Identity of Gaseous Toxicants from Organic Sulfur Fungicides[1]

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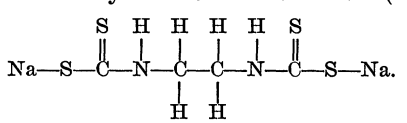
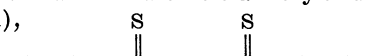
It is well established that certain organic sulfur compounds are toxic to fungi. Among them are thiuramdisulfides, metal dithiocarbamates, and metal alkyl bisdithiocarbamates. During recent years there has been considerable speculation concerning the mode of action of these compounds.

Tisdale and Williams (*1*) have proposed that the group  is responsible for fungicidal activity. On the other hand, Goldsworthy, Green, and Smith (*2*) found no conclusive evidence that fungicidal action is directly attributable to this group. They pointed out that the fungicidal properties may be modified by the nature of the alkyl groups. Parker-Rhodes (*3*) inferred, on the basis of dosage-response data, that fungicidal activity might be attributed to the decomposition products carbon disulfide and dialkylamine. He did not, however, report chemical tests indicating the presence of these compounds. Davies and Sexton (*4*) found that sulfur and oxygen analogs of various organic fungicides behaved somewhat similarly and concluded that sulfur, per se, makes no contribution to fungicidal activity, but reached no conclusion as to how the dithiocarbamates affect fungi. McCallan and Wilcoxon (*5*) developed the theory that hydrogen sulfide is responsible for the toxicity of sulfur to fungi. Barrett and Horsfall (*6*) extended this theory to include the dithiocarbamates. They worked with disodium ethylene bisdithiocarbamate (nabam[2]),



Rich and Horsfall (*7*) have recently reported studies on the toxicity of gaseous emanations from nabam and other organic sulfur compounds. They showed that solutions of purified samples of nabam, which do not liberate hydrogen sulfide readily, do liberate some other gaseous toxicant capable of preventing germination of spores of *Sclerotina fructicola* and *Stemphylium sarcinaeforme* in hanging drops of water exposed to the gas. Their experiments showed that the volatile toxicant was not hydrogen sulfide, sulfur dioxide, or ethylene thiourea, which has been reported as a decomposition product of nabam *in vitro*. Furthermore, they reported that the slope of the dosage-response curve of the volatile toxicant indicates that it is not volatile nabam itself. These authors pointed out that the identity of the fungicidal gas from nabam and other organic sulfur compounds was still in question, and also that "no completely satisfactory theory has yet been offered to explain the fungicidal mechanism of nabam." They voice the hope that a clue will be found by attempting to isolate and identify the gaseous toxicants from nabam solutions.

We have worked with tetramethylthiuramdisulfide (thiram),  , and, to a more limited extent, with nabam and other related fungicides. Sisler and Cox (*8*) reported that when spores of *Fusarium roseum* Lk. are in contact with purified thiram in solution or suspension, a volatile toxicant is liberated which reduces the rate of respiration and inhibits or prevents germination of other spores of this *Fusarium* at a distance from the thiram. No inhibition in germination or reduction in rate of respiration is observed when spores are exposed to the atmosphere over aqueous solutions or suspensions of thiram alone. The toxicant was not hydrogen sulfide. More recently the same authors (*9*) reported with regard to this volatile toxicant (*a*) that spores and mycelia of several fungi and certain other plant cells may release it from thiram, (*b*) that its release is apparently under enzymatic control since it occurs only in the presence of cells and is prevented by heating the cells to a temperature between 50° and 60° C for 5 min, and (*c*) that it contains carbon disulfide. Carbon disulfide was identified by a positive reaction to two different chemical tests: the test of Vogel, described by Dennis and Nichols (*10*), in which the carbon disulfide is trapped in alkaline absolute alcohol, acidified with acetic acid, and treated with dilute copper sulfate solution to give a yellow precipitate of cuprous xanthate; and the test described by Viles (*11*), in which the gas is bubbled into an ethanol solution of triethanolamine, diethylamine, and copper acetate to form cupric diethyldithiocarbamate, which is yellow-brown in color.

Volatile toxic materials other than carbon disulfide have not been found in atmospheres over suspensions of thiram and living cells, and no volatile toxicant has been found over suspensions of thiram alone. Details of these experiments will be published elsewhere.

When this study was extended to related organic sulfur compounds, it was observed that the atmosphere over solutions of Dithane D-14 (a proprietary fungicide containing 25% disodium ethylene bisdithiocarbamate hexahydrate as its active ingredient) gave positive chemical tests for both hydrogen sulfide and carbon disulfide. These gases were liberated spontaneously and did not require the presence of living cells. The atmosphere above solutions of various concentrations of two purified samples of nabam[3] was also

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[2] The common names nabam and thiram were coined by a special committee and have been accepted by the American Phytopathological Society to designate, respectively, disodium ethylene bisdithiocarbamate and tetramethylthiuramdisulfide.

[3] One sample was provided by the Rohm & Haas Company, Philadelphia; the other was purified in our laboratory by recrystallization from a commercial sample of Dithane D-14.

tested for hydrogen sulfide and carbon disulfide. As in the experiments of Rich and Horsfall (*7*) no hydrogen sulfide was detected. However, solutions of both samples began to liberate measurable quantities of carbon disulfide shortly after they were prepared and continued to liberate this gas for several days, the gas being released for a longer time from the higher than from the lower concentrations. A series of concentrations of purified nabam in water of 0.01, 0.1, 1.0, and 10.0 g/l was prepared. These solutions were used to set up spore germination tests similar to those described by Rich and Horsfall (*7*) in which glass slides, each bearing a drop of spore suspension, were inverted over the mouths of vials of about 67-ml capacity containing 15 ml of the various nabam solutions, so that the drops hung in the air space above the surface of the liquid. The slides were sealed to the rims of the vials with silicone stopcock grease. As controls, spores were exposed over 15 ml of distilled water. Spores were also similarly exposed in vials containing 0.5 ml of carbon disulfide covered by 14.5 ml distilled water. Each treatment was tested in duplicate. The spores were incubated for 24 hr at room temperature. The test fungi were *Colletotrichum phomoides* (Sacc.) Chester, and *F. roseum*. The results of one of the experiments are summarized in Table 1. Several similar experiments yielded comparable results.

TABLE 1

EFFECT OF GASEOUS TOXICANTS FROM NABAM SOLUTIONS AND OF CARBON DISULFIDE ON SPORE GERMINATION*

Toxicant	Nabam (conc in g/l)	Percentage germination in drop of water suspended above solutions	
		Colletotrichum phomoides	*Fusarium roseum*
None (distd water)	—	> 90†	> 90†
Carbon disulfide–water mixture‡	—	0	0
Nabam (purified)	10.	0	9
	1.	8	34
	0.1	36	96
	0.01	45	—

* Percentage germination expressed as mean of duplicate determinations; 150–250 spores counted in each case.

† Exact determination of percentage germinated over distilled water impossible because of extensive mycelial growth.

‡ Spores exposed to gas over 0.5 ml CS_2 covered by 14.5 ml water.

The percentage of spores germinated in drops exposed over water was above 90%. None of the spores germinated over the carbon disulfide–water mixture. No spores of *C. phomoides* and a low percentage of *F. roseum* germinated over the 10 g/l concentration of nabam. The percentage of spores germinating over the other solutions was intermediate between these extremes and increased with decreasing concentrations of nabam. There were also differences in the length of germ tubes in the various treatments, the higher concentrations inhibiting their growth more strongly than the lower.

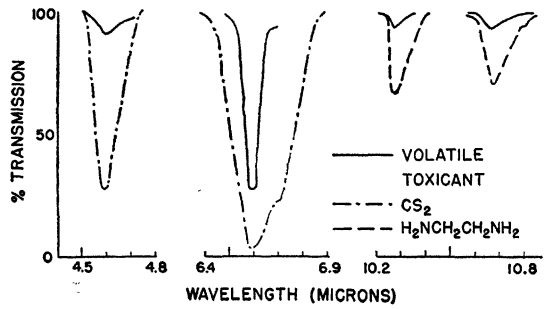

FIG. 1. Infrared absorption bands of the volatile toxicant over nabam solution in comparison with those of carbon disulfide and ethylene diamine.

An attempt was made to determine whether nabam might exist as a gas over a solution of the fungicide and be dissolved in the spore drops in such quantities as to inhibit germination of spores. Disodium ethylene bisdithiocarbamate in solution will react instantaneously with silver nitrate to produce a brownish-yellow compound. With a 1% solution of silver nitrate as an indicator, as little as 4 ppm nabam in solution may be detected by a color change readily visible to the unaided eye.

Five-ml lots of the silver nitrate solution in evaporating dishes were exposed for 72 hr in sealed 2.5-liter desiccators containing 500 ml of 1% nabam solution. The exposure period was three times that required to produce the toxic effects that have been observed when fungus spores are exposed in hanging drops of water over nabam solutions of similar concentration. No color change occurred. This should probably be expected, since a consideration of the chemical composition of nabam would exclude the possibility of its having an appreciable vapor pressure, especially at room temperature and over aqueous solutions the molarity of which is in the order of 0.04 or less.

On the basis of this evidence and that of Rich and Horsfall (*7*), it seems quite unlikely that volatile nabam may be an important component of the gaseous toxicants which form over solutions of the fungicide.

The vapors emanating from a solution of nabam were investigated by infrared spectroscopy. One liter of solution containing 10 g of purified nabam was placed in a 4-liter Erlenmeyer flask. The flask was closed and allowed to stand for 24 hr at room temperature. A 10-cm infrared gas cell was then filled with the vapor and the spectrum was run on a Perkin-Elmer infrared spectrograph equipped with sodium chloride optics. The vapor showed only four absorption bands other than those of water and carbon dioxide (Fig. 1). Also shown in Fig. 1 are the absorption bands of carbon disulfide and of ethylene diamine at partial pressures of 366 and 13.8 mm Hg, respectively. It is apparent that the atmosphere over the nabam solution contained these two compounds. Application of Beer's law gave the result that the partial pressure of carbon disulfide in the vapor was 26.3 mm Hg and that of ethylene diamine, 2.3 mm. It

should be pointed out that the spectrum of ethylene diamine in this region resembles closely that of ammonia. On the basis of the chemical structure of nabam, however, it is probable that the compound in question is ethylene diamine.

Dimond and Horsfall (*12*) showed that small quantities of dimethyl amine greatly increased the toxicity of carbon disulfide to *Sclerotinia fruiticola*.

Results of the present experiments lead to the conclusion that the gaseous toxicants from nabam solutions are carbon disulfide and ethylene diamine. The effect at a distance from the solutions, observed by Rich and Horsfall (*7*) and confirmed herein, is thus to be attributed to these compounds. It may be inferred that carbon disulfide, in cooperation with certain amines, is responsible, at least in part, for the effect of the fungicidally active dithiocarbamic acid derivatives.

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Ascorbic Acid, a Coenzyme in Tyrosine Oxidation[1]

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The crystalline antiscorbutic vitamin has been available since its isolation was announced in 1932. However, no adequate explanation of the chemistry of its biological functions has been established. Now at least one of its functions may be understood, for it has been found to be a necessary coenzyme in the metabolic oxidation of the amino acid tyrosine. Following the discovery of its need for complete utilization of tyrosine in the intact animal (*1*), it was shown that liver slices from scorbutic guinea pigs were unable to oxidize tyrosine (as normal slices were) unless the crystalline vitamin were added (*2*). With the *in vitro* addition of crystalline synthetic ascorbic acid, scorbutic liver slices were indistinguishable from normal slices in their ability to oxidize tyrosine as measured by oxygen consumption. Since surviving cells were present, an uncertainty remained as to whether the action of the vitamin was a direct enzymatic participation or some indirect effect.

Studies with cell-free scorbutic liver preparations gave indefinite answers, for with large numbers of animals the average values for tyrosine oxidation by deficient livers were almost equal to the average values obtained with normal livers. In occasional animals, the degree of tyrosine oxidation was increased by the addition of the vitamin. Painter and Zilva (*3*) have observed the increased disappearance of the tyrosine phenolic group with the addition of ascorbic acid to scorbutic liver preparations, which also were capable of extensive tyrosine metabolism.

In contrast, enzyme preparations from even normal animals completely lose the ability to oxidize tyrosine if the preparation is made in such a fashion that the total concentration of both free and bound ascorbic acid is reduced to an insignificant value. This removal of ascorbic acid may be accomplished by thorough dialysis, by certain types of acetone desiccation, by dialysis of other types of acetone powders, and by specific protein fractionations. With preparations of this sort the role of ascorbic acid is readily demonstrated. In order to achieve tyrosine oxidation, it is necessary to add α-ketoglutaric acid as amino group acceptor in the first stage of the reaction. This first stage is probably a nonoxidative transamination, as shown by experiments with tyrosine marked with heavy nitrogen in our own work and by use of ketoglutaric acid (*4*) and other experiments dealing with the transamination enzymes (*5, 6*).

The tyrosine oxidation was carried out by means of the usual manometric procedure, as previously described (*7*). Appropriate solutions of the enzymes combined with the necessary additives were incubated with tyrosine (5 μm), the oxygen consumption observed being corrected by subtraction of suitable controls. With the addition of the ketoglutarate in stoichiometric proportions (5–10 μm) and ascorbic acid in catalytic quantities (150–200 μg), the oxidation of tyrosine occurs with the uptake of 4 atoms of oxygen per mole of tyrosine present. Without the addition of the vitamin the oxidation proves insignificant, with much less than 1 atom of oxygen being consumed. With the total system present in adequate quantity the oxidation proceeds to completion in 1 hr.

From these results it may be argued that ascorbic acid is necessary to the enzyme responsible for the first oxidative step, as may be concluded from the results obtained with liver slices (*2*). In light of this finding it also may be concluded that the vitamin is a coenzyme in this step, a conclusion which is further supported by the finding that the velocity of the oxidation is dependent upon the concentration of ascorbic acid present. In fact, it has been possible to calculate the dissociation constant between the coenzyme and the apoenzyme by the classical procedures of enzyme chemistry (as will be described elsewhere).

Since transamination appears to be the first step in the reaction sequence, it is obvious that *p*-hydroxy-

[1] The generous assistance of the Nutrition Foundation is gratefully acknowledged.

[2] Deceased.