concentration of mercuric chloride required for total growth inhibition of P.erythroseptica.

The latter figure of 33 p.p.m. mercury was obtained by extrapolation of the dosage-response curve drawn from data of Table II. Since available mercury in the cupferron complex amounts to only 4.5 p.p.m. and less than 1.0 p.p.m. in the phenyl thiohydantoic acid complex, it may be assumed that available mercury plays little part in fungistatic action. Nevertheless, there is the possibility that under certain conditions, more may become available, and the toxic action of mercury become apparent.

The possibility of further mercury becoming available was determined by investigating the relative stability constants of the chelate metal complexes. Mellor and Maley (32), have reported that the stability order of divalent metals with a number of chelates fall in the following order of decreasing stability:

$$Pb > Cu > Zn > Fe > Mg.$$

In a following review, Mellor and Maley (33) discussed additional evidence to show that this order is probably similar for all organic compounds forming similar complexes. Since there has been no report on the relative stability of mercury in chelate complexes, this was determined by a study of displacement reactions with cupferron.

Pfeiffer (41) has pointed out that if some metal complexes

are less stable than others, then under suitable conditions, a metal should displace another from a less stable complex. By a method based upon the above assumption, the relative stabilities of metal complexes of cupferron were determined. The procedure involved the addition of metal salts to cupferron suspensions, and a determination of the amount of mercury displaced. Twenty ml. of metal chloride solution, containing an equivalent of 1.5×10^{-3} M metal, were added to 20 ml. of aqueous suspensions of cupferron mercury complex in Erlenmyer flasks. These flasks were then tightly corked and shaken at 20°C for 24 hours.

After filtering, the amounts of mercury displaced by the metals were determined by analysis of filtrate aliquots by the colormetric para-dimethylomino-benzal-rhodanine method (48). Metals were selected which did not interfere with color developed by mercury under the conditions of the test. In the presence of .01N. nitric acid, neither iron, zinc, copper nor magnesium at a concentration of 1.5×10^{-3}M interfered with the colormetric reagent.

The amounts of mercury displaced from the cupferron complex by these four metals are shown in Table IV.

Table IV

The Relative Amounts of Mercury Displaced from Suspension of Cupferron-mercury Complex by Divalent Metals at Concentrations of 1.5×10^{-3} M.

Displacing Metal	Concentration of Displaced Mercury in p.p.m.
None	4.5
Magnesium	16.8
Iron	40.
Zinc	60.
Copper	100.

Two conclusions may be drawn from these results. First, it appears that the mercury complex is less stable than the complexes formed from the other four metals. This follows as a result of all metals being able to displace mercury. The second conclusion is based upon the assumption that the metal forming the most stable complex will displace the most mercury. It follows from this assumption, that the stabilities of metal complexes of cupferron decrease from copper to magnesium. This is precisely the same order of stability as Mellor and Maley (31) report for these metals with other chelates.

As a result of these stability studies, the following conclusion may be made regarding the fungistatic action of available

mercury in the cupferron complex. Although in distilled water the available mercury is insufficient to play an important role in fungistatic action, in the presence of traces of magnesium or iron, such as are likely to be present in organic media, sufficient mercury may be displaced from the complex to produce a mercury toxicity.

Although it is concluded what part mercury may play in the toxicity of the cupferron complex, no consideration has been taken of the organic radical, itself a powerful fungistat. Information on the fungistatic action of the chelate radical was obtained by comparing dosage-response curves of the complex with the curves of its component parts, cupferron and mercuric chloride. These curves, shown in Fig.2 were obtained with P.erythroseptica as test organism according to previously described methods.

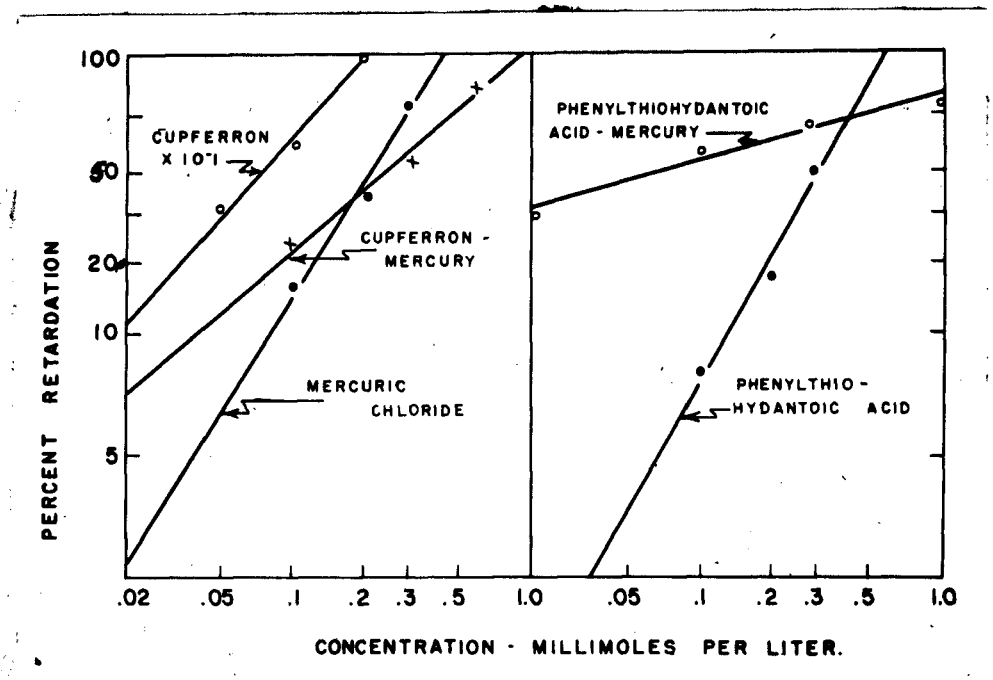


Fig.2. Dosage response-curves of fungicides calculated from data on the inhibition of mycelial growth P erythroseptica on agar plates. Points represent the mean growth retardation resulting from five replicates.

If the inhibitory action of the Mercury-complex was due to ionized mercury only, the slope of the dosage-response should be similar to that of mercuric chloride. This follows from Bateman's (8) work indicating that the slopes of dosage-response curves of salts of a toxic metal are similar regardless of anion. Since Fig.2A shows that these slopes are not similar, it follows that the cupferron portion of the molecule is also involved in the fungistatic action.

The displacement of mercury from phenyl-thio-hydantoic acid complex by metals, was examined as with cupferron. Apparently, mercurous mercury forms a very stable complex with this chelate, since mercury was not displaced by any of the metals added. If ionized mercury plays no part in the fungistatic action of this complex, is the action due solely to the chelate radical? Reference to dosage-response curves of Fig.IIB again, will answer this question. If the action of the mercury complex was due to the chelate molecule only, then the slopes of both the chelate and mercury-complex curves should be similar. However, the curve of the complex is considerably flatter than that of phenyl-thiohydantoic acid. It appears, therefore, that the mercurous complex of phenyl-thiohydantoic functions

neither by virtue of the chelate radical, nor by mercury alone, but rather as an undissociated molecule.

A second procedure was used to determine the effect upon toxicity of introducing mercury into the chelate molecule. This method involved a determination of the relative growth and respiration inhibitory powers of the chelates, compared to those of their mercury complexes.

The concentrations of two chelates, their mercury complexes, and mercuric chloride required to produce 50% growth inhibition, were obtained by interpolations from the dosage-response curves of Fig.2. The corresponding concentrations of these compounds required to reduce respiration of P.erythroseptica by 50%, were determined in the standard Warburg manometer by methods based on those of Nickerson (40) and Bronfrebrenner et al (6).

For this purpose, shake cultures of P.erythroseptica were grown for manometer respiration measurements in the following medium:

Glucose	50 Gm.
Dipotassium phosphate	1.0 "
Magnesium Sulphate	0.02 "
Calcium Sulphate	0.02 "
Asparagine	4.0 "
Thiamin Hydrochloride	0.2 mg.

Trace metals - Cu.05 p.p.m. Zn 1. p.p.m. Fe.1. p.p.m.
Mn.05 p.p.m.

Water to make 1 liter

pH 6.5 adjusted with hydrochloric acid.

Cultures were shaken until mold growth had reached a stage suitable for pipetting. The mold mycelium was then filtered off, washed with Sorensen's buffer of pH 6.5, and finally re-suspended in buffer. By means of a pipette, equivalent amounts of mold were then added to manometer flasks.

Fungistatic agents were added to a side arm, aqueous potash to the centre well, and finally sufficient water added to the main flask to produce a total liquid volume of three ml. The flasks were then placed in the water bath at 28°C, and shaken for an equilibration period of 10 min.

The rate of oxygen uptake was determined during the linear respiration period for each concentration of chemical. The percentage inhibition was then plotted against concentration, and a smooth curve drawn through the points. Concentrations producing 50% respiration inhibition were then interpolated from these curves. The resulting concentration of compounds which produced 50% growth inhibition and 50% respiration inhibition are shown on Table V.

Table V

Concentrations of Compounds Producing 50% Growth and Respiration Inhibition of P.erythroseptica

Compound	Concentration in moles per liter ($x10^{-5}$)	
	50% Growth Inhibition	50% Respiration Inhibition
Mercuric Chloride	20.	23.
Cupferron	.7	120.
Cupferron Mercury	10.	90.
Phenyl-thio-hydantoic acid	45.	1000.
Phenyl-thio-hydantoic acid mercury	2.5	105.

Table V shows that introducing mercury in both chelates increased the action of these compounds against respiration. Nevertheless, both chelates and their mercury complexes are more effective inhibitors of growth, than of respiration. This fact may explain why chelate fungicides are generally fungistatic rather than fungicidal. Obviously Nickerson's method (40) of estimating fungistatic activity based upon respiration inhibition, cannot be applied to these types of compounds.

Part II

Toxic Gases evolved from Chelate Fungicides Related to Dithiocarbamic Acid

The instability of the dithiocarbamate fungicides has been recognized for some time. Carbamic acid and dithiocarbamic acid are both unstable compounds: the former acid has not been isolated in the free state (46), and the latter decomposes in water (38). Chernikov and Dobkina (9) showed that the stability of the dithiocarbamate metal complexes paralleled that of the sulphides. Heuberger (18) concluded that the zinc salts were more stable to heat and light, than the sodium, iron and calcium salts, and that these metallic salts are rather unstable to the action of acids. Apart from Dithane, however, the evolution of toxic gases produced during decomposition has not been reported. Only one of the two gases evolved from Dithane has been identified (45), and the conditions responsible for the decomposition of this compound do not appear to have been clearly understood.

During an attempt to measure the inhibitory action of sodium diethyl-dithiocarbamate against the enzyme carboxylase in the Warburg manometer, it was noticed that a gas was evolved from this chelate. Due to the importance attached by Rich and Horsfall (45) to gases evolved from the related compound Dithane, an attempt was made to identify the gas from sodium diethyl-dithiocarbamate. Since the identification was successful, the study was expanded to include

other dithiocarbamate fungicides, including Dithane. The following experiments were designed to identify the gases given off by these compounds, and to determine the conditions of gas evolution.

The sources of the dithiocarbamates used in the following experiments were:

Sodium diethyl-dithiocarbamate, Eastman Kodak Co.

The copper, iron and zinc complexes of dimethyl-dithiocarbamate, by courtesy of Canadian Industries Ltd.

Dithane (di-sodium ethylene-bis-dithiocarbamate), by courtesy of Dr.J.G.Horsfall, Connecticut Agricultural Experiment Station.

Measurements of gas evolution were made in all experiments in the conventional Warburg manometer. Except in specified cases, the following manometer technique was adopted.

One ml. of 10^{-2} x 3 M dithiocarbamate was added to the manometer side arm, and 0.4 ml. of buffer placed in the main flask. Water was finally added to the main flask to produce a total liquid volume of 3.0 ml. After an equilibration period of 10 minutes in the water bath, the dithiocarbamate was dumped into the buffer, and gas evolution recorded at 5 minute intervals. All experiments were carried out at 28°C.

Sodium Diethyl-dithiocarbamate

The effect of pH upon gas evolution from sodium diethyl-dithiocarbamate was first determined. For this purpose, a series of Sorensen's phosphate buffers ranging from pH 4.9 to 7.4 were

prepared. Fig.3 shows the relative rate of gas evolution from the above compound in the presence of these buffers. Since the gas was later identified as carbon disulphide, gas volumes in Fig.3 were calculated on a basis of a carbon disulphide manometer constant. (K_{CS_2}).

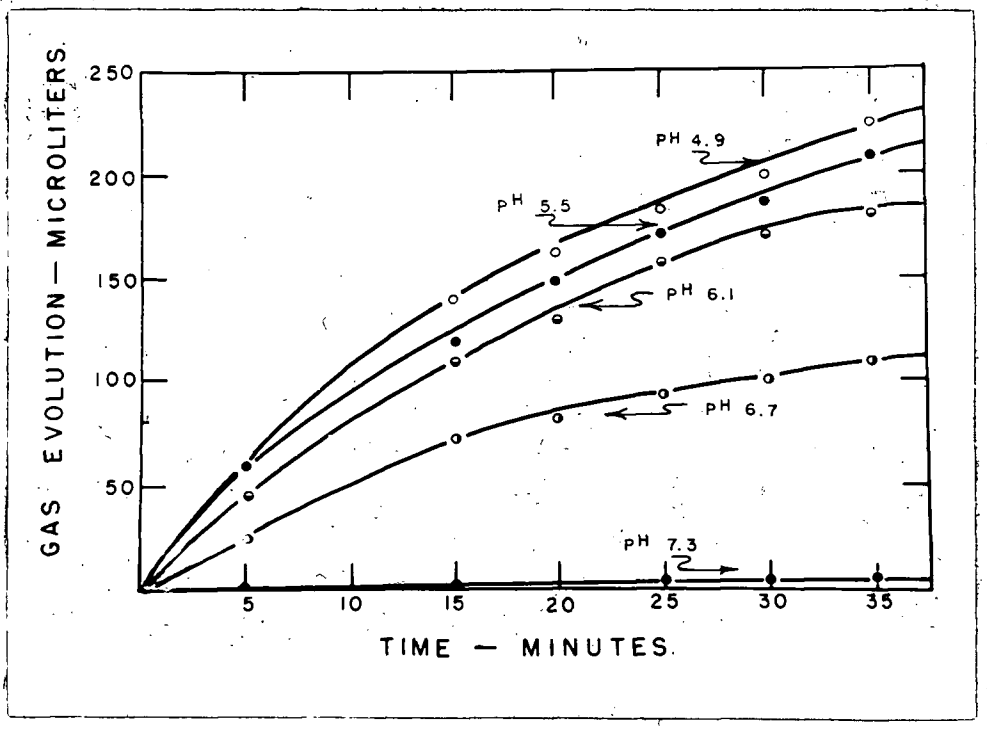


Fig.3. The effect of pH of Sorensen's phosphate buffer upon gas evolution from diethyl-dithiocarbamate.

To determine that gas evolution was not limited to the action of phosphate buffer, a comparison was made between the action of citrate and phosphate buffer on gas evolution. In order to obtain greater manometer readings, the buffer content was increased to 6 ml. These results are shown in Fig.4. Figs.3 and 4 indicate conclusively

that gas evolution is a result of buffer acidity.

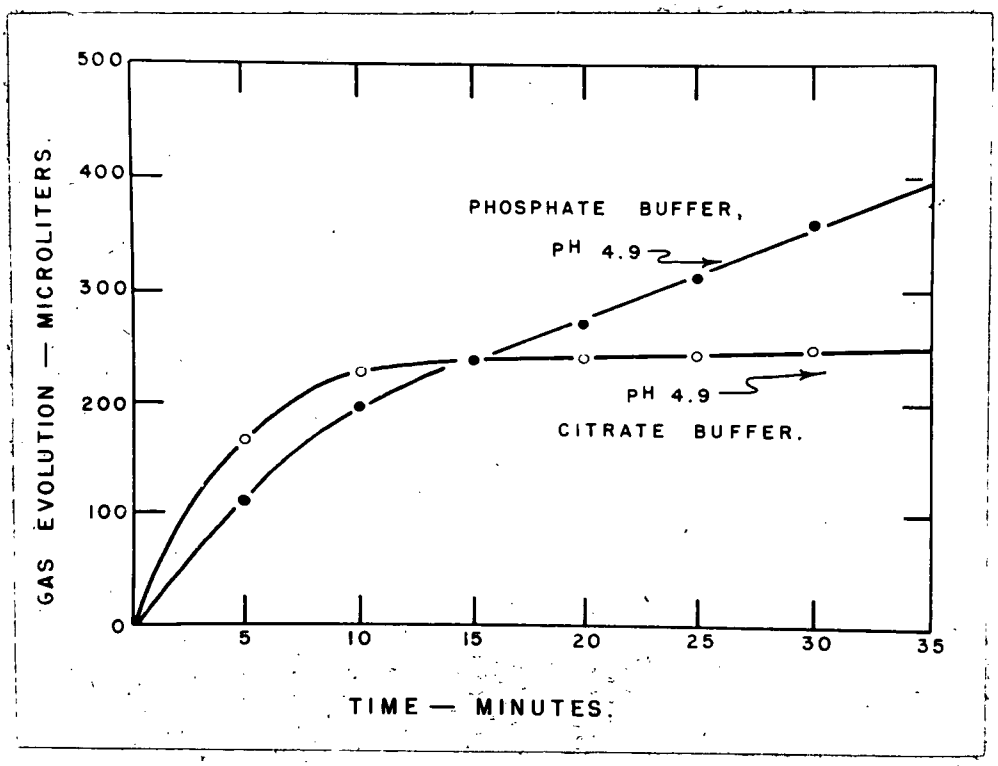


Fig.4. The effect of phosphate and citrate buffers upon gas evolution from sodium diethyl dithiocarbamate.

The interaction of buffer with dithio-carbamate was examined further by measuring the gas evolved in the presence of increased amounts of buffer. At the same time, an attempt was made to identify the gas by its action on aqueous and alcoholic potash solutions. For the latter purpose, 0.2 ml of a 5.0% aqueous solution of potash were added to the centre well of the manometer flask. In a corresponding flask, a 5.0% solution of potash in absolute alcohol was added to the centre well. Filter papers were added in the standard manner

to increase the rate of gas absorption. The results are shown in Fig.5.

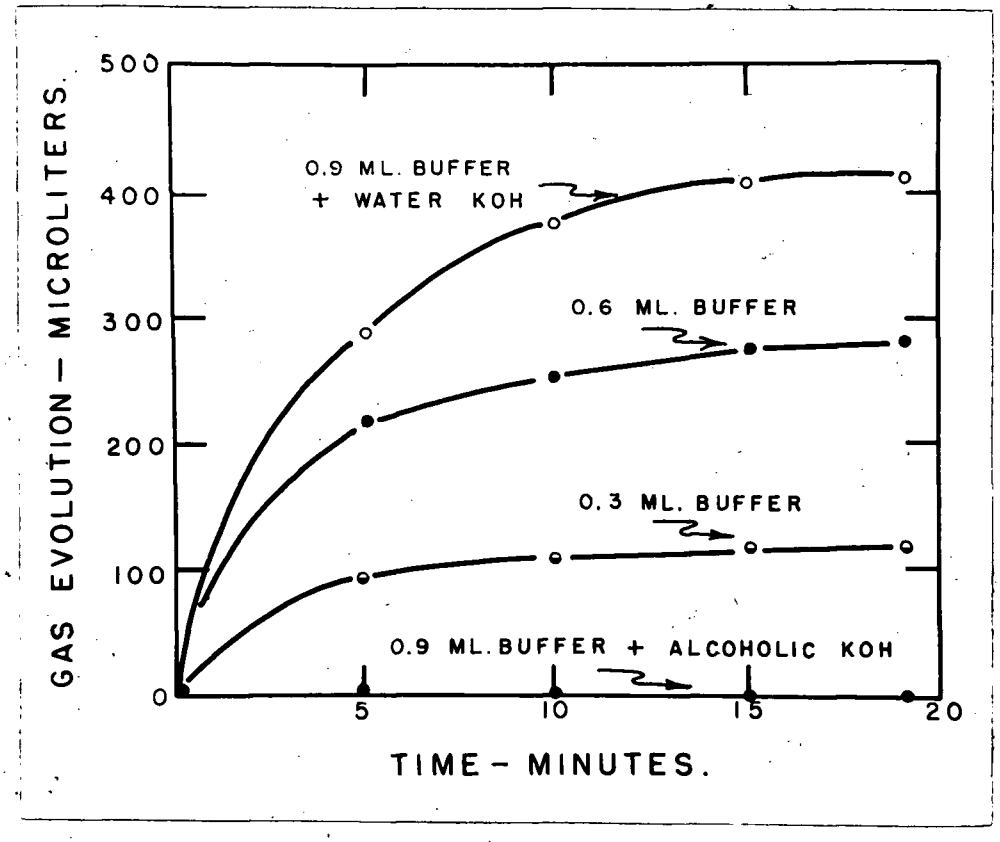


Fig.5. The effect of increasing amounts of phosphate buffer of pH 4.9 upon gas evolution from sodium diethyl-dithiocarbamate, and the relative absorption of the gas by aqueous and alcoholic solutions of potash. The gas evolution resulting from 0.9 ml. buffer in the presence and absence of aqueous potash was identical, and hence only one curve is shown. Volumes calculated on a basis of K_{CS_2}.

Fig.5 shows that since the gas is absorbed instantly by alcoholic potash, but not by aqueous potash, the gas is not hydrogen sulphide. Confirming this point was the inability of a 20% aqueous solution of lead acetate to absorb the gas. These reactions are, however, characteristic of carbon disulphide (53). As a result,

Reith's confirmatory test for the presence of carbon disulphide (44) was done on the alcoholic potash solution.

One drop of 1/50N copper acetate and 0.2 ml. of 4N acetic acid were added to the alcoholic potash solution. The characteristic bright yellow color of copper zanthogenate confirmed the presence of carbon disulphide.

Metal Complexes of Dimethyl-dithiocarbamate

An attempt was then made to determine if gas evolution took place under similar conditions, from other closely related fungicide derivatives. For this purpose, the copper, zinc, and iron complexes of dimethyl-dithiocarbamate, were examined.

One ml. suspensions of these compounds were added to the manometer side arms, and two ml. of Sorensen's phosphate buffer pH 4.9 placed in the main flask. Gas evolution was then measured in the usual manner. No carbon disulphide evolution could be detected from this copper complex. The relative evolution of gas from the iron and zinc complexes are shown in Fig.6.

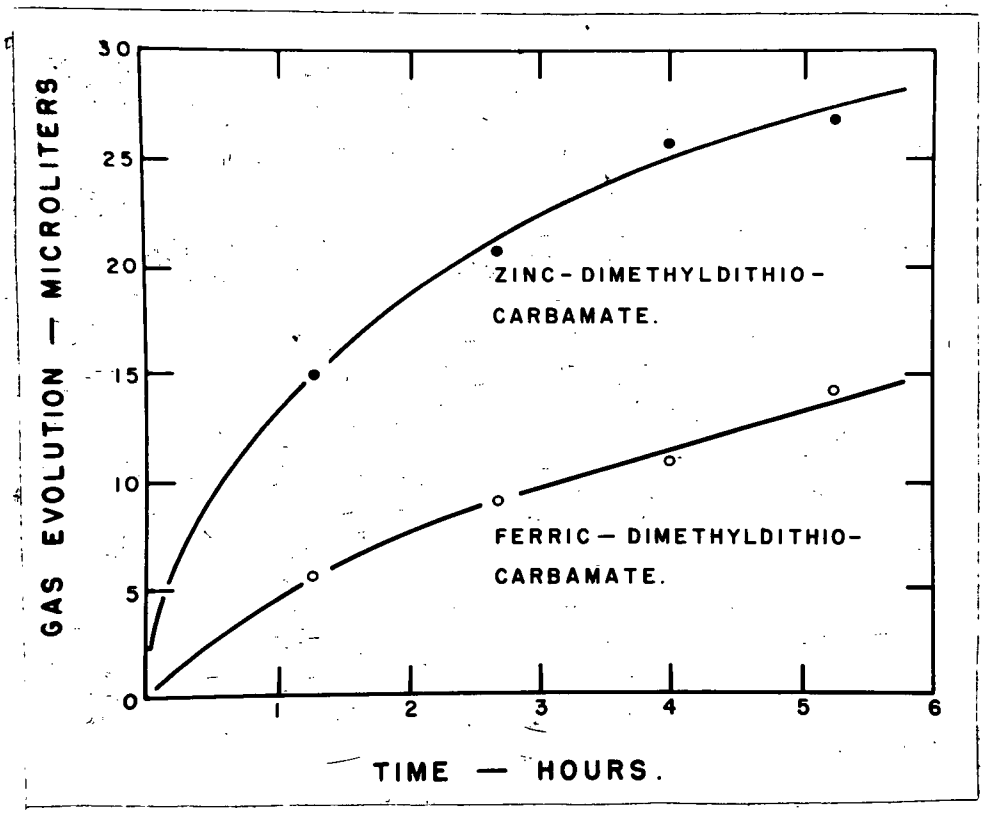
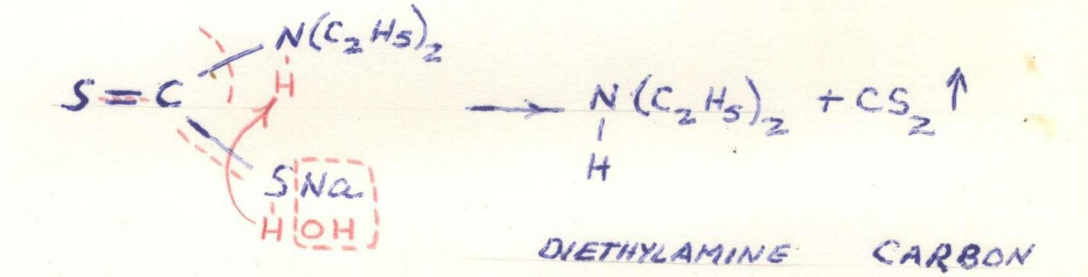


Fig.6. Gas evolution from metal complexes of dimethyl-dithiocarbamic acid in the presence of phosphate buffer of pH 4.9. Volumes calculated on basis of K_{CS_2}.

The gas given off from both these chelate complexes was identified by the methods previously described as carbon disulphide. At the same time it was noticed that the decomposition of these two neutral compounds was accompanied by a rise in pH of the buffer mixture. It was considered possible, therefore, that an anine might remain in the buffer solution as a secondary decomposition product. As a result, an attempt was made to isolate and identify the product resulting from the decomposition of the soluble sodium diethyl-dithiocarbamate in the following manner:

Sodium diethyl-dithiocarbamate was decomposed with evolution of carbon disulphide by adding slowly .01N hydrochloric acid until pH of 3.0 was produced. The solution was then evaporated to dryness, and the residue washed with ether. After drying a second time, the residue was then extracted with chloroform. On evaporation, the chloroform solution left a perfectly white, semi-crystalline mass producing the characteristic reaction of an amine salt (42). The compound produced a sharp melting point of 118.2°C, which compares closely to that of 119-20°C reported for diethylamine hydrochloride (21).

It was concluded, therefore, that the dialkyl series of dithio-carbamate fungicides decompose in the presence of weakly acidic buffers in the following manner:



HYDROLYSIS OF SODIUM DIETHYL-DITHIOCARBAMATE

Hydrolysis first takes place. Probably as a result of dithiocarbamic acid itself being unstable the derivative formed on hydrolysis then breaks down to form a dialkyamine, and simultaneously splits off carbon-disulphide.

Fig.6 illustrates another interesting feature regarding the relative rate of decomposition of the metal complexes. In Part I of this paper, it was mentioned that the stability order of divalent metals with chelates appeared to be similar, regardless of the chelate radical. If this fact applies also to the complexes of dimethyl dithiocarbamates, the rate of carbon disulphide evolution from the three compounds examined should fall in the following increasing order from copper to iron:

$$Cu < Zn < Fe.$$

It was already mentioned that no carbon disulphide could be detected from the very stable copper compound. However, Fig.6 indicates that the stabilities of the zinc and iron complexes are reversed, since the zinc compound decomposes more rapidly than the iron compound. However, Pfeiffer et al, (41), reported the ferric complex of bis-salacylaldehyde-ethylenediamene more stable than the zinc complex. Mallor and Maley (33), commenting on the reversal of the stability of the iron complex, concluded that this was undoubtedly due to the iron being in the ferric state. As the iron complex of dimethyl dithiocarbamate examined was also in the ferric state, it appears that the rate of decomposition of the three dithiocarbamate complexes agrees with the general order of metal chelate complex stabilities.

Dithane

Tests were finally made in the Warburg manometer for evolution of gases from Dithane in the presence of an acid buffer. The

concentration of fungicide and other conditions were identical to those described for the iron and zinc complexes. Flasks containing aqueous and alcoholic potash were included as previously described. The results of this experiment are shown in Fig.7.

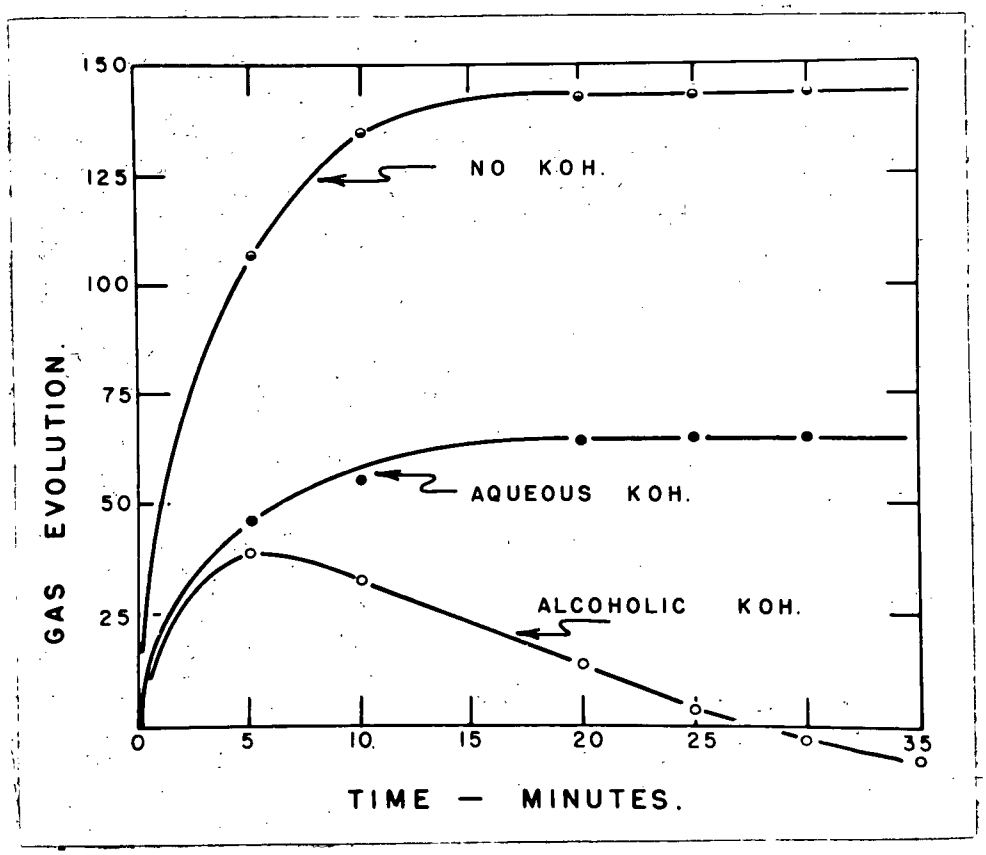


Fig.7. The evolution of gases from Dithane in the presence of Sorensen's phosphate buffer of pH 4.9, and the relative absorption of the two gases by aqueous and alcoholic potash. For method of calculating volume of gas mixture see text.

Since the gas evolved from Dithane was a mixture, later identified as carbon sulphide and hydrogen sulphide, the volume of the total gas could not be estimated directly. This follows as a result of the manometer constant being based upon the solubility

of the gas being measured. The total gas volume of Fig.7 was therefore calculated in the following manner:

From manometer A, containing aqueous potash, the volume of carbon disulphide was calculated, using K_{CS_2}. From manometer B, containing no potash, the total manometer change due to evolution of both gases was recorded. The manometer change of B, corresponding to the volume of CS_2 obtained from A, was next calculated. Then the total manometer change of A, less the calculated manometer change due to CS_2, equalled the change due to H_2S. This result, multiplied by K_{H_2S} for manometer B, gave the hydrogen sulphide evolved.

Fig.7 shows that the gas is only partially absorbed by aqueous potash. Addition of lead acetate to the aqueous potash solution produced a black precipitate of lead sulphide, indicating that the portion of gas absorbed by aqueous potash was hydrogen sulphide.

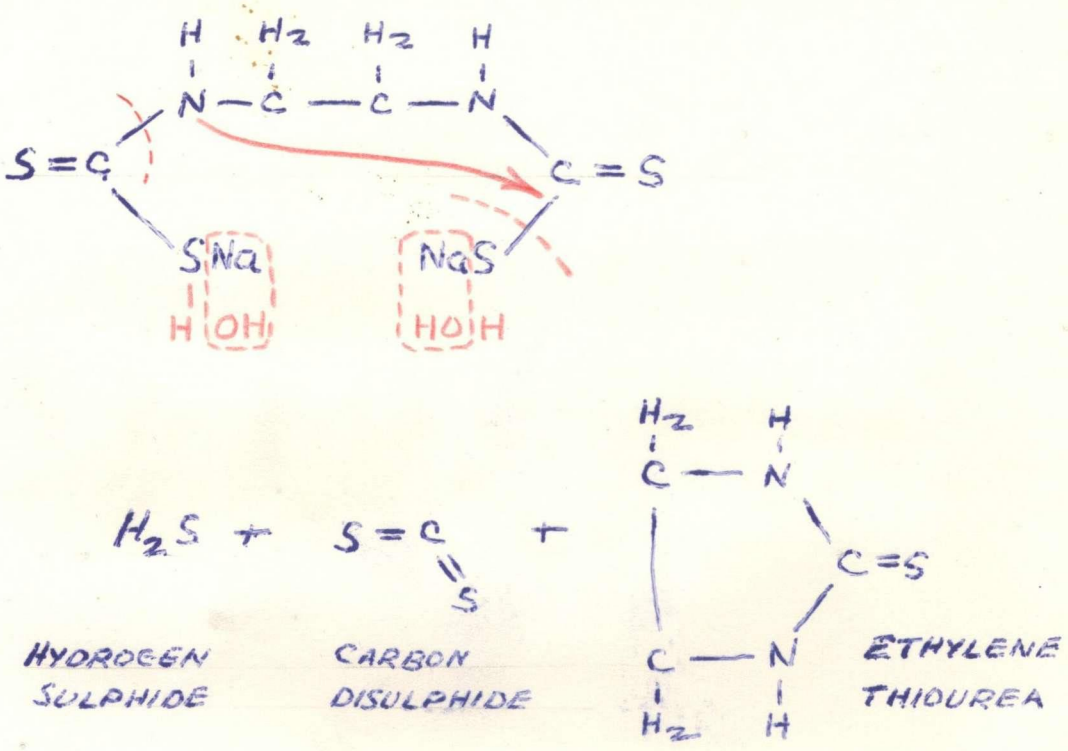
Fig.7 indicates also that although the gas is eventually totally absorbed by alcoholic potash, there is a considerable lag period. This contrasts with the immediate absorption of carbon disulphide by this solution in previous experiments. Since, however, traces of water interfere with absorption of carbon disulphide by alcoholic potash, it was considered possible that hydrogen sulphide might do the same. As a result, it was decided to test for the presence of carbon disulphide in the alcoholic solution, by Reith's Procedure (44).

Since Reith's test for carbon disulphide cannot be carried

out in the presence of hydrogen sulphide, it was first necessary to remove this gas. This was accomplished by adding aqueous potash to the centre well of the manometer in the usual way. At the same time, however, a folded filter paper strip was placed in a side arm of the flask and the side arm stopped with a vented plug. Dithane was added to the second side arm, and buffer placed in the main flask as before.

The evolution of gas was recorded until no further change in readings occurred. In order to absorb the last trace of hydrogen sulphide, shaking was continued for a further half hour. By means of the vented plug, 0.2 ml of alcoholic potash solution was then run into the side arm, and shaking was again continued for another half hour. Reith's test for the presence of carbon disulphide in the alcoholic potash was then made by adding the acetic acid and copper acetate solutions directly to the side arm containing the paper. The characteristic yellow color indicating carbon disulphide was produced.

As a result of these experiments, it was concluded that Dithane decomposes with evolution of hydrogen sulphide and carbon disulphide in the presence of weakly acid buffer in the following fashion.



Following hydrolysis, ethylene bis-dithiocarbamic acid is formed. This compound, inheriting the instability of dithiocarbamic acid, breaks down to form ethylene-thiourea, carbon disulphide, and hydrogen sulphide.

This mode of breakdown is substantiated by two additional facts. First, it has already been mentioned that ethylene-thiourea is the only compound identified in the breakdown debris of Dithane (45). And second, the one to one ratio of hydrogen sulphide to carbon dioxide evolved in theory, approaches the 1.2 to 1.0 obtained in practice. (See Fig.7). This latter ratio may be considered an approximation only, since it is calculated from readings involving two flasks, and hence the pipetting of two supposedly equal portions of insoluble Dithane in the form of water suspensions.

DISCUSSION

The results of Part I of this paper show that active fungistatic compounds may be prepared by combining fungistatic chelates with metals. Observations from Part II, indicate that probably all active dithiocarbamate fungicides decompose with evolution of toxic gases. The implications of this fact are that sulphur compounds enjoy a particularly favourable position in the chelate fungicide field. For economic reasons already discussed, the two mercury complexes examined in this paper must of necessity be limited to use as seed protectants. On the other hand, the relatively inexpensive sodium and iron complexes of the dithiocarbamates, in spite of the absence of a toxic metal, are active fungicides which can be used economically both as seed protectants and as field protectant fungicides. The effectiveness of this latter type of compound appears undoubtedly to be due to toxic gas evolution.

A search for other sulphur chelates evolving toxic gases upon decomposition, would appear to be a practical application of these studies. Reference to the chelate stability order of Mellor and Maley should facilitate the preparation of metal complexes from these sulphur chelates possessing the desired fungistatic activity. The great value of this metal stability order in both explaining the predicting the action of this type of fungicide has not yet been appreciated.

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