Fungicidal Action of Metallic Alkyl Bisdithiocarbamates

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FUNGICIDAL ACTION OF METALLIC ALKYL BISDITHIOCARBAMATES[1]

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This is the report on seven years of investigation on the fungicidal action of the bisdithiocarbamates. It covers the screening of many molecular configurations, the selection of disodium ethylene bisdithiocarbamate and the problems encountered during its development as a practical fungicide, the mode of toxic action of the dithiocarbamates, and the effect of molecular configuration on this action. This monograph serves to emphasize the point, that in fungicide research, more than the inherent toxicity of a compound must be considered.

This is the story of a fungicide that almost died at birth, the history of its growing pains, and its present stature. Actually, as a composition of matter, the compound is older than seven years. Hester first synthesized it in 1935. He sent it to this laboratory and it was tested as a candidate fungicide in January, 1940. It failed to kill spores of *Stemphylium sarcinaeforme,* despite the fact that the fungus was known to be sensitive to other dithiocarbamates. Therefore, a new sample was tested in 1941 and it was found to be a very potent spore killer. Later studies indicated that the sample synthesized in 1935 had deteriorated prior to testing.

During the search for the cause of this deterioration, information was obtained on how the compound acts to kill spores and to protect plants against disease. This paper records that information.

The chemical was developed first under the pre-name code number of He 175. The trade now calls it "Dithane", but we shall abbreviate it here to DSE as a shortened form of disodium ethylene bisdithiocarbamate.

The first publications on DSE were made in 1943 when Hester (15) described the compound and its preparation and we described its fungicidal action (6). Heuberger, having been interested in the compound from the first (6), continued his interest after moving to the Delaware Experiment Station. In 1943, he and Manns (16) published a significant note on the salutary influence of zinc sulfate and lime [$Ca(OH)_2$] on the field performance of DSE—the so-called "Heuberger effect".

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[1] This project has been conducted in cooperation with the Crop Protection Institute. Much of the work, especially on the analogues, would have been impossible without the cooperation of Dr. W. F. Hester and associates of the Rohm and Haas Co., who synthesized most of the compounds studied. Dr. Ralph Connor (4) has been very helpful on the sulfur chemistry, but he must not be charged with any errors of fact or opinion. Research associates of the Crop Protection Institute who have aided the project are: A. E. Dimond, W. D. Henry, J. W. Heuberger and S. Rich. The technicians were Mrs. Pauline Baxendale, Mrs. Winifred Butterfield, Miss Edith Converse, Miss Katherine Cooke, Miss Doris Lowe, Miss Florence MacWilliam, and Mrs. Louise Nutile. To all of these we express our indebtedness.

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This observation cast a new light on DSE, and saved the compound from almost certain failure. He suggested that the effect may have been due to an improvement in tenacity or that a new compound may have been formed. In any case, it now seems safe to say that the fungicide would never have reached the grower's spray tank had the zinc sulfate-lime effect not been discovered. The elucidation of this synergistic action has been an intriguing study that has been pursued ever since the observation was first published. It now seems clear that the formation of a new compound is the real explanation, but the mechanism of the reaction is elusive and complex.

DSE was one of the first compounds observed to be more active against fungus spores than copper sulfate. It has an LD 50 in solution against spores of *Stemphylium* (*Macrosporium*) *sarcinaeforme* of 3.3×10^{-6} moles, as compared with about 38×10^{-6} moles of copper sulfate.

Even after it was determined to possess high fungicidal properties, DSE was not expected to succeed as an agricultural fungicide because of its water solubility. The textbooks all agreed that water solubility and resistance to rain are mutually exclusive. Therefore, it was with some astonishment that it was learned the dried spray residues resist washing. This peculiar tenacity is interwoven with the matter of toxicity and stability.

TECHNIQUES

All of the research reported herein was conducted in the laboratory, although much of it is supported by field work. The test fungus used throughout was *S. sarcinaeforme.*[1] The chemicals were assayed either as dried spray deposits on cellulose-nitrate coated glass slides, or as solutions. The spray deposits were applied with a compressed air atomizer as described in previous publications (19 and 20). The solutions were applied with a graduated 1-ml. syringe to the flat-bottomed circular depressions in so-called culture slides. The depressions were 15 mm. in diameter and had a capacity of 0.4 ml. of liquid. Two-tenths of a milliliter of double strength solution of the compound was placed in the depression. To this was added 0.2 ml. of double strength spore suspension. This filled the depression, thus eliminating the meniscus effect. The spores settled on the bottom of the depression, apparently according to the Poisson distribution.

As previously shown (2), bioassays are best interpreted in terms of the dosage-response curve. Therefore, in all cases, several doses of each chemical were applied either by varying the concentration in the case of solutions, or the spray time in the case of spray deposits. Two counts of 50 spores each were made for each dose. Data were expressed as percentage of spores that failed to germinate after a 16-hour incubation period. No correction for natural mortality seemed necessary since the untreated spores almost invariably germinated from 98 to 100 per cent. When the controls fell below this level, the assay was disregarded. Toxicity data plotted as the logarithm of the dose against the mor-

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[1] This fungus has been known in the literature for many years as *Macrosporium sarcinaeforme* Cav. Presumably, it is desirable to change the generic name to *Stemphylium* as Wiltshire (32) proposed and as McCallan (26) has reminded us.

tality in probits give a straight line if the data follow the usual biological dosage-response curves. A simplification of this is to plot dose against response on the specially designed logarithmic-probability paper. This technique was used throughout the research. Such dosage-response curves have two characteristics—position and slope. Certain notable exceptions to the straight line, *i.e.,* the "TMTD" curve will be discussed below.

Position is usually considered (20) as a measure of the potency of a compound. It may be used as a measure of the purity, particle size, *etc.* The position of a dosage-response curve is expressed in terms of the dose required to give some level such as a 50 or 90 per cent response. The accepted abbreviation is LD 50 or LD 90, *i.e.,* the lethal dose for those responses. It must be remembered then that a high LD value means a low potency. In this paper LD values for spray deposits are expressed as micrograms per square centimeter of sprayed surface. In the case of solutions it is given as moles per liter.

The slope of the dosage-response curve is considered as an indication of mode of action. This is a fairly new concept in toxicology. It is reasonable to assume on the basis of this concept that materials that inhibit spores alike will have similar slopes. The converse is less certain but still reasonable, *i.e.,* the materials with the same slope act alike.

Materials with different slopes, therefore, should act differently. In general, this is a fair statement except that altering a toxicant to make it insoluble seems at times to diminish the slope without necessarily changing the mode of action. Nevertheless, in the absence of data to the contrary, it will be assumed that materials with different slopes have different modes of toxic action and that materials of equal slopes act alike. It should be emphasized that these generalizations apply most rigidly when the materials are assayed in the same test.

Slope is given herein in terms of the LD 50 divided by the LD 90. In such terms an infinitely steep slope would equal unity and an infinitely flat slope would equal zero. In other words, the larger the decimal fraction, the steeper the slope. Since slope is a ratio, the mean slope for a compound is calculated from the average LD values rather than from the separate slopes.

FUNGICIDAL VALUE

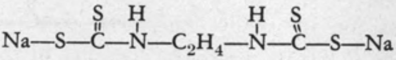
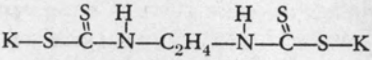
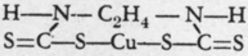
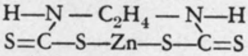
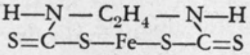
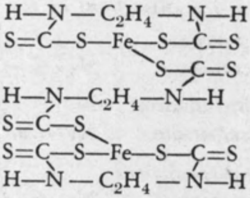
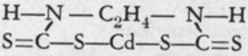
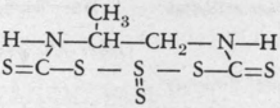
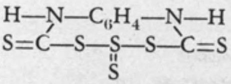
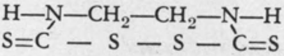
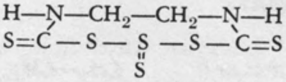
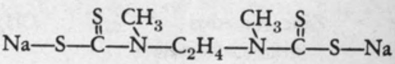
As discussed in greater detail elsewhere (20), the assets of a successful foliage fungicide are protective value and fungicidal value. Protective value is the ability to control disease in the field. Fungicidal value is the ability to kill or inhibit the fungus. Fungicidal value is concerned with the physical and chemical factors involved in solubilizing a spray deposit, permeation into the spore and inherent toxicity. It is best measured in the laboratory.

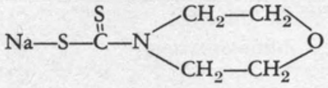
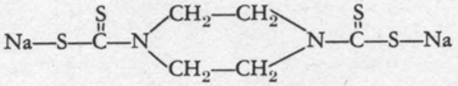
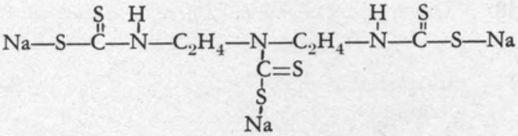
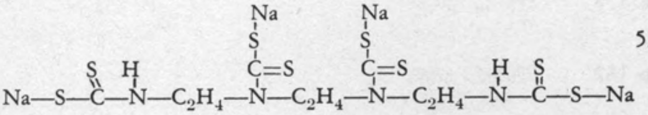
Effect of Analogues and Homologues

Some 36 analogues and homologues of disodium ethylene bisdithiocarbamate have been investigated during the seven years of work. The names, structural formulas and molecular weights are listed in Table 1.

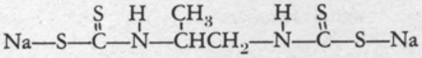
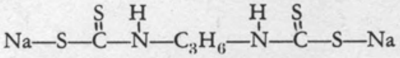
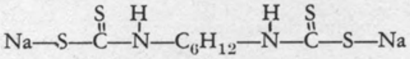
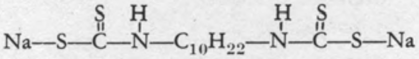
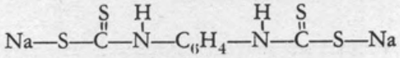
The most desirable arrangement of the data would be to place all results from each test together. To save space, however, the results are assembled by com-

TABLE 1. NAME, CODE, STRUCTURAL FORMULA, AND MOLECULAR WEIGHT OF ANALOGUES AND HOMOLOGUES OF DSE

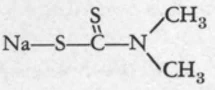
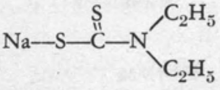
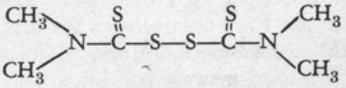
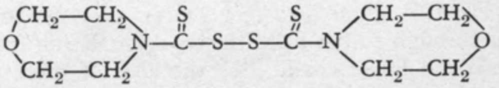
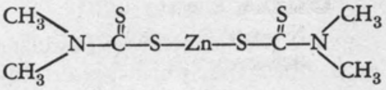
Code No.	Name	Structural formula	Mol. wt.
		Metal Salts	
DSE	Disodium ethylene bisdithiocarbamate	 $Na-S-\overset{S}{\overset{\parallel}{C}}-\overset{H}{\overset{\vert}{N}}-C_2H_4-\overset{H}{\overset{\vert}{N}}-\overset{S}{\overset{\parallel}{C}}-S-Na$	256
He 1138	Dipotassium ethylene bisdithiocarbamate	 $K-S-\overset{S}{\overset{\parallel}{C}}-\overset{H}{\overset{\vert}{N}}-C_2H_4-\overset{H}{\overset{\vert}{N}}-\overset{S}{\overset{\parallel}{C}}-S-K$	288
He 177	Cupric ethylene bisdithiocarbamate		274
He 178	Zinc ethylene bisdithiocarbamate		275
He 182	Ferrous ethylene bisdithiocarbamate		266
He 181	Ferric ethylene bisdithiocarbamate		742
He 184	Cadmium ethylene bisdithiocarbamate		322
		Cyclic polysulfides	
He 905	Propylene thiuram tetrasulfide		288
He 909	1, 4—phenylene thiuram tetrasulfide		322
He 183	Ethylene thiuram disulfide		210
He 257	Ethylene thiuram tetrasulfide		274
		Alterations of the secondary amines	
D 25	Disodium ethylene N—methyl bisdithiocarbamate	 $Na-S-\overset{S}{\overset{\parallel}{C}}-\overset{CH_3}{N}-C_2H_4-\overset{CH_3}{N}-\overset{S}{\overset{\parallel}{C}}-S-Na$	284

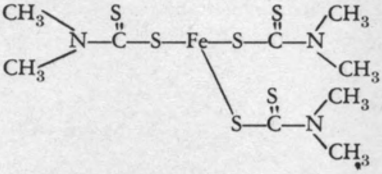
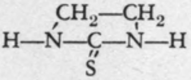
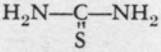
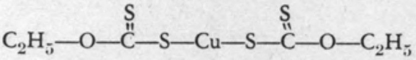
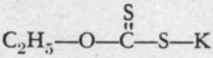
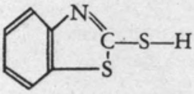
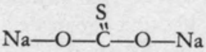
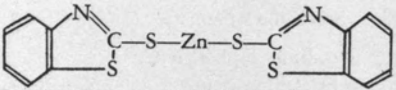
Code No.	Name	Structural formula	Mol. wt.
He 901	Sodium morpholine dithiocarbamate		185
He 956			282
He 1050		 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}C_2H_4{-}\underset{\substack{\vert\\C{=}S\\\vert\\S\\\vert\\Na}}{N}{-}C_2H_4{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	397
He 1051		 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}C_2H_4{-}\overset{\substack{Na\\\vert\\S\\\vert\\C{=}S\\\vert}}{N}{-}C_2H_4{-}\overset{\substack{Na\\\vert\\S\\\vert\\C{=}S\\\vert}}{N}{-}C_2H_4{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	538

Alterations in the aliphatic chain

Code No.	Name	Structural formula	Mol. wt.
He 902	Disodium propylene bisdithiocarbamate	 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{CH_3}{\vert}}{C}HCH_2{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	270
D 28	Disodium trimethylene bisdithiocarbamate	 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}C_3H_6{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	270
D 31	Disodium hexamethylene bisdithiocarbamate	 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}C_6H_{12}{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	312
D 33	Disodium decamethylene bisdithiocarbamate	 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}C_{10}H_{22}{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	370
He 907	Disodium *p*-phenylene bisdithiocarbamate	 $Na{-}S{-}\overset{\overset{S}{\Vert}}{C}{-}\overset{\overset{H}{\vert}}{N}{-}C_6H_4{-}\overset{\overset{H}{\vert}}{N}{-}\overset{\overset{S}{\Vert}}{C}{-}S{-}Na$	304

Other dithiocarbamates

Code No.	Name	Structural formula	Mol. wt.
He 1046	Sodium dimethyl dithiocarbamate		143
613	Sodium diethyl dithiocarbamate		171
TMTD	Tetramethyl thiuram disulfide		240
MOTD	Morpholine thiuram disulfide		324
Zimate or Zerlate	Zinc dimethyl dithiocarbamate		305

Code No.	Name	Structural formula	Mol. wt.
Fermate	Ferric dimethyl dithiocarbamate		416
	Other related organic sulfur compounds		
He 1054	Ethylene thiourea		102
——	Thiourea	 $H_2N-\underset{\overset{\parallel}{S}}{C}-NH_2$	76
He 166	Cupric ethyl xanthate	 $C_2H_5-O-\overset{\overset{S}{\parallel}}{C}-S-Cu-S-\overset{\overset{S}{\parallel}}{C}-O-C_2H_5$	306
——	Potassium ethyl xanthate	 $C_2H_5-O-\overset{\overset{S}{\parallel}}{C}-S-K$	160
Captax	Mercaptobenzothiazole		167
——	Sodium thiocarbonate	 $Na-O-\overset{\overset{S}{\parallel}}{C}-O-Na$	122
Zn Captax	Zinc salt of mercaptobenzothiazole		397

pound (Table 2) and dated. Reference to the dates will indicate whether any given chemicals were tested together. In Table 3, the compounds are arranged according to the mean slope of the dosage-response curve and also according to the LD 50 of the dosage-response curve.

The compounds fall into two large groups of structures: (1) the salts of the open chain compounds which have a monovalent cation on each end, DSE being the typical example, (2) those compounds without a monovalent cation. Such molecules have unsatisfied valence bonds on the two terminal sulfur atoms. There are four possibilities for satisfying these end valencies: (1) the two end sulfur atoms may combine to form a cyclic disulfide (see He 183, Table 1); (2) the two may be combined through a divalent metal to form a metallic salt (see He 178, Table 1); (3) the ends of two molecules may become linked either through sulfur to sulfur or through sulfur to metal to sulfur to form a polymerized linear chain; (4) the ends of these polymerized chains may then join to form cycles of many units.

The open chain compound postulated under (3) must have unsatisfied valencies at either end, which presumably would be occupied by a monovalent

Table 2. Fungicidal Value of Spray Deposits of Analogues and Homologues of DSE. LD Values are in Micrograms/cm^2
Slope Values are in Terms of LD 50/LD 90

Code	Date tested	LD50	LD90	Slope
DSE-A[1]	2/25/41	< 2.400	< 2.400	
DSE-A	5/ 5/41	.168	.276	
	5/ 6/41	.150	.249	
	5/20/41	.310	.742	
DSE-B	1/ 5/44	.235	.336	
	1/14/44	.128	.347	
	2/18/44	.080	.172	
	2/28/44	.112	.248	
	3/25/44	.057	.235	
DSE-C	3/ 2/45	.016	.038	
	3/27/45	.062	.124	
	11/21/45	.011	.025	
	11/30/45	.032	.070	
	12/31/45	.043	.132	
	1/29/46	.008	.019	
	2/13/46	.027	.055	
	3/ 8/46	.014	.028	
	Mean	.089	.193	.46
D 14[2]	11/29/45	.011	.025	
	11/31/45	.032	.070	
	12/31/45	.043	.131	
	3/ 2/45	.017	.038	
	3/27/45	.062	.124	
	Mean	.033	.079	.43
He178A	12/19/44	.305	1.201	
He178B	3/19/45	.225	.465	
He178C	2/13/46	.041	.338	
He178D		.707	1.591	
He178E		.484	1.149	
He178F		1.367	2.302	
He178C	3/18/46	.175	.308	
He178D		.457	1.196	
He178E		.603	1.181	
He178F		1.177	2.241	
He178G	2/11/47	.674	1.034	
He178G	2/13/47	.570	.921	
He178G	2/14/47	.236	.414	
	Mean	.540	1.109	.486
He184	2/25/41	3.168	4.704	
	4/ 8/41	2.256	3.808	
	Mean	2.720	4.256	.64
He905	2/26/41	3.712	8.192	
	4/ 8/41	3.840	4.608	
	Mean	4.257	6.400	.67
He909	2/26/41	3.648	9.953	
	3/31/41	.763	6.080	
	Mean	2.208	8.016	.28
He1046	12/17/45	.544	1.312	
	12/31/45	.588	1.728	
	Mean	.566	1.520	.37
He183	3/31/41	< 12.800	< 12.800	
	4/ 7/41	1.257	1.808	
	2/18/41	.102	.265	
	2/28/44	6.049	11.521	
	3/27/45	.344	.540	
	4/ 6/45	.192	.495	
	Mean	1.589	2.819	.56
He257	3/19/40	5.760	8.320	
	4/19/40	3.328	7.920	
	5/23/40	2.784	5.408	
	6/12/40	2.672	3.552	
	Mean	3.632	6.304	.59
Zimate	12/23/43	1.025	2.145	
	12/27/43	3.025	6.161	
	1/ 4/44	3.025	4.481	
	1/11/44	3.361	7.777	
	Mean	2.609	5.137	.51
Zimate (home-made)	2/11/47	.735	1.809	
	2/13/47	.294	1.030	
	2/14/47	.655	1.729	
	Mean	.562	1.521	.37
Fermate				
A	9/23/42	.833	1.953	
A	9/25/42	.545	.977	
A	11/18/42	1.873	3.153	
B	1/ 3/44	4.673	10.610	
B	1/ 6/44	1.825	6.081	
	Mean	1.953	4.545	.46
He1054	12/17/40	81.601	——	
	2/31/45	>102.401	——	
He1051	2/17/42	3.408	8.400	.41
He902	2/17/42	.512	1.040	
He902A	2/28/44	.083	.244	
He902A	3/ 2/45	.024	.046	
	Mean	.206	.443	.47
D 28	6/24/46	.030	.060	
	7/ 3/46	.041	.091	
	Mean	.036	.076	.47
D 31	6/24/46	4.160	7.520	
	7/ 3/46	1.904	4.432	
	Mean	3.040	5.984	.52
He1138	1/ 5/44	.100	.167	

Code	Date tested	LD50	LD90	Slope	Code	Date tested	LD50	LD90	Slope
	3/ 2/45	.016	.038			Mean	.123	.724	.18
	Mean	.059	.102	.58	613	12/17/45	4.289	8.641	
He177	3/27/41	1.888	2.256	.84		12/31/45	1.521	3.970	
D 25	11/29/45	6.721	12.961			Mean	2.913	6.305	.46
	11/31/45	5.761	15.041		TMTD	5/22/42	1.361	3.201	
	1/29/46	6.561	15.841			5/24/42	.337	.513	
	Mean	6.353	14.609	.43		9/23/42	1.345	2.113	
He901	2/18/44	1.152	11.040	.10		9/25/42	.369	2.369	
He956	12/ 7/45	24.320	58.240			Mean	.852	2.049	.42
	12/17/45	126.400	249.600		MOTD	9/ 1/42	3.009	6.401	
	Mean	75.300	153.920	.49		9/14/42	1.441	3.681	
He1050	2/17/42	3.105	5.792	.44		9/23/42	1.921	7.041	
He182	2/25/41	2.912	4.320			9/25/42	.929	2.561	
	4/ 1/41	1.009	1.441			Mean	1.825	4.929	.37
	Mean	1.968	2.880	.68	He166	3/27/41	.905	1.68	.54
He181	2/25/41	2.544	3.296			4/ 7/41	1.100	> 1.100	
	4/ 1/41	1.840	2.560		Captax	4/20/42	6.273	11.201	
	Mean	2.192	2.920	.75		4/22/42	12.081	20.961	
D 33	6/24/46	>16.000	>16.000			5/ 6/42	8.561	11.201	
	7/ 3/46	29.600	>32.000			5/11/42	5.249	14.961	
He907A	2/16/42	.131	.240			5/21/42	11.761	17.441	
He907A	2/19/42	.033	.512			8/20/42	3.489	4.642	
He907A	2/18/44	.113	1.088			Mean	7.378	13.345	.61
He907A	2/28/44	.291	1.360		Zn				
He907B	3/ 2/45	.073	.422		Captax	8/20/42	8.257	15.121	.54

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[1] Capital letters at the end of the code indicate batch numbers.

[2] D 14 is liquid DSE (mother liquor).

cation. Dr. H. J. Fisher of this Station has kindly analyzed three samples of the zinc salt made from the sodium compound. He obtained no precipitate with magnesium uranyl acetate, indicating less than 0.01 per cent of sodium. This means that if the polymer is an open chain, it must have at least 84 bisdithiocarbamate units in order to exist with less than 0.01 per cent of sodium. This number of units seems unlikely.

Since the precise form of the compounds is still uncertain, we shall refer to them as cyclic for the sake of brevity. Whatever is said below regarding the biological activity of the compounds should be construed as applying to either possibility until more is known about their spatial arrangement.

Effect of Cyclic and Open Chain Structures

The most striking fact in Table 3 is that the cyclic compounds have steeper slopes than the others. This is true whether the sulfur atoms are separated by divalent metals or not. Three comparisons are possible: (1) the propylene cyclic compound (He 905) has a steeper slope (0.67) than the parent open chain compound (He 902, slope 0.47); (2) the ethylene cyclic compound (He 183) has a slope of 0.56, whereas, the open chain analogue (DSE) has a slope of only 0.46; (3) the 1,4-phenylene compound (He 909) has a slope of 0.28 as compared with 0.18 for the open chain analogue (He 907).

Difference in slope exists among the metallic cyclic compounds. The slope for the zinc salt (0.49) is the flattest of those tested. Insufficient data are available to distinguish the slopes of the cupric (He 177), ferrous (He 182) and cadmium (He 184) salts. The ferric salt (He 181) exhibits a steeper slope (0.75) than the ferrous salt (0.68) as can be seen from the data in Table 2. The ferric salt exists as a cyclic molecule containing three bisdithiocarbamate units while the ferrous salt contains only one bisdithiocarbamate unit.

On the basis of the data, it is unwise to distinguish between the slopes of the open chain salts except disodium 1,4-phenylene bisdithiocarbamate (He 907). This salt has a much flatter slope than its aliphatic analogue, DSE. The difference in slope between the ethylene and phenylene compounds is not unexpected since aromatic and aliphatic compounds often do act differently.

The compounds do not group themselves in the same way for potency (LD 50) as they do for slope. In the three direct comparisons of cyclic versus open chain compounds the LD 50 values are in reverse order to that for slope.

TABLE 3. ANALOGUES AND HOMOLOGUES OF DSE ARRANGED IN THE ORDER OF LD 50 AND SLOPE (MEAN LD50/MEAN LD90)

Order of LD50		Order of slope	
Code	LD50 γ/cm^2	Code	Slope
D 14	0.03	He 177	.84
D 28	0.04	He 181	.75
He 1138	0.06	He 182	.68
DSE	0.09	He 905	.67
He 907	0.12	He 184	.64
He 902	0.21	Captex	.61
He 178	0.54	He 257	.58
Zimate[2]	0.56	He 1138	.58
He 1046	0.57	He 183	.56
TMTD[1]	0.85	Zn Captex	.54
He 183	1.59	He 166	.54
MOTD	1.82	D 31	.52
He 177	1.89	Zimate	.51
Fermate[1]	1.95	He 956	.49
He 182	1.97	He 178	.49
He 181	2.19	He 902	.47
He 909	2.21	D 28	.47
Zimate	2.61	DSE	.46
He 184	2.72	613	.46
613	2.91	Fermate[1]	.46
D 31	3.04	He 1050	.44
He 1050	3.10	D 25	.43
He 1051	3.41	D 14	.43
He 257	3.63	TMTD[1]	.42
He 905	4.26	He 1051	.41
D 25	6.35	He 1046	.37
Captex	7.38	MOTD	.37
Zn Captex	8.26	Zimate[2]	.37
D 33	29.60	He 909	.28
He 956	75.30	He 907	.18
He 1054	81.60	He 901	.10

[1] Compound with "TMTD" curve. See page 29.

[2] Zimate prepared in this laboratory.

The cyclic propylene compound (He 905) has an LD 50 of 4.26, but the corresponding open chain compound (He 902) has an LD 50 of 0.512. The ethylene cyclic compound (He 257) and the open chain DSE have LD 50 values of 3.63 and 0.09, respectively. And finally the cyclic (He 909) and open (He 907) phenylene compounds have LD 50 values of 2.21 and 0.12, respectively.

So far as the zinc salts are concerned, the LD 50 values of the closed (He 178) and open chain (Zimate) were 0.54 and 0.56, respectively. On account of the relatively flatter slope of the latter, however, the differences at LD 90 are much larger. The LD 50 values of the copper, ferric, ferrous, and cadmium salts probably do not differ significantly, but the zinc salt is definitely more potent than the others, presumably because it is more soluble.

It may be concluded from a study of the open and closed chain systems that the closing of the chain or the polymerizing of the compound steepens the slope, presumably because it changes the mode of action. Closing the chain seems to have no consistent effect on potency, as measured by LD 50. This probably means that the percentage of spores inhibited is about the same whether they are affected by one mechanism or by another.

Effect of Length of Polysulfide Linkage

There was only one direct comparison of numbers of sulfur atoms in the sulfur to sulfur linkage. The slopes of ethylenethiuram disulfide (He 183) and ethylenethiuram tetrasulfide (He 257) did not differ significantly, but the disulfide compound was about six times as toxic as the tetrasulfide.

Effect of Length of the Aliphatic Chain

The effect of altering the length of the central aliphatic chain was studied. Chain length seems to have no effect on slope, but as the chain is lengthened (D 28, D 31, D 33) the LD 50 increases very rapidly, *i.e.,* potency falls. Likewise, sodium diethyl dithiocarbamate (613) is only about one-fifth as toxic as the corresponding dimethyl salt (He 1046). As already mentioned, the substitution of a benzene ring for the ethylene nucleus increases the LD 50 (decreases toxicity) by about 1.5-fold. The reduction in potency with increase in number of carbons in the central linkage is presumably due to a decrease in solubility.

Effect of the Two Amine Hydrogen Atoms

Each of the two nitrogens in the molecule of DSE has a replaceable hydrogen, which may very well be involved in the fungicidal action of the compound. This possibility may be explored by examining compounds D 25 and He 956. In these materials the hydrogens have been substituted. In D 25 they have been replaced by methyl groups, and the slope has not been changed (0.43 as compared with 0.46). The toxicity, however, is reduced by some 71-fold. Obviously, the amine hydrogens are important in the potency of the compound, but apparently not in its mode of action. In He 956 the two nitrogen atoms are present in a heterocycle and thus have no replaceable hydrogen. The slope is 0.49 as compared with 0.46 for the unsubstituted compound, but the toxicity has been reduced by some 840-fold. Here again only the potency is affected, not the mode of toxic action.

He 1050 and He 1051 are of some interest here. He 1050 is made from diethylene triamine, and He 1051 is made from triethylene tetramine, instead of from mono ethylene diamine. He 1050 and He 1051 are quite similar to DSE except that some of the nitrogens bear hydrogen atoms and some do not. The compounds have essentially the same slope as DSE, *i.e.,* 0.44 and 0.41, respectively, as compared with 0.46, but they are much weaker, by 34.2 and 38.2-fold, respectively. Here, again, the mode of action seems similar but the potency is reduced by a larger molecule.

Discussion of Mechanisms of Fungicidal Action

Literature

Very little has been published on the mechanism of the action of any dithiocarbamate material on a fungus. Davies and Sexton (5) reported experiments on the effect of structure on fungicidal action using the agar technique. Data were expressed as minimum lethal dose only and, hence, nothing is available about slope of the dosage-response curve. The doses were such that no difference smaller than five-fold could be detected. The authors concluded that sulfur *per se* makes no contribution to the activity. This was based on the equivalence of sulfur and oxygen analogues. In connection with the discussion above, relative to amine hydrogen, it is interesting that they deduced that thiuram disulfide was less potent than tetramethyl- or tetraethylthiuram disulfide. Davies and Sexton ventured no opinion as to how dithiocarbamates could kill fungi. The limitations of the agar technique for assaying slight differences among fungicides need not be discussed.

Parker-Rhodes (29) also using *S. sarcinaeforme* spores, decided that the activity might be due to liberation of carbon disulfide and dialkylamine. He found, as we have, that neither compound has a very high level of toxicity. He drew no conclusions as to what biological function or group of functions in the fungus was disturbed by the dithiocarbamates.

Goldsworthy *et al* (10) investigated a series of dithiocarbamates in the laboratory and field, but they were concerned with differences in field performance rather than with the mechanisms of toxicity.

Present Research

Mechanisms of fungicidal action may be considered from the point of view of the fungus or of the chemical. Biologists are likely to take the former view, the chemists the latter. Neither alone is sufficient, however. "Mechanism" is here defined as the method by which some essential biological activity of an organism (respiration, for instance) is interrupted.

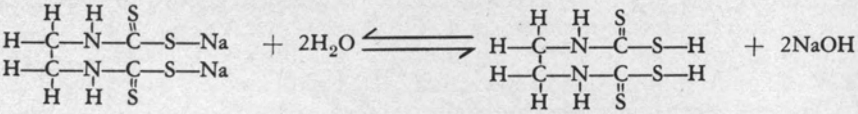
Two chemical reactions of DSE are known: (1) it may spontaneously liberate H_2S; and (2) it forms metal salts by substitution of the sodium. On the basis of these two reactions, two mechanisms are proposed by which DSE poisons spores: (1) the proteins of the spore may be altered by the addition of sulfhydryl groups; (2) the trace metals which the fungus needs in its metabolism are made unavailable either through the formation of metallic sulfides or the formation of metallic salts of ethylene bisdithiocarbamic acid. These mechanisms could operate singly or simultaneously.

The precipitation of metals by H_2S is recalled from sophomore courses in qualitative analysis. The precipitation of metals by the sodium salt of dithiocarbamic acid is commonly used in quantitative analysis, as witness the use of sodium diethyl dithiocarbamate as a reagent for copper. The thought that dithiocarbamates precipitate metals in the fungus is an extension of Zentmyer's (33) theory. He proposed that 8-hydroxyquinoline compounds inhibit spores by such a mechanism.

The conclusions just summarized were derived primarily by studying how analogues of DSE act on the fungus, but also from an examination of the possible and probable chemical reactions of DSE. These will now be discussed.

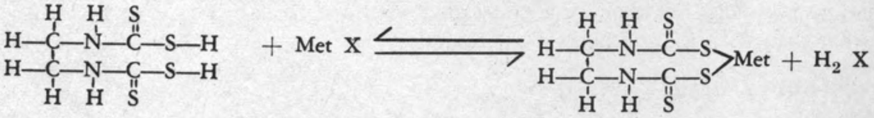
Hydrolysis of DSE

Hydrolysis at the sodium bond to form ethylene bisdithiocarbamic acid is an easy first step. The hydrolysis proceeds as follows:



Clearly, any substitution in the molecule that would prevent or retard this hydrolysis should reduce the initial potency of the compound. On the basis of usual chemical theory, the cyclic or polymeric forms of the compound such as He 183 (Table 1) should hydrolyze much more slowly than the open chains. As already mentioned, they are less toxic. Likewise, preformed insoluble metal salts such as He 178 (Table 1) are less readily hydrolyzable than the soluble salts. They should, therefore, be less fungicidal, and that is the case. The only metal salt that is as toxic as the sodium salt is the potassium salt, and it is equally as soluble.

Once hydrolysis is accomplished, the formation of salts with the metals in the fungus should follow the usual course:



"Met" in the above equation represents an essential divalent trace metal and "X" represents an unknown anion in the fungus.

It should be mentioned that, if salt formation by this reaction were the only mechanism by which DSE could inhibit spores, the insoluble metallic salts should not be active as fungicides, but they are active. The possibilities to account for this are given below.

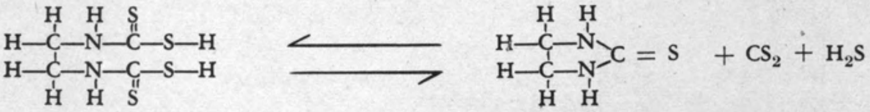
Liberation of Hydrogen Sulfide

It has been known since the days of Pollacci (30) that hydrogen sulfide is toxic to fungi. McCallan and Wilcoxon (27) have shown that hydrogen sulfide gives a 50 per cent inhibition of spores of our test fungus (*S. sarcinaeforme*) at a concentration of 1.48×10^{-5} molar in the spore suspension.

Experimenters need no documentary evidence that DSE liberates H_2S. Containers emit the stench. When a solution of DSE is placed in a Van Tiegham cell, spores are killed in a hanging drop above it. In fact the LD 50 is of the order of 7.4×10^{-4} molar. That this quantity is 50 times as large as that found by McCallan and Wilcoxon (27) is accounted for by the fact that the H_2S must diffuse through the free air space and into the hanging drop. Presumably, the concentration of H_2S in the hanging drop is also near 1.48×10^{-5} molar as McCallan and Wilcoxon found. If an insoluble metal salt (*e.g.*, zinc salt) is substituted for DSE in the Van Tiegham cell, few spores are killed in the hanging drop. Furthermore, it is worth noting that H_2S liberation is accelerated if yeast is mixed with DSE or its analogues. McCallan and Wilcoxon (27) first showed this phenomenon with elemental sulfur.

Three hypotheses expressed as chemical equations have been written to account for the liberation of H_2S from the molecule of DSE in the presence of fungus spores. Two of them start from the hydrolyzed molecule discussed above.

Equation I postulates the formation of ethylene thiourea and CS_2 as well as H_2S.

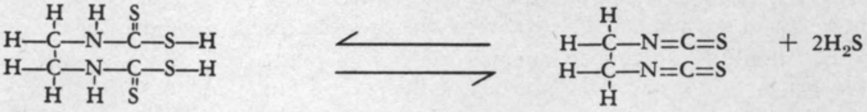


Equation I

Ethylene thiourea has been identified among the "debris" when DSE decomposes *in vitro*. Carbon disulfide has not been positively identified. The ethylene thiourea (see He 1054 in Table 2) and the CS_2 are both very poor spore killers.

Under the conditions where the LD 50 of H_2S is about 7.4×10^{-4} molar, the LD 50 of CS_2 is 1.25×10^{-2} molar. Hence, H_2S is about 17 times as potent as CS_2.

The difficulty with equation I is that it fails to account for the poor performance of those analogues such as D 25 in which the amine hydrogen has been substituted. Equation I does not postulate the participation of the amine hydrogen. Equations II and III account for such participation.



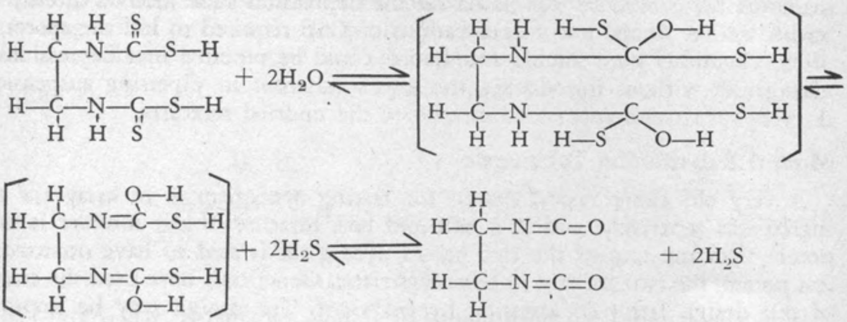
Equation II

Even though equation II shows how the amine hydrogen could be involved some chemists think that it represents a very unlikely reaction. The reaction of primary aromatic amines with carbon disulfide gives thioureas presumably through isothiocyanates, whereas aliphatic primary amines give dithiocarbamates. This would indicate that an isothiocyanate from an aliphatic dithiocarbamate is unlikely, at least *in vitro*.

Another difficulty with equation II is that the isothiocyanate postulated is unstable. It will be noted that equation II differs from equation I in that it provides twice as much hydrogen sulfide. Although it may not be admissible to compare data of McCallan and Wilcoxon (27) with ours because of differences in conditions, it is interesting, nevertheless, to note that they say the LD 50 for H_2S is 14.8×10^{-6} molar. In our data (see Table 5 below) the LD 50 of DSE is about 3.3×10^{-6} molar. Allowing for a two-fold error, one would still need two molecules of H_2S from each molecule of DSE to bring the toxicities of the two compounds into line.

Whether equation II represents a valid reaction or not, the evidence demands that an equation be written to account for the participation of the amine hydrogen. Equation III proposes a different reaction involving the amine hydrogen.

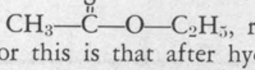
Equation III postulates a method by which the =C=S group may participate in the activity of the dithiocarbamate and thiuram disulfide compounds. The activity of the =C=S group has been reported on in some detail already (20).



Equation III

Equation III postulates ethylene di-isocyanate and H_2S as the end products. Equation III provides four times as much H_2S as equation I and twice as much as equation II. This agrees very closely with the amount of H_2S required on the basis of data of McCallan and Wilcoxon (27).

Equation III also accounts for the action of the cyclic or polymerized compounds including the metallic ones which presumably hydrolyze to dithiocarbamic acid slowly if at all. The hydrolysis of the =C=S group probably occurs in the thiuram disulfide and thion esters, probably not in the dithiocarbamate salts, but possibly in dithiocarbamic acid. It is the best equation yet written, however, to account for the toxicity of the divalent metallic compounds.

Equation III explains an anomaly previously published (20), but not explained, with respect to the potency of the =C=S group. An oxygen atom attached to the carbon atom as in  $CH_3—\overset{\overset{S}{\parallel}}{C}—O—C_2H_5$, reduced the fungicidal activity. Presumably, the reason for this is that after hydrolysis of the =C=S group, the compound could split out either ethyl alcohol or H_2S. This would reduce the possible amount of H_2S liberated, and hence would reduce potency.

Having in mind the various chemical reactions, the data on slope begin to make a pattern. It will be recalled that the steep slope materials are the cyclic or polymeric compounds. Since they probably do not hydrolyze readily to dithiocarbamic acid, they probably are capable of acting mainly by liberation of H_2S (by equation III). Therefore, perhaps, one can say that steep slope for a compound implies H_2S as the primary cause of death of the spores. The flat slope materials are the open chain materials. These are capable of forming metal salts directly by substitution. They are capable also of liberating H_2S. Hence, the flat slopes probably result from the concurrent effects of both reactions, direct salt formation and H_2S liberation.

The substituted nitrogen compounds such as D 25 need further consideration here. D 25 like DSE can form metal salts. Perhaps, it can liberate H_2S by the first stage of equation III.

Effect of Electrolytes in Solution

Because of the significance of the effect of zinc sulfate and lime on DSE as mentioned above, a study was made of the general effects of electrolytes in solution on its toxicity. The effect of electrolytes on fungicides has been given but little attention. Clark (2 and 3) studied mixtures of electrolytes with $HgCl_2$ and $CuSO_4$ and Lin (25) worked likewise with $CuSO_4$. DSE offered an opportunity to study mixtures of electrolytes with an organo-metallic molecule. The study dovetailed with our research on the general problem of synergism and antagonism. A few aspects of this problem have already been published (20 and 21).

Two techniques are currently used in bioassay of synergism and antagonism: (1) the mutual substitution technique and (2) the "titration" technique. The manipulative procedure was based on the depression slide method already described above. At the low concentrations of DSE required to kill the spores, all of the chemicals were soluble and, hence, could be pipetted into depressions in glass slides without introducing the errors inherent in pipetting suspensions. A spore suspension was added directly to the undried mixtures.

Mutual Substitution Technique

A very old experimental design for testing synergism is to assay the two ingredients separately, and in a half and half mixture. If the mixture is more potent than the sum of the two halves, synergism is said to have occurred; if less potent, the two are said to be antagonistic. Geneticists have used the essence of this design freely in assessing hybrid vigor. The design may be improved materially by using several mixtures with varying proportions of ingredients.

A graphic demonstration of results of a test of DSE and $ZnSO_4$ is given in Table 4 and in Figure 1. The dotted line is the hypothetical line of no inter-

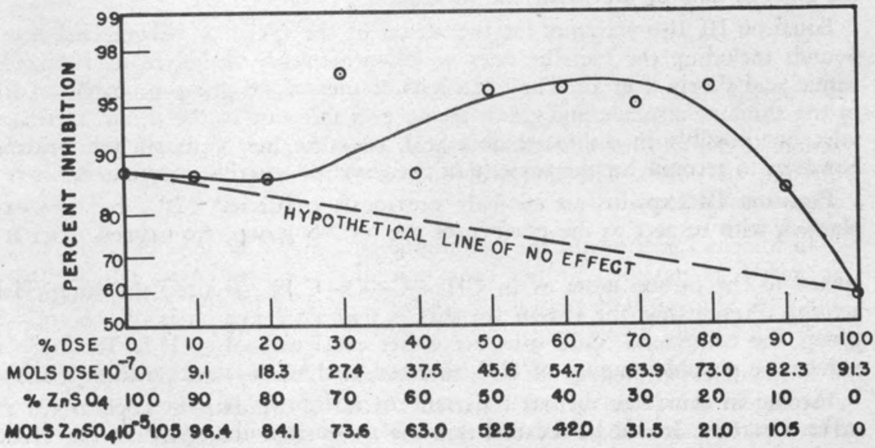


Figure 1. The mutual substitution technique for studying synergism between $ZnSO_4$ and DSE.

TABLE 4. FUNGICIDAL ACTION OF MIXTURES OF DSE AND ZINC SULFATE USING THE MUTUAL SUBSTITUTION TECHNIQUE. DATA EXPRESSED AS PERCENTAGE OF SPORES INHIBITED.

Ingredients mixed					Ingredients alone			
$ZnSO_4 \cdot 1H_2O$		Disodium ethylene bisdithiocarbamate			$ZnSO_4 \cdot 1H_2O$		Disodium ethylene bisdithiocarbamate	
molarity X10−6	per cent of mixture	molarity X10−6	per cent of mixture	spores killed per cent	molarity X10−6	spores killed per cent	molarity X10−6	spores killed per cent
1050	100	0	0	88	2105	98	18.30	100
946	90	.91	10	87	1890	98	16.45	100
841	80	1.83	20	86	1690	98	14.60	98
736	70	2.74	30	97	1470	97	12.80	98
630	60	3.75	40	87	1360	89	11.00	94
525	50	4.56	50	96	1050	88	9.15	58
420	40	5.47	60	96	841	75	7.31	43
315	30	6.39	70	95	631	74	5.50	24
210	20	7.30	80	96	421	67	3.66	11
105	10	8.23	90	84	211	41	1.83	1
0	0	9.13	100	58	0	0	0	0
631	100	0	0	74				
566	90	.55	10	100				
502	80	1.09	20	83				
442	70	1.64	30	86				
379	60	2.19	40	95				
315	50	2.73	50	93				
251	40	3.28	60	89				
189	30	3.82	70	84				
126	20	4.37	80	78				
63	10	4.92	90	70				
0	0	5.47	100	24				

action. If the two ingredients are wholly substitutable, a straight line will pass through all points. The line will be convex if synergism occurs, concave if antagonism occurs.

In toxicity experiments, the two ingredients should be used in a dose to give as nearly as possible 50 per cent mortality; *i.e.,* equitoxic, not equimolar, amounts. Only so is it possible to measure increase or decrease in toxicity for each ingredient.

Such an experimental design was used by LePelley and Sullivan (24) on insecticidal synergism between pyrethrum and rotenone and by Dimond and Horsfall (7) on fungicidal synergism between metallic oxides and sulfur. It has been used here to study the effect of zinc sulfate on disodium ethylene bisdithiocarbamate. (Table 4 and Figure 1).

The results give all the symptoms of synergism between the substances. The dosage-response curve balloons upward as postulated for synergism. Calculations showed that LD 90, based on the area of the depression (15 mm. deep and 0.2 cc. of liquid) was 0.28 γ/cm^2 of DSE. This is well within the range of that obtained when employing the spray assay technique as set forth in Table 2.

Perhaps the experimental design starting with approximately equitoxic doses is not sound. As Finney (9) and others have pointed out such a design requires that the ingredients have parallel dosage-response curves. This necessitates that each loses potency at equal rates as it is diluted. Actually, DSE has a much steeper dosage-response curve than zinc sulfate. Nobody, as yet, seems to know quite how to handle cases of synergism between compounds of dissimilar slopes. The original data will be made available to any statistician who wishes to explore them.

Titration Technique Holding DSE Constant

Another experimental design already discussed (20) was used. Basically, it is the standard titration design of the chemist, using ingredients on a molar basis.

TABLE 5. EFFECT OF INCREASING AMOUNTS OF ELECTROLYTES ON TOXICITY OF DSE USED AT 3.33 X 10−6 MOLAR. DATA EXPRESSED AS PERCENTAGE OF SPORES INHIBITED.

Electrolyte		February 6, 1945		March 12, 1945			February 27, 1945
moles per liter X10−6	molar ratio[1]	$ZnSO_4$	Na_2SO_4	$CaSO_4$	Na_2SO_4	equal[2]	$Ca(OH)_2$
80.00	24	63	64	42	35	66	54
40.00	12	43	50	48	50	52	38
20.00	6	31	41	49	35	53	52
13.35	4	29	45	52	23	51	69
10.00	3	35	43	42	40	34	45
6.65	2	51	39	35	39	33	45
5.00	1.5	60	35	63	46	54	38
4.65	1.4	61	30	39	26	59	29
4.35	1.3	54	26	48	34	54	46
4.00	1.2	63	32	41	37	61	49
3.65	1.1	67	28	41	24	43	43
3.35	1.0	49	42	39	25	32	55
3.00	.9	58	32	59	22	33	61
2.65	.8	57	28	58	32	33	62
2.30	.7	44	9	42	57	32	56
2.00	.6	42	8	37	52	29	55
1.65	.5	34	8	36	42	30	44
1.35	.4	21	15	50	47	16	61
1.0	.3	28	37	43	29	58	55
.65	.2	18	30	36	36	49	59
Mean	....	45	32	45	37	44	52
DSE alone	0	51	51	40	40	40	57

[1] Molar ratio $= \frac{\text{moles electrolyte}}{\text{moles DSE}}$

[2] Equal moles of $CaSO_4$ and Na_2SO_4

TABLE 6. EFFECT OF INCREASING AMOUNTS OF $ZnSO_4$ MIXTURES ON TOXICITY OF DSE USED AT 3.33 X 10^{-6} MOLAR. DATA EXPRESSED AS PERCENTAGE OF SPORES INHIBITED.

$ZnSO_4$ X10^{-6} molar	Molar[1] ratio $ZnSO_4$ to DSE	Molar ratios - $ZnSO_4/CaSO_4$					Molar ratios - $ZnSO_4/Ca(OH)_2$[2]									
		4/5/45					4/11/45					4/17/45				
		4	2	1	0.5	$ZnSO_4$ alone	4	2	1	0.5	$ZnSO_4$ alone	4	2	1	0.5	$ZnSO_4$ alone
80.00	24	45	48	30	44	42	81	68	64	100	72	74	61	81	98	86
40.00	12	27	47	31	34	46	76	49	50	52	58	68	43	68	62	63
20.00	6	30	70	32	34	61	58	42	43	35	47	48	18	44	27	42
13.35	4	35	45	51	57	67	62	44	43	53	28	30	8	42	38	42
10.00	3	35	46	61	57	80	66	44	47	42	30	29	17	34	24	44
6.65	2	35	39	66	49	75	73	66	49	49	26	16	20	62	24	48
5.00	1.5	29	39	63	43	74	87	63	50	39	36	32	10	47	33	50
4.65	1.4	40	31	61	46	72	67	56	36	41	59	18	8	39	40	59
4.35	1.3	33	43	54	36	72	77	43	53	40	52	28	10	37	29	66
4.00	1.2	27	45	35	37	84	66	43	38	38	57	21	6	54	20	62
3.65	1.1	27	47	62	59	78	67	49	36	39	42	35	21	25	25	37
3.33	1.0	30	64	68	69	76	81	49	31	34	60	45	23	37	41	25
3.00	.9	25	60	68	59	78	69	62	27	45	58	37	22	30	38	17
2.65	.8	26	40	64	46	73	70	57	20	43	51	38	17	39	31	19
2.30	.7	40	54	62	56	75	61	28	25	39	53	26	21	20	26	27
2.00	.6	25	49	53	51	72	67	37	16	44	56	17	17	28	12	27
1.65	.5	16	47	54	48	80	73	41	22	37	57	29	13	22	29	16
1.35	.4	30	37	67	46	62	76	29	23	34	45	17	9	19	21	30
1.00	.3	37	19	66	43	49	57	46	17	34	41	19	20	23	27	23
.65	.2	23	40	65	55	45	63	38	17	40	25	22	14	25	21	11
Mean	....	29	46	56	49	68	70	48	34	34	34	33	19	39	33	40
DSE alone	0	71	71	71	71	71	34	34	34	34	34	4	4	4	4	4

[1] $\frac{\text{molar } ZnSO_4}{\text{molar DSE}}$ in the field = 0.30, based on 1 to 1.5 lbs. each per 100 gals.

[2] $\frac{\text{molar } ZnSO_4}{\text{molar } Ca(OH)_2}$ in the field = 4.9, based on 1 to 0.5 lbs. each per 100 gals.

The material to be "titrated" was DSE. Varying molar solutions of electrolyte were added to a series of solutions containing a constant amount of DSE. The concentration of DSE was calculated to give as nearly as possible 50 per cent spore inhibition (3.33 X 10^{-6} molar). The range of final concentration of electrolyte in distilled water was 0.65 X 10^{-6} to 80 X 10^{-6} molar. In practice the mixtures were made in small vials using equal portions of quadruple strength ingredients. Then 0.2 cc. of the mixture was placed in the depression in the slide and 0.2 cc. of double strength spore suspension was added. The double dilution thus brought the concentration of electrolyte, DSE and spores to that desired.

The effect of electrolytes on toxicity was striking. No simple relationship emerged. The "titration" curves for the most part were polymodal as already reported briefly by Gries *et al* (11). Although the results were similar in many respects to those obtained by Lin (25) and by others for metallic poisons plus electrolytes, still it required several months of work to convince us that the data were sound and that the polymodality could be reproduced. Basic data for several typical experiments are given in Tables 5 and 6.

Detailed data will be made available to anyone really interested in unraveling the phenomenon. Representative curves smoothed by inspection are shown in Figures 2A to 3C.

Effect of Zinc Sulfate

Data on the effect of zinc sulfate will be presented first because of its general interest in connection with DSE. From the curves in Figures 2A, 3A, 3B and 3C, it will be seen, in general, that as the amount of zinc sulfate increases, the toxicity of the mixture diminishes slightly. With further addition of zinc sulfate, the toxicity then climbs steadily, reaching a peak somewhere near 3×10^{-6} molar. By definition, synergism had occurred as in the experiment using the mutual substitution technique. As still more zinc sulfate is added, the toxicity declines from its peak near 3×10^{-6} molar and reaches a valley floor near 10×10^{-6} molar. Toxicity then increases again.

In searching for an explanation, the possibility of forming the zinc salt would come to mind. It will be shown below that the zinc salt is formed when zinc sulfate is mixed with DSE. From the empirical equation on page 14, it follows that the reaction proceeds on an equimolar basis. If so, the toxicity should diminish with the first increments of zinc sulfate and continue to diminish until the 1 to 1 molar ratio is reached. This is patently not the case because the toxicity *rises* until a ratio of approximately 0.8 to 1 is reached. Two escapes from this dilemma suggest themselves: (1) the equilibrium may not be far in the direction of the zinc salt because the dilution is so great that all salts are completely soluble; (2) the sodium sulfate formed by the reaction may increase the permeability of the spore membrane to the mixture of zinc sulfate, zinc ethylene bisdithiocarbamate, and DSE itself. It is conceivable, therefore, that the increased permeation of the mixture to the spore membrane more than offsets the decreased toxicity of the zinc salt. If so, the apparent toxicity should increase as long as additional sodium sulfate is formed (22).

Presumably, the increasingly large amount of inherently less toxic zinc salt finally overbalances any salutary influence of the increased permeability and, therefore, the toxicity falls rapidly. As might be expected, then, there is a carryover in the permeability effect and the floor of the valley is reached not at a 1 to 1 but at about a 2 to 1 ratio. If the amount of zinc sulfate is raised still further, the range of zinc toxicity is reached and the curve rises again.

Effect of Sodium Sulfate

This theory should be tested with sodium sulfate alone, although the situation is not quite comparable because of the absence of the zinc salt. It will be noted from the curves in Figures 2A and 2B that sodium sulfate produces more peaks and valleys than zinc sulfate. There appear to be two or three well-defined peaks

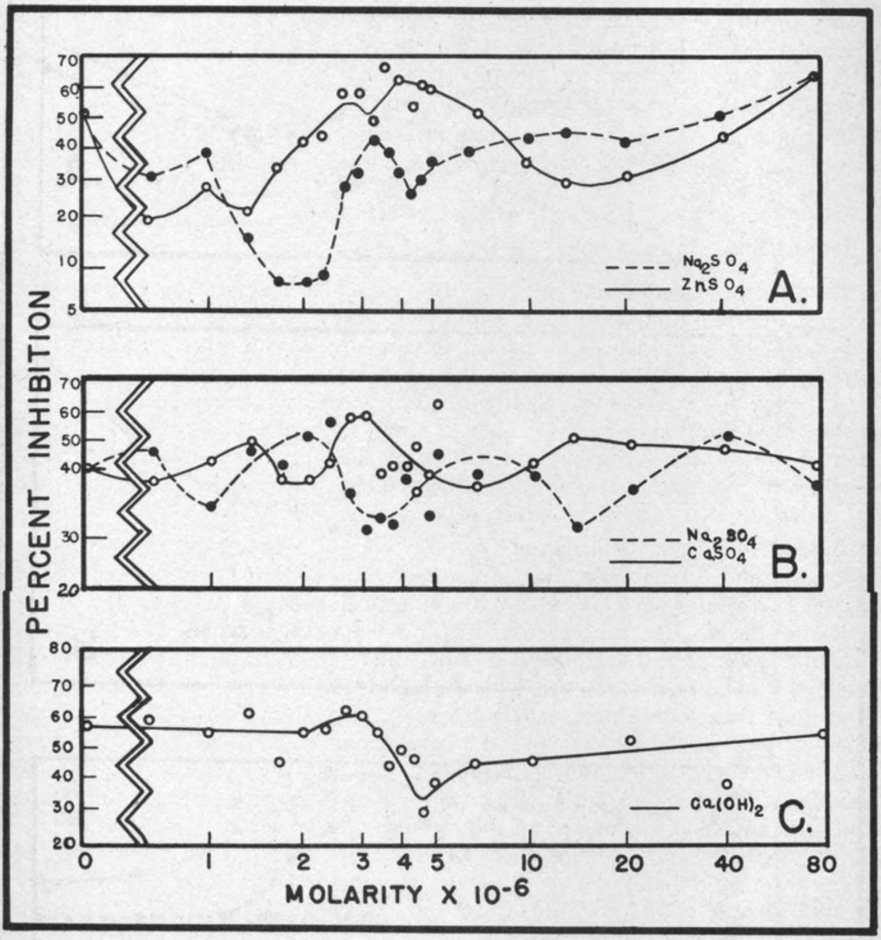


Figure 2. A. Effect of "titrating" DSE with $ZnSO_4$ and Na_2SO_4. Test of February 6, 1945.
B. Effect of "titrating" DSE with $CaSO_4$ and Na_2SO_4. Test of March 12, 1945.
C. Effect of "titrating" DSE with $Ca(OH)_2$. Test of February 27, 1945.

and as many valleys. These peaks and valleys are not strictly reproducible from test to test. Therefore, some may hold that they are simply cases of heterogeneity. They occur in so many experiments, however, that such an explanation seems untenable.

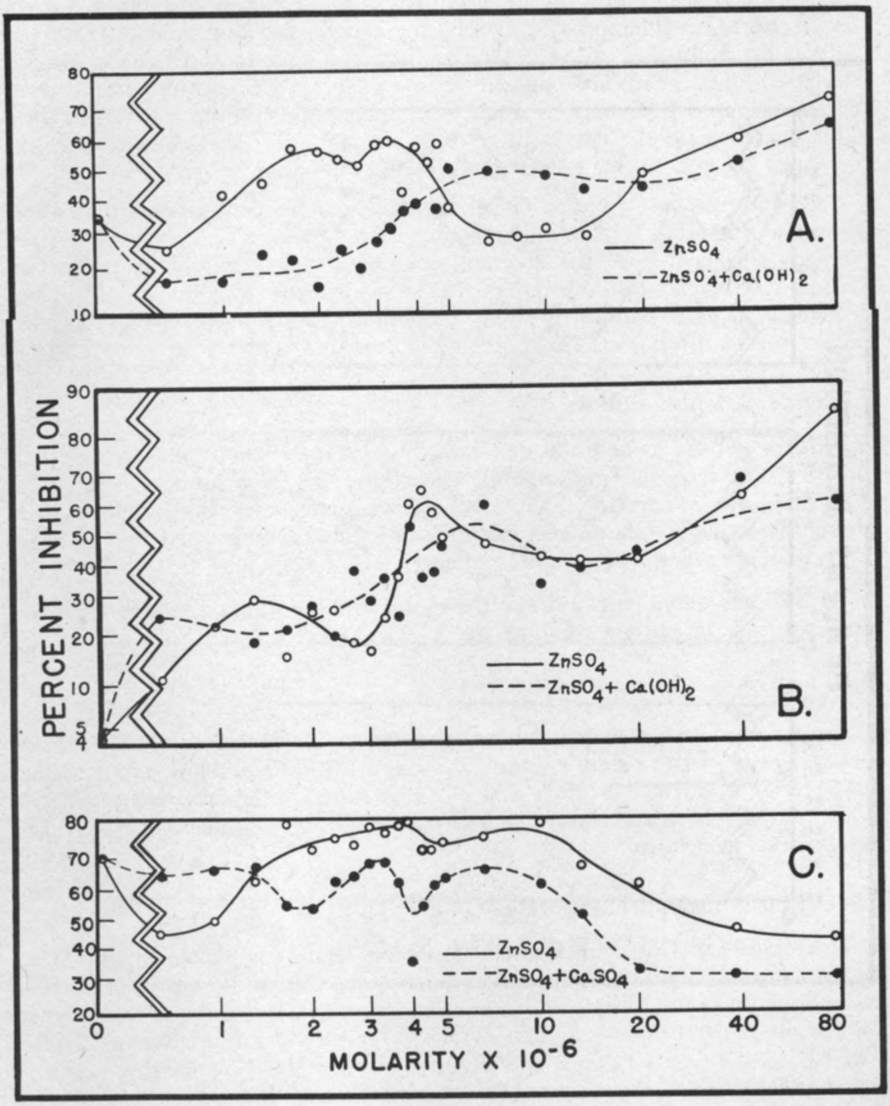


Figure 3. A. Effect of "titrating" DSE with $ZnSO_4$ and with $ZnSO_4 + Ca(OH)_2$ mixtures. Test of April 11, 1945.

B. Effect of "titrating" DSE with $ZnSO_4$ and with $ZnSO_4 + Ca(OH)_2$ mixtures. Test of April 17, 1945.

C. Effect of "titrating" DSE with $ZnSO_4$ and with $ZnSO_4 + CaSO_4$ mixtures. Test of April 5,. 1945.

Although the effect of both sodium and zinc sulfate follows a periodic relationship to concentration of the electrolyte, the position of the peaks and valleys seems to be different, as shown by the experiment illustrated in Figure 2A. This is no doubt due to the difference in the cations. In general, the peaks of the zinc sulfate curve are opposite to the valleys of the sodium sulfate curve except that they both tend to reach a common peak near a 1 to 1 molar ratio. This tendency for the curves to reach agreement may be due to the fact that the curve for zinc sulfate reflects the presence of sodium sulfate as well.

One curious effect appears. Generally speaking, the curve for sodium sulfate lies below that for zinc sulfate showing that the inhibition of spores is less. Perhaps this is due to the common ion effect between sodium sulfate and disodium ethylene bisdithiocarbamate, in which the sodium sulfate reduces hydrolysis of DSE, curtails its ability to produce H_2S and, therefore, reduces its ability to kill the spores. This point will come up again below.

Effect of Calcium Sulfate

It is the general assumption that calcium salts and sodium salts act oppositely on permeability. Data from a single test appear in Table 5 and in Figure 2B. The peaks and valleys for calcium sulfate were almost *exact mirror images* of those for sodium sulfate. The calcium and sodium salts acted oppositely as suggested by general permeability theory.

It should be noted here also that the sodium sulfate mixture kills fewer spores than the calcium sulfate mixture.

Effect of Other Anions

To determine whether the polymodal curve is due to anions or cations, one test was made with calcium chloride and one with sodium chloride, but neither gave clean-cut polymodal curves. Calcium hydroxide, in the only test made, gave what seems to be a good polymodal curve (see Table 5 and Figure 2C). The results are inconclusive.

Effect of Mixtures of Electrolytes

The mixture of DSE witn a single electrolyte seems complex enough. It must be remembered that the "Heuberger effect" was obtained with a three-way mixture of DSE, zinc sulfate and lime. Therefore, this mixture was tested twice using four molar proportions of $Ca(OH)_2$ for each concentration of $ZnSO_4$; 4x, 2x, 1x, and ½x (Table 6 and Figures 3A and 3B). For brevity, only the equal molar proportions are graphed for the two tests. It seems significant that the calcium hydroxide reverses the peaks and valleys for the zinc sulfate in both cases. This is additional evidence that the final effect of the zinc sulfate may be to shift permeability.

The mixture of zinc sulfate and calcium sulfate was assayed once (Table 5 and Figure 3C). The results were essentially in agreement with those for calcium hydroxide—the peaks and valleys were out of phase. The tapering downward at the high doses apparently is due to the fact that the zinc sulfate alone was not as toxic as usual.

Titration Technique Holding Electrolyte Constant

In one experiment $CuSO_4$ at 1.54 X 10^{-4} molar was titrated with DSE ranging from 0.04 X 10^{-6} molar to 82 X 10^{-6}. The dosage-response curve was polymodal with a maximum at 1.0 X 10^{-6} molar DSE, but the waves were less well defined than those obtained in the reciprocal experimental design.

In another experiment $FeSO_4$ at 1.4 X 10^{-5} molar was titrated with DSE. A hint of a double peak appeared. The dosage-response curve in the presence of $FeSO_4$ flattened in the same molar range of DSE as for the $CuSO_4$ in the experiment above, but unfortunately the range of DSE did not extend low enough to cover a possible maximum at about 1.0 X 10^{-6} molar.

Other experiments were conducted with thiourea. When used alone, the dosage-response curve gave a hint of a secondary peak but, in the presence of copper sulfate at 1.54 X 10^{-4} and 7.7 X 10^{-5} molar (Figure 4), peaks and

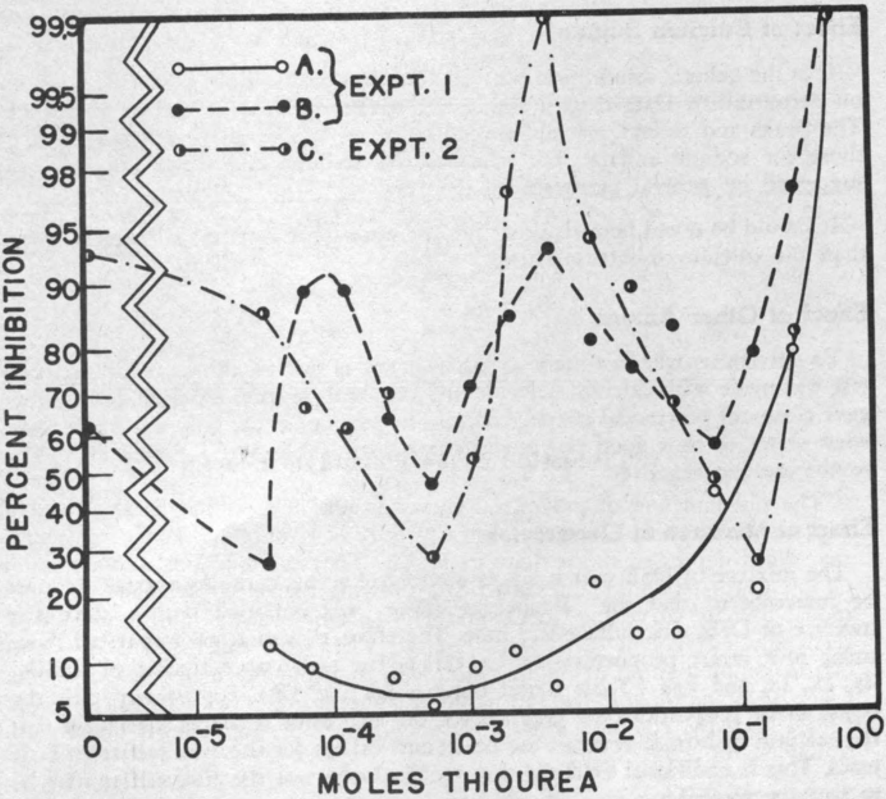


Figure 4. Effect of "titrating" $CuSO_4$ with thiourea. Test of May 25, 1944.
Note also ethylene thiourea in Fig. 10.
A. Thiourea only.
B. Thiourea plus $CuSO_4$ 1.54 X 10^{-4} molar.
C. Thiourea plus $CuSO_4$ 3.08 X 10^{-4} molar.

valleys definitely appeared. The peaks on the curves for the two concentrations of $CuSO_4$ coincided at the same molar concentrations of thiourea, not at the same molar ratios.

Finally, one test was made titrating five levels of copper sulfate with sodium diethyl dithiocarbamate. The range used gave excellent polymodal curves. The complete data are given in Table 7 and two typical curves are shown in Figure 5. Sodium diethyl dithiocarbamate alone does not give a straight line dosage-response curve on log-probability coordinates as expected. The addition of copper sulfate exaggerates the departure from linearity (see Figure 5). The major peak occurs at 1.5×10^{-3} molar sodium diethyl dithiocarbamate regardless of the concentration of $CuSO_4$. Again, the concentration and not the molar ratio is the important factor.

TABLE 7. EFFECT OF "TITRATING" $CuSO_4$ AND SODIUM DIETHYL DITHIOCARBAMATE (CODE 613) AGAINST EACH OTHER. DATA EXPRESSED AS PERCENTAGE OF SPORES INHIBITED.

$CuSO_4$ X10^{-4} molar	Sodium diethyl dithiocarbamate X 10^{-4} molar						
	117	58.5	29.3	14.6	7.3	3.7	0
12.32	96	62	83	86	27	96	100
6.16	87	83	90	90	19	25	91
3.08	94	90	86	87	57	2	25
1.54	96	91	88	96	62	5	10
.77	95	99	98	91	81	12	4
0	99	96	95	93	84	56	0

Discussion of the Electrolyte Effect

The phenomenon of polymodal curves is not new, although no satisfactory explanation has been advanced. As early as 1897 Kronig and Paul (23) showed that NaCl depressed the toxicity of $HgCl_2$. They ascribed this to the common Cl ion. Clark (2 and 3) presented data showing that electrolytes induce a polymodal toxicity curve for $CuSO_4$ and $HgCl_2$. Obviously, Kronig and Paul had seen only a fragment of the whole curve. Clark contended against the theory of the common anion. It might cause depression but not a polymodal response. Clark advocated double salt formation at some ratios. Hawkins (13) vigorously vetoes both the common ion theory and the double salt theory, but admitted to having no better theory.

Lin (25) produced polymodal toxicity curves for copper sulfate with several electrolytes. This certainly serves to destroy Kronig and Paul's theory. Lin inhibited spores with copper sulfate and then resuscitated them with electrolytes. His ingenious explanation was that the electrolyte removes the copper by displacement from the locus of toxicity. This explanation, however, accounts only for the depressive action of the electrolyte. It does not account for the subsequent rise in toxicity.

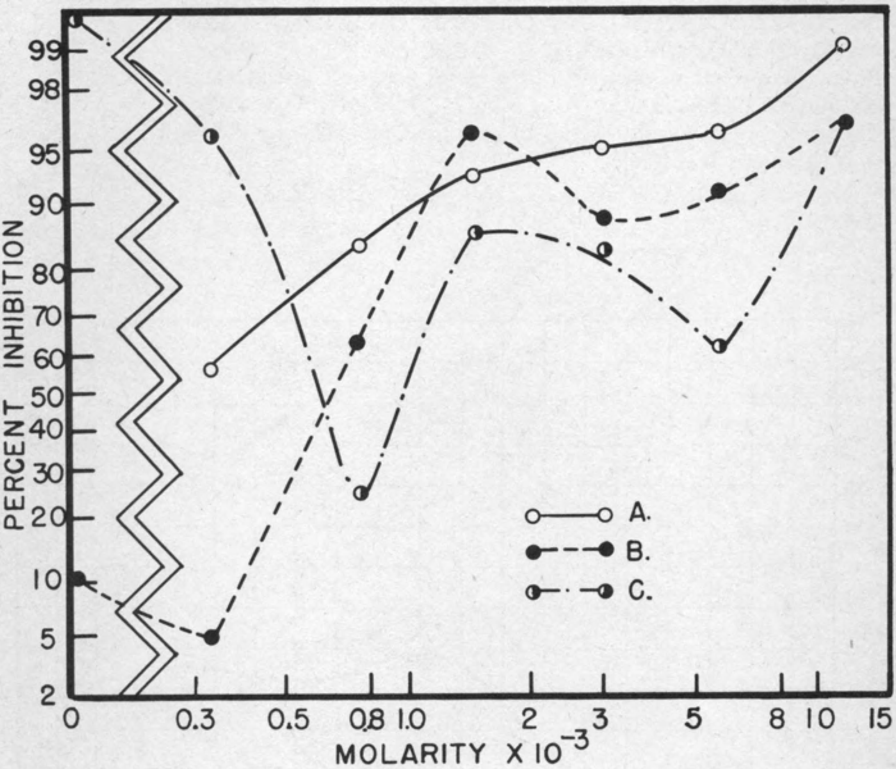


Figure 5. Effect of "titrating" $CuSO_4$ with sodium diethyl dithiocarbamate.

A. Sodium diethyl dithiocarbamate alone.

B. Sodium diethyl dithiocarbamate plus 1.54 X 10^{-4} molar $CuSO_4$.

C. Sodium diethyl dithiocarbamate plus 1.23 X 10^{-4} molar $CuSO_4$.

This paper adds two or three additional facts to what is known: (1) that electrolytes produce polymodal curves for organic as well as for inorganic toxicants, (2) in some cases at least, the electrolytes merely exaggerate a tendency already present, (3) different cations produce different types of curves, (4) cations appear to be more important in producing polymodal curves than anions, (5) there appears to be a lyotropic series of cations with respect to total kill, (6) in some cases at least, the existence of polymodal curves is independent of, but may be confused with, molecular proportions of the ingredients.

All of the electrolytes tested on DSE produced the wavy response curve, but the position of the crests and the troughs varied with the cation. There was some evidence that the chloride anion was less effective in producing the polymodal response than the sulfate anion.

The mean toxicity of the DSE seemed to vary somewhat with the cation. The following information is available on effect of cation on over-all toxicity: $Na_2SO_4 < ZnSO_4$, $Na_2SO_4 < CaSO_4$, $NaCl < CaCl_2$, $CaSO_4 < ZnSO_4$, $MgSO_4 < MnSO_4 < Al_2(SO_4)_3$.
From these separate comparisons a series can be constructed as follows: $Na_2SO_4 < CaSO_4 < ZnSO_4$. Also $MgSO_4 < MnSO_4 < Al_2(SO_4)_3$.
This suggests a lyotropic series of cations. One each of the following comparisons of anions was made:

$NaCl < Na_2SO_4$, $Ca(OH)_2 < CaSO_4$, $Ca(OH)_2 < ZnSO_4$.

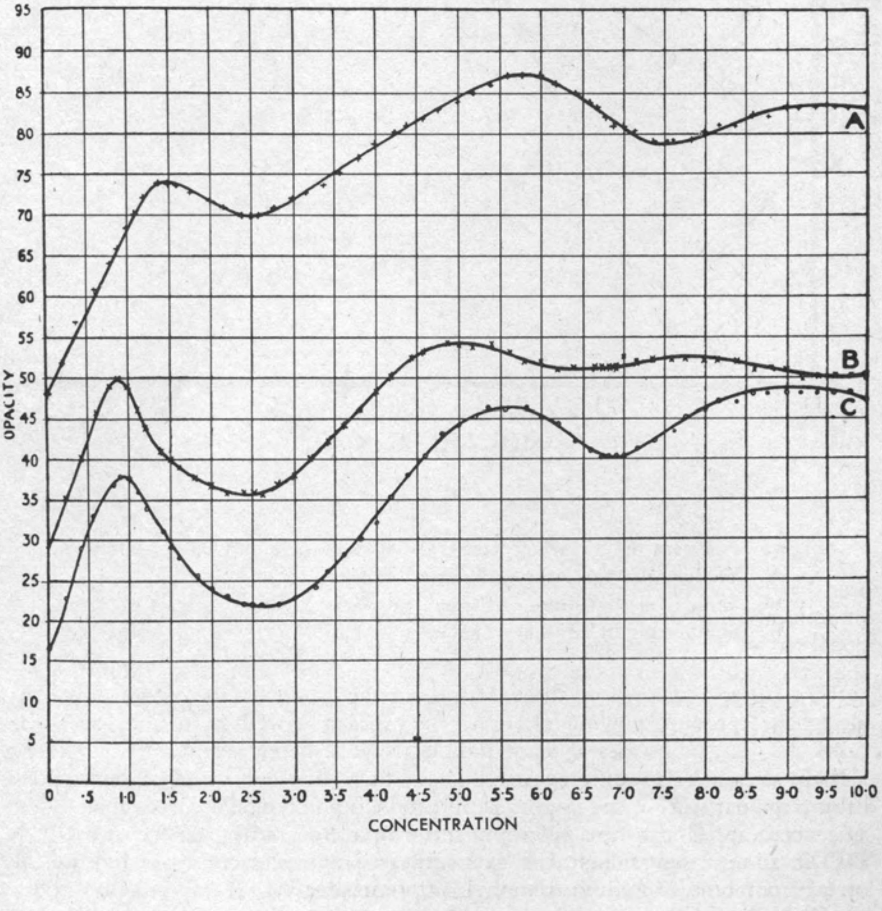


Figure 6. Effect of concentration of sodium chloride on opacity of blood serum to light. Data after Holker (18). A, B, and C are different sera.

All of the facts lead one to conclude that the phenomenon is one of a periodic effect of the electrolyte on permeability of the cell to the toxicant (22). The apparent existence of a lyotropic series of cations is one of the strongest reasons for this hypothesis. Another bit of evidence is that the effect seems largely associated with the concentration of electrolyte, not molar ratio with the toxicant. If there were only a single peak and a single valley and if it were associated with the theoretical combining molar ratios, one would expect the action to be due to an extracellular reaction of the chemicals. Otherwise, the effect must be due to the action of the electrolyte on the cell itself.

Holker (18) working with blood serum (an assemblage of colloids) discovered that its opacity to light followed a polymodal curve with respect to the concentration of sodium chloride. His curve is reproduced in Figure 6. Holker's work and ours can be harmonized by assuming that the protoplast changes not in opacity to light but in "opacity" to the toxicant with changes in the concentration of electrolyte. Presumably Holker was observing periodic variation in the precipitation of the colloids of the blood serum. We are observing periodic variations in the permeability of the membrane of the cell, perhaps due to the precipitation of the colloids.

Hedges and Myers (14) have suggested that Holker's phenomenon is related to the well-known Liesegang rings in which precipitation rings in a gelatin film occur at intervals outward from a center. It should be noted that calling attention to the analogy with Liesegang rings does not "explain" the phenomenon.

Some good explanation for the periodicity is necessary. If the problem is one of precipitation of colloids, then electric charge must be involved. Perhaps there are various colloids in the system each having a different isoelectric point and hence each one precipitated by a different concentration of electrolyte.

The "TMTD" Curve

The polymodal curve just discussed in the last section is a phenomenon related to the "TMTD" curve. Dimond *et al* (8) described a polymodal curve for tetramethylthiuram disulfide used alone. Therefore, in our laboratory polymodal curves are nicknamed "TMTD" curves after the prime letters in the name. The existence of such a curve for TMTD has been confirmed by Montgomery and Shaw (28) in England. The original TMTD curve of Dimond *et al* (8) recalculated to γ/cm^2 is reproduced in Figure 7.

It has already been noted (pages 25 and 26) that thiourea and sodium diethyl dithiocarbamate show hints of TMTD curves when tested alone at different concentrations. During the research on the action of dithiocarbamates, a whole TMTD curve or fragments of it have appeared one or more times for the following compounds: sodium dimethyl dithiocarbamate (He 1046) (see Figure 8), ferric dimethyl dithiocarbamate (Fermate) (see Figure 9), He 1051 (see Figure 10), He 909, and He 166 (cupric ethyl xanthate). Dimond *et al* (8) reported that DSE exhibited curves with double maxima, but this is rare unless the substance is contaminated with electrolytes as described above. Of

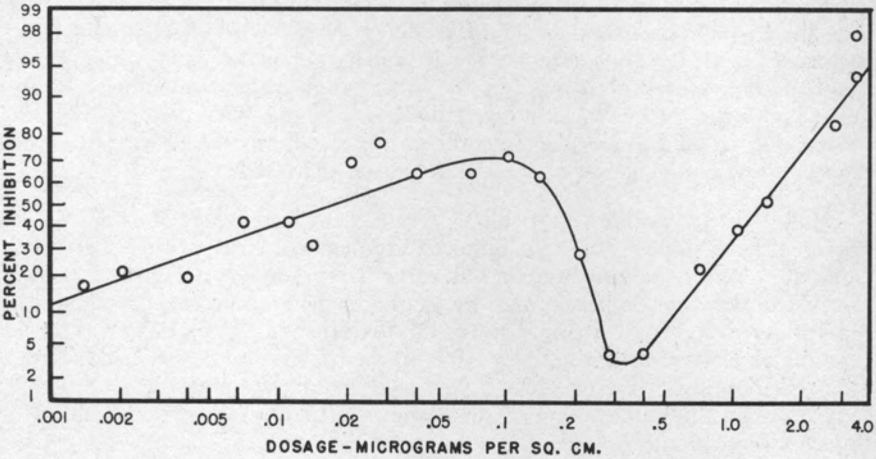


Figure 7. Original "TMTD" curve of Dimond *et al* (8) recalculated to γ/cm^2.

course, He 1051 and He 909 are derivatives of DSE. The only other non-organic sulfur material for which it has been observed is 8-hydroxyquinoline sulfate. In fact, it was observed in this case before it was observed in TMTD. Montgomery and Shaw (28) report that the phenomenon occurs in five thiuram sulfide derivatives and in six dithiocarbamate derivatives.

Undoubtedly, the curve exists in the data of many toxicity experiments, but it is masked either because doses are too far apart or because the range covered is too narrow. TMTD curves may at times be dismissed as due to poor technique or to heterogeneity. Ferric dimethyl dithiocarbamate seldom shows the curve except after the deposits have been washed by the Heuberger (16) technique. This is illustrated for three separate experiments in Figure 9.

The original TMTD curve itself (Figure 7) is worth restudying in connection with an explanation of the phenomenon. Dimond *et al* (8) suggested that the peak of toxicity in the high range of concentration was probably due to the fungicidal action of undissociated molecules of TMTD. And that the peak in the low range of concentration was probably due to the action of dissociated dithiocarbamate ions. This may be stated in reverse by saying that the hypothetical undissociated molecule has a lower potency than the dissociated molecule.

Further reference to Figure 7 will show that the "down side" of each of the peaks has a different slope. It is steep for the peak at the high level and flat for the peak at the low level.

Several deductions that are germane here were made in connection with the fungicidal action of DSE (see page 16): (1) that slowly hydrolyzable molecules (He 178) have a lower potency than readily hydrolyzable molecules

(DSE), (2) that slowly hydrolyzable molecules probably act only by liberating H_2S, (3) that readily hydrolyzable molecules probably act also by direct salt formation with metals in the fungus, (4) that steep slopes probably refer to H_2S liberation from the =C=S group, and (5) that flat slopes probably refer to a composite of H_2S liberation and direct salt formation. All of this lends additional assurance that the explanation advanced by Dimond *et al* (8) was accurate as far as it went.

To complete the picture, then, the explanation for the TMTD curve *might* read as follows: At high concentrations, TMTD acts by liberating H_2S from the =C=S groups. Dilution reduces the amount of TMTD, and hence the amount of H_2S available. Dilution and perhaps the reducing action of H_2S on the molecule tends to encourage decomposition at the -S-S- linkage. This makes available a second reaction for inhibiting spores, *viz.,* salt formation. When enough dithiocarbamate units have been made available, the downward trend of dilution is counterbalanced and potency rises in the active range of the second reaction. With further dilution, the potency of the second reaction declines.

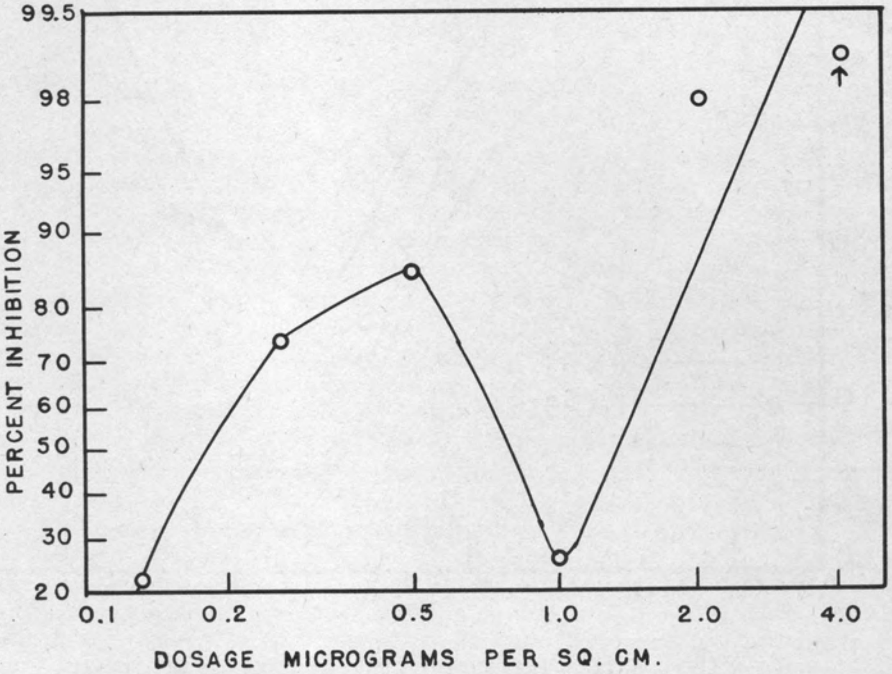


Figure 8. "TMTD" curve for sodium dimethyl dithiocarbamate (He 1046) mean of two assays. Similar curves occurred in almost every test for this material.

Reference has been made above (page 29) of the possible role of electric charge in the generation of polymodal curves. Turner (31) of this Station has reported a TMTD curve for the killing of aphids with derris dust extended with pyrophyllite. Turner's explanation was based on the proposition of electrostatic charge set up by friction as the pyrophyllite and derris particles emerge

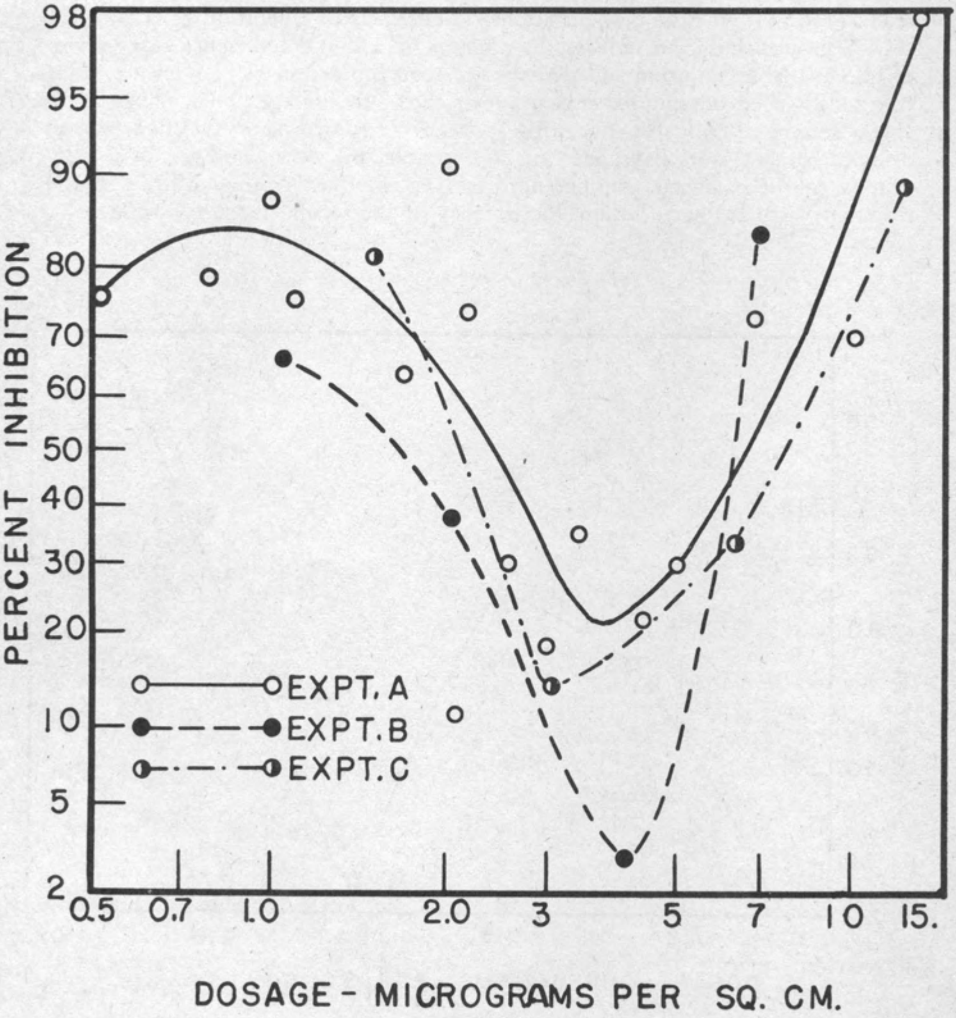


Figure 9. "TMTD" curves for 3 separate tests of washed deposits of Fermate. In this case peaks and valleys were remarkably constant.

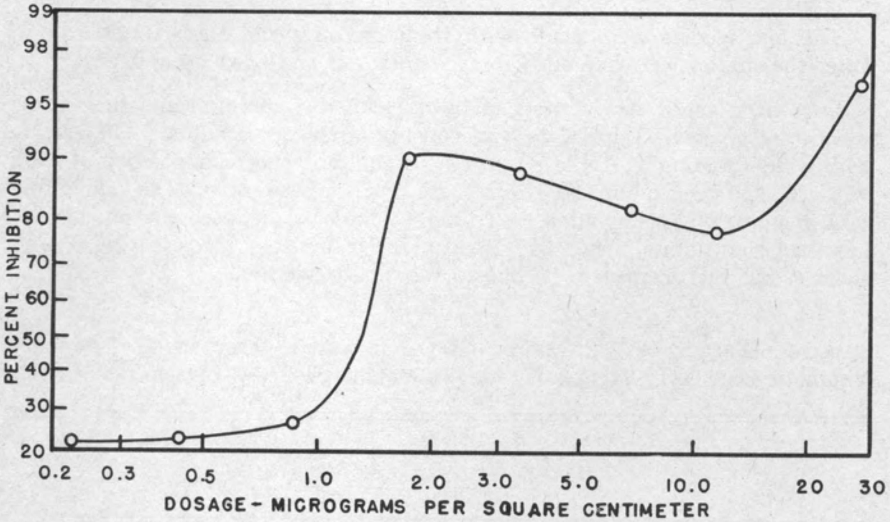


Figure 10. "TMTD" curve for ethylene thiourea. Note also thiourea in Figure 4.

from the dust nozzle. The electric charge causes the derris to cling to the insect, thus increasing the effective dose. In essence his theory is that, as he diluted his derris with pyrophyllite, he reduced the toxicity but, simultaneously, he increased the charge on the particles and thus tended to compensate for loss in concentration. Eventually, the charge increased faster than the dilution factor on the derris. This increased the amount of derris clinging to the insect and actually increased the dose on it. As a result, the toxicity increased. The toxicity soon reached the second peak and then declined with further dilution of the derris by the pyrophyllite. In Turner's case, the effect of the charge was not to increase the dose in the organism by changing the permeability, but rather by changing the amount that clings to its outside.

From all that has been said, it follows that TMTD curves seem to be generated by the interaction of two factors acting at different rates or in opposite directions. The four interactions just discussed are: (1) competition for the locus of toxicity (see Lin 25), (2) relative effects of permeation and inherent toxicity of a compound, (3) relative significance of two modes of toxicity exhibited by a single compound, (4) relative effects of clinging to an insect and poisoning it. Obviously, when more than two factors operate simultaneously, the curves will be more jagged than when only two are involved. It is even conceivable that some of the peaks or valleys will be damped out.

Effect of Zinc Sulfate and Lime in Fresh Spray Deposits

As mentioned above, Heuberger and Manns (17) published a paper showing the salutary influence of $ZnSO_4$ and $Ca(OH)_2$ on the field performance of DSE. Upon the appearance of their paper, laboratory research was begun in an effort to elucidate the phenomenon. The remainder of this bulletin will be devoted to that research.

The first studies were made with fresh mixtures and fresh spray deposits. Later the studies were extended to aged mixtures and aged spray deposits.

Data from a few typical tests of fresh Heuberger mixtures and their ingredients, are given in Table 8. Several conclusions seem warranted: (1) calcium hydroxide (column 2, Table 8) had no significant effect on potency of DSE; (2) zinc sulfate (column 3) reduced the potency between two- and three-fold; (3) a mixture of zinc sulfate and lime (column 4) reduced the potency but less than zinc sulfate alone; (4) zinc oxide (column 5) increased the potency slightly, but this seemed to be due to the zinc oxide alone.

TABLE 8. EFFECT OF ZINC SALTS, $CA(OH)_2$, AND A MIXTURE ON FUNGICIDAL VALUE OF DSE. DATA EXPRESSED AS LD 90 VALUES IN γ/CM^2 OF SPRAYED SURFACE.

Date of test	DSE alone	DSE + $Ca(OH)_2$	DSE + $ZnSO_4$	DSE + ZnO	DSE + Zinc-lime[1]
Dec. 22, 1943	0.264	....	....	0.111	....
Jan. 25, 1944	0.112	....	....	0.171	0.220
Jan. 27, 1944	0.083	0.096	0.221	0.068	0.110
Feb. 2, 1944	0.137	0.173	....	....	....
Feb. 17, 1944	0.212	....	....	0.276	0.487

[1] Zinc-lime means a mixture of $ZnSO_4 + Ca(OH)_2$

A consideration of the equilibria of the chemical reactions that occur in the mixtures will aid in understanding the results of the bioassay in Table 8. To save space, the ethylene bisdithiocarbamate part of the molecule will be indicated below by EB. The solid arrow will indicate the direction of major reaction toward the least soluble ingredient. Approximate solubilities in grams per 100 grams of water are indicated in parentheses below each molecule. The symbol VS denotes very soluble.

$$\underset{(42)}{\mathrm{Na_2EB}} + \underset{(0.18)}{\mathrm{Ca(OH)_2}} \rightleftharpoons \underset{(VS)}{\mathrm{CaEB}} + \underset{(50)}{\mathrm{2NaOH}} \qquad \text{(column 3, Table 8)}$$

$$\underset{(42)}{\mathrm{Na_2EB}} + \underset{(86)}{\mathrm{ZnSO_4}} \rightleftharpoons \underset{(0.001)}{\mathrm{ZnEB}} + \underset{(49)}{\mathrm{Na_2SO_4}} \qquad \text{(column 4)}$$

$$\left.\begin{array}{l} \mathrm{ZnO} + \mathrm{HOH} \longrightarrow \mathrm{Zn(OH)_2} \\ \underset{(42)}{\mathrm{Na_2EB}} + \underset{(2.6X10^{-7})}{\mathrm{Zn(OH)_2}} \rightleftharpoons \underset{(0.001)}{\mathrm{ZnEB}} + \underset{(50)}{\mathrm{2NaOH}} \end{array}\right| \qquad \text{(column 5)}$$

$$\left.\begin{array}{l} \underset{(42)}{\mathrm{Na_2EB}} + \underset{(86)}{\mathrm{ZnSO_4}} \rightleftharpoons \underset{(0.001)}{\mathrm{ZnEB}} + \underset{(49)}{\mathrm{Na_2SO_4}} \\ \underset{(0.001)}{\mathrm{ZnEB}} + \underset{(0.18)}{\mathrm{Ca(OH)_2}} \rightleftharpoons \underset{(VS)}{\mathrm{CaEB}} + \underset{(2.6X10^{-7})}{\mathrm{Zn(OH)_2}} \\ \underset{(VS)}{\mathrm{CaEB}} + \underset{(49)}{\mathrm{Na_2SO_4}} \rightleftharpoons \underset{(42)}{\mathrm{Na_2EB}} + \underset{(0.18)}{\mathrm{CaSO_4}} \end{array}\right| \qquad \text{(column 6)}$$

Considering the reaction of DSE with $Ca(OH)_2$ for column 3 of Table 8, the equilibrium is on the side of the $Ca(OH)_2$ so that little or no reaction will occur. This agrees with the result in Table 8 that little or no effect on toxicity occurred. Examination of the reaction with $ZnSO_4$ for column 4 indicates an equilibrium favoring the formation of ZnEB. This agrees with the two- to threefold reduction in potency, for as shown in Table 2 in this study ZnEB is less potent than Na_2EB by a large factor. The equilibrium reaction for ZnO for column 5 predicts from the insolubility of $Zn(OH)_2$ that no reaction will occur. Again this agrees with the results obtained experimentally. The equilibrium for $ZnSO_4 + Ca(OH)_2$ in column 5 has been shown to be independent of the order of addition of the ingredients. The final compounds will be Na_2EB, $CaSO_4$ and $Zn(OH)_2$ plus perhaps a small amount of ZnEB. This checks with the experimental observations of only a slight reduction in potency.

The reaction for column 6 warrants further investigation. Some filtration experiments are in order, if the precipitation of ZnEB by zinc sulfate is prevented by the presence of calcium hydroxide. The toxicity should reside in the precipitate (filter cake) when zinc sulfate alone is used, but in the filtrate when zinc sulfate plus calcium hydroxide is used.

It was learned that the reactions had to be carried out with fairly concentrated mixtures in order to exceed the solubility limits. The precipitates and filtrates were diluted after filtration to the levels within the range of toxicity and assayed by spraying as usual.

Data are given in Table 9. The results were as expected from the above reactions. If zinc sulfate alone were added, the toxic substance remained on the filter paper. If lime also were added, the toxic substance passed through the filter paper and was recovered in the filtrate. The question arose as to whether or not the toxic material was still DSE. Since DSE is the only known soluble dithiocarbamate possessing tenacity, a tenacity trial should answer the question. The filtrate when dried was just as tenacious as DSE, but other soluble dithiocarbamate salts like sodium diethyl dithiocarbamate had no tenacity.

TABLE 9. EFFECT OF FILTRATION ON THE HEUBERGER EFFECT. DATA EXPRESSED AS LD 90 VALUES = γ/CM^2 SPRAYED SURFACE

Date of test	DSE alone	Original spray fluid DSE plus				Filter cake of DSE plus		Filtrate of DSE plus			
		$Ca(OH)_2$	$ZnSO_4$	Zinc-lime[1]	ZnO	$ZnSO_4$	Zinc-lime[1]	$Ca(OH)_2$	$ZnSO_4$	Zinc-lime[1]	ZnO
1/27/44	0.083	0.096	0.221	0.110	0.068	....	....	0.078	no kill	....	0.063
1/28/44	0.162	0.228	....	0.174	....	....	....	0.218	....	0.268	....
1/31/44	0.328	....	....	0.748	....	....	....	....	no kill	0.970	....
2/ 3/44	0.253	....	....	....	....	1.140[2]	no kill	....	no kill	0.376	....
2/ 9/44	0.080	....	....	....	....	0.615	no kill	....	no kill	0.296	....

[1] Zinc-lime means a mixture of $ZnSO_4 + Ca(OH)_2$

[2] If this precipitate was resuspended with lime as in case where lime was used and refiltered, there was no kill of spores.

The conclusion is, therefore, inescapable that the synergistic effect of zinc sulfate and lime on DSE, *i.e.,* the "Heuberger effect", is not due to the immediate formation of the zinc salt. The lime in the mixture prevents it.

The clarity of this conclusion was beclouded at the time by the results of another experimental approach. A test was conducted in which an unsprayed slide was placed alongside a sprayed slide in a petri dish moist chamber with a water seal. Spores in suspension were placed on the unsprayed slide to measure the H_2S evolving from the damp sprayed slide nearby. This is a modified version of the Van Tiegham technique. Table 10 shows the data obtained when one series of slides was sprayed with DSE alone and another with DSE plus zinc sulfate and lime. The additives reduced the inhibition of the spores, presumably by reducing the evolution of H_2S. From the results given on page 14 one could infer that this reduction in the evolution of H_2S was due to the formation of the zinc salt. The hiatus can be explained by assuming that the H_2S liberated from the DSE reacts with the calcium sulfate giving calcium sulfide, which is less soluble than calcium sulfate by a factor of 30. Further discussion on the probable formation of the zinc salt will be made under the heading "stability".

TABLE 10. EFFECT OF ZINC SULFATE AND LIME ON VOLATILE GAS PRODUCTION FROM DSE. DATA EXPRESSED AS PERCENTAGE OF SPORES INHIBITED.

Concentration of DSE, molarity X 0.687	DSE alone	DSE plus zinc sulfate and lime
0.01	100	8
0.001	99.5	6.5
0.0001	84	0
0.00001	61	0
0.000001	42	0
None	0	0

The research so far, however enlightening, led away from rather than toward a solution of the puzzling result that zinc sulfate and lime improved the field performance of disodium ethylene bisdithiocarbamate.

PROTECTIVE VALUE

Having wrung the data dry on the spore killing properties of DSE, it is time to examine its protective value. The fact that a water-soluble chemical can cling to a sprayed surface during rain has been puzzling from the start (6).

Tenacity

Effect of Drying and Aging on Tenacity

Perhaps the most puzzling pair of facts is that dry fresh crystals of the chemical are readily water soluble, but dried thin spray deposits are not. The best theory to harmonize these facts and to explain the tenacity of DSE was given to us by Dr. D. G. Clarke of the Rohm & Haas Co. He proposed that, while drying, some of the molecules decompose into a water-insoluble compound, which binds the undecomposed portion into a water-resistant deposit. Later on we shall show that the molecule does decompose. A newly dried deposit in one experiment showed a tenacity coefficient of 0.24 but, if the deposit were stored for 24 hours in a humid environment before testing, the tenacity coefficient increased to 0.61. Apparently, the deposit had "set" or hardened in some way. The material seems to exhibit wider variations in tenacity coefficient from test to test than, say, bordeaux mixture. Perhaps, this variation may be explained by variation in the degree of decomposition into the tenacious form. Furthermore, old crystals in some of the earlier batches of DSE tend to decompose and become insoluble.

Effect of Zinc Sulfate and Lime on Tenacity

Since zinc sulfate and lime react to give a colloidal suspension of zinc hydroxide, their synergistic action on DSE in the field might be due to increasing the tenacity. When Heuberger and Manns (17) discovered the effect of zinc sulfate and lime, they were seeking to improve the tenacity of DSE. Several bioassays on the effect of the colloidal zinc sulfate-lime mixture on tenacity were made. Results of a typical test are given in Table 11. In no case did the additives improve tenacity. To check this point further, several other colloidal materials—gelatin, aluminum gel and bentonite were also tried. None improved the tenacity. Next, the colloid was sprayed onto the surface and the DSE was sprayed on top but the tenacity was not improved. The conclusion is inescapable that zinc sulfate and lime have no effect on tenacity.

Stability

Most chemicals experience difficulty "between the test tube and the tank car". DSE is no exception. In its early days the compound sometimes failed to control plant diseases. Such failures were traceable to unanticipated variations in stability among different batches. The word "stability" needs clarification in this context. Strictly speaking a stable compound is one that does not decompose. Such a compound is said to have stability. It a freshly prepared fungicide kills a fungus but fails to do so after storage, the experimenter must assume that the chemical is not stable. If the addition of any substance increases the length of active life, such a substance may be called a "stabilizer". The mechanism for stabilizing may be: (1) physical, to prevent decomposition, or (2) chemical, to form a new compound that does not decompose. In the data to be presented below, these two possibilities will be differentiated.

TABLE 11. EFFECT OF ZINC SULFATE AND LIME ON TENACITY OF DSE

Deposit	DSE alone					DSE plus zinc sulfate and lime				
DSE	Not washed		Washed		T.C.[1]	Not washed		Washed		T.C.[1]
γ/cm^2	Spores killed %	LD50 γ/cm^2	Spores killed %	LD50 γ/cm^2		Spores killed %	LD50 γ/cm^2	Spores killed %	LD50 γ/cm^2	
0.90	100		100			100		84		
0.45	100		67			98		50		
0.225	94		47			88		15		
0.113	56		13			45		6		
0.0563	20	0.091	0	0.25	0.36	5	0.128	0	0.450	0.29

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[1] Tenacity Coefficient

The problem of stability was found to have two facets: instability of the material in the container, and instability of sprayed deposits on the foliage.

Originally DSE was marketed in a dry crystalline form called Dithane A 10 (activity greater than 75%). Although many batches deteriorated but little in the container and performed well in the field, others deteriorated as much as 97 per cent and gave ineffective spray solutions.

It was found that the batches of mother liquor remained uniformly stable in storage and shipment. The practical problem was solved, therefore, by shipping the liquid material. It was found to be as good a fungicide as the dried form and no more phytotoxic. The trade knows this product as Dithane D 14 (25 per cent active).

Effect of Moisture on Stability

Moisture is important in the decomposition of DSE. Although DSE is stable in a concentrated solution (mother liquor), it decomposes almost overnight to a non-toxic level when diluted to spray strength (0.2%). Laboratory assays show losses up to 90 per cent of the toxicity in 26 hours in dilute solutions. Even the liquid product (D 14) decomposes rapidly when diluted to the concentration of 3×10^{-6} molar necessary for spore germination assays. The presence of oxygen does not seem to be essential for decomposition but increases the rate.

If the material decomposes in dilute solution, it might be expected to decompose even more rapidly when sprayed deposits, which offer a tremendous surface for reaction, are exposed to humid air. This was put to test by a technique described elsewhere.[1] Twenty-four replicates, each replicate being a 1 X 3″ glass slide, were sprayed with a series of concentrations of DSE. Eight slides of each concentration were stored dry, eight were sprayed daily for 10 seconds with distilled water in the laboratory sprayer and allowed to dry, and eight were stored in a saturated atmosphere. After 0, 1, 2, 4, 8, 16, 29 and 48 days storage, one slide of each concentration (16 for each assay) from each treatment was assayed by the usual spore germination technique. The results plotted as the logarithm of the LD 90 in micrograms per square centimeter against time in days are graphed in Figure 11. A standard sample of DSE was run in each assay to measure the day to day variation. The values used in the figures and tables of this section have been weighted to correct for this variation. The slides stored in a saturated atmosphere fell to 10 per cent of their original activity in 1.5 days, those sprayed daily in 3.5 days, while those stored dry fell to 10 per cent activity only after more than 30 days.

Thus the second facet of the stability problem became apparent. DSE was bound to give widely varying field results and thus was doomed to failure if decomposition occurred at such a rapid rate on the plant. Even more significant, the compound was failing under the identical conditions favoring fungus infection. The varying field results would depend upon the time elapsing between spray application and inoculation.

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[1] Barratt, R. W. A laboratory assay for stability of organic fungicide residues. Phytopath. **36**:679. 1946.

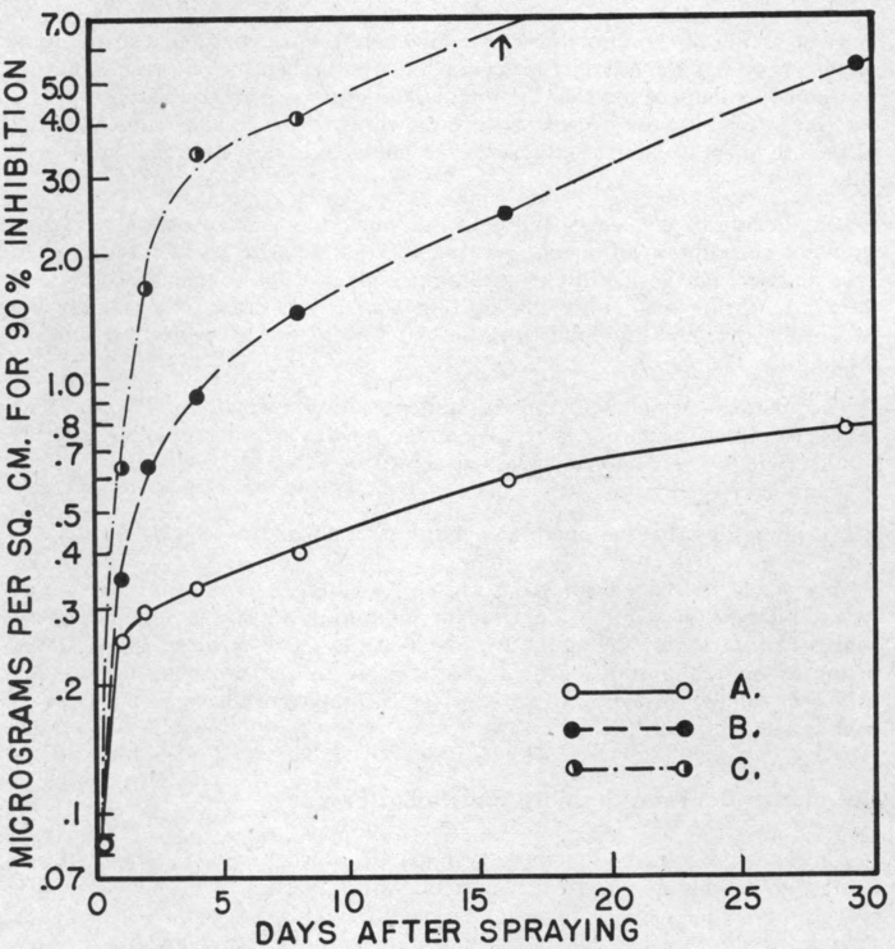


Figure 11. Effect of moisture on decomposition of DSE on a sprayed surface.
A. Stored dry.
B. Sprayed for 10 seconds daily.
C. Stored in saturated atmosphere.

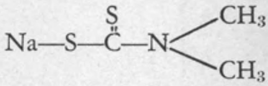
Effect of Molecular Structure on Stability

Several of the homologues and analogues mentioned above in the section on mechanism of toxicity were tested for stability. The soluble metallic salts like the potassium salt are as unstable as the sodium salt itself, whereas the insoluble ones like those of zinc and iron are relatively stable. On account of its stability the zinc salt is rapidly replacing the sodium salt as a practical material in the field.

The stability of the insoluble salts is of interest when considered in the light of the theories advanced in the section on the mechanism of toxic action. It was noted in that section that hydrolysis is the first step in the action and also that the insoluble salts hydrolyze more slowly than the soluble salts. It is not surprising, then, to find that the insoluble salts are more stable than the soluble ones.

Lengthening of the central linkage in the molecule from two to three carbon atoms or substitution of a benzene ring for the aliphatic chain was found to have no effect on the stability of the compounds. Yet the compound containing the benzene ring was rather insoluble in water. This seems to eliminate the possibility that the insoluble metallic salts were stable merely because of insolubility.

The molecule could be stabilized quite readily, however, by changing the nitrogens from a secondary to tertiary amine type in accordance with the structures for He 901, He 956, and D 25 as shown in Table 1.

It is interesting that sodium dimethyl dithiocarbamate  $Na{-}S{-}\overset{\overset{S}{\|}}{C}{-}N\begin{matrix}CH_3\\CH_3\end{matrix}$ is very stable indeed on sprayed surfaces. Its nitrogen is of a tertiary amine type. This type of stabilization is again interesting when one reconsiders the suggested function of the amine hydrogen in the mode of toxic action. If the hydrogen on the nitrogen actually participates in the decomposition giving hydrogen sulfide, then one would expect the compounds above to be stable as well as relatively non-toxic.

Correlation Between Stability and Initial Potency

Almost without exception those compounds which have the greatest initial toxicity to fungus spores are the most unstable. Changes in the molecule that reduce the initial toxicity increase the stability. This is strong evidence that high reactivity with consequent decomposition is involved in toxicity.

Effect of Zinc Sulfate and Lime on Stability

At this point it seemed reasonable to turn again to the "Heuberger effect", *i.e.,* the effect of zinc sulfate and lime. Perhaps they act to stabilize DSE. They were found to exert no stabilizing effect on a dilute solution of DSE. They might prevent the decomposition of DSE on the leaf, however. Figure 12 gives the results of an assay for stability of DSE plus zinc sulfate and lime on a sprayed cellulose nitrate surface. The experimental design was similar to that outlined above for the effect of moisture on DSE. The zinc sulfate and lime were added in the usual field ratio of 1.0 DSE to 1.5 to 0.86 molar. While the effect of moisture was in the same direction, the magnitude of loss in the presence of zinc sulfate and lime was *vastly less*—requiring some 16 days for 90 per cent loss in

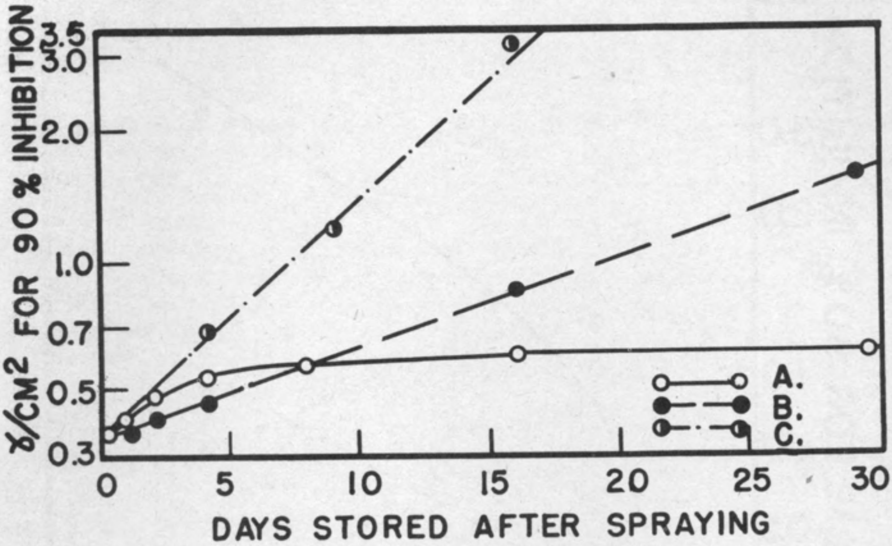


Figure 12. Effect of zinc sulfate and lime on the stability of DSE on a sprayed surface.
A. Stored dry.
B. Sprayed for 10 seconds daily.
C. Stored in saturated atmosphere.

activity under the same conditions that caused a similar loss of DSE alone in 1.5 days (see Figure 11). Figure 13 shows the comparative stability of DSE and of DSE plus zinc sulfate and lime in the same assay. The results confirm the data in Figures 11 and 12. Thus, the action of zinc sulfate and lime on DSE is to stabilize the compound on the plant.

The stabilizing action may be due to two causes: (1) the zinc sulfate and lime mixture may act as a protective colloid (physical action), (2) a stable compound may be formed (chemical action).

If the mixture of zinc sulfate and lime acts as a protective colloid to prevent physically the DSE from deteriorating, then other colloidal materials like gelatin, water-glass (sodium silicate), bentonite and aluminum hydroxide gel should work equally as well for stabilizing. Table 12 shows the relative stability of 1 to 50 mixtures of DSE plus water-glass, DSE plus aluminum hydroxide, DSE plus zinc sulfate and lime, and DSE alone. The results show that water-glass and aluminum hydroxide gel have no effect in stabilizing DSE. Similar results were obtained with gelatin, buffered magnesium silicate and aluminum silicate. Therefore, the stabilizing action of zinc sulfate and lime is not due to any colloid.

Whether or not a chemical reaction is involved in stability can be determined by testing the stability: (1) of DSE filtered free from the colloid, (2)

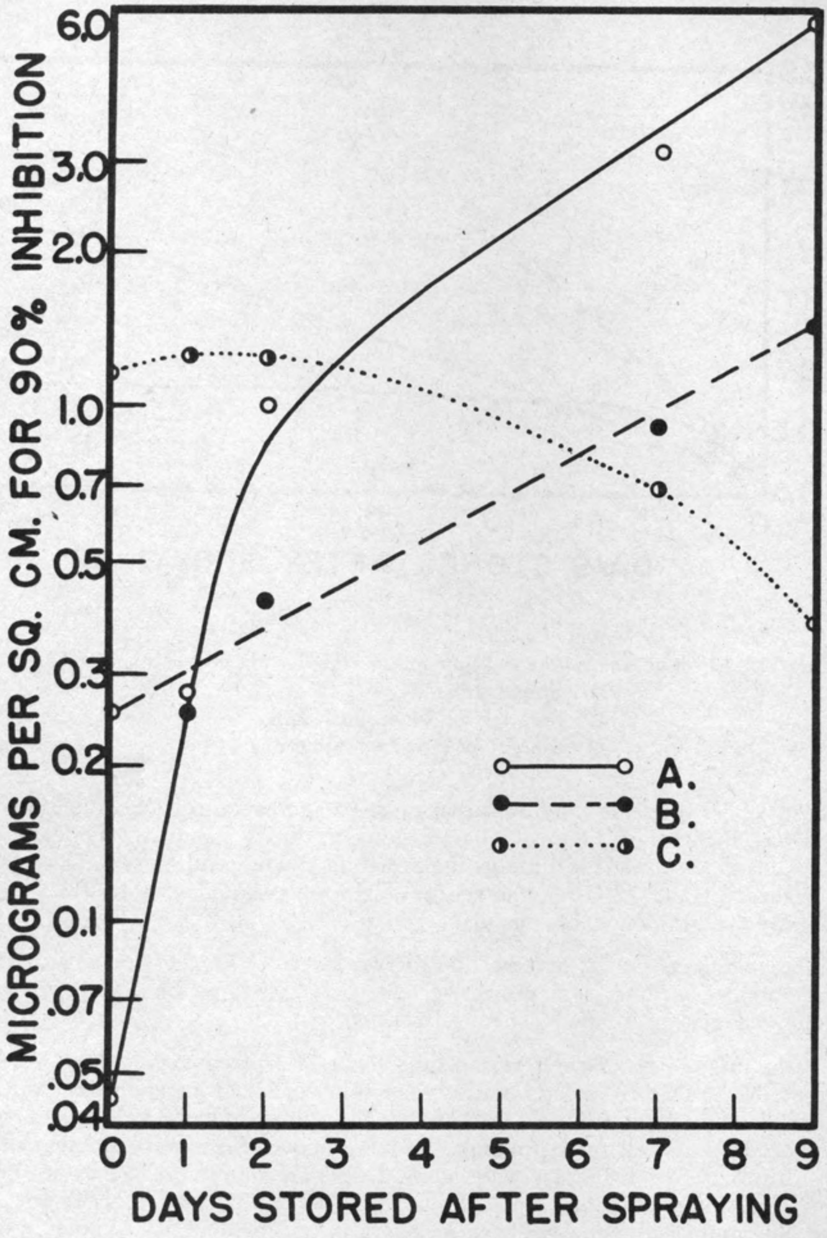


Figure 13. Stability of zinc ethylene bisdithiocarbamate and effect of zinc sulfate plus lime on stability of DSE on a sprayed surface.

A. DSE alone.
B. DSE + zinc sulfate + lime.
C. Zinc ethylene bisdithiocarbamate.

Table 12. Effect of Physical Agents on the Stability of DSE

Material	Micrograms/cm^2 to give 90 per cent inhibition of spores				
	0 day	1 day	2 days	5 days	10 days
DSE	0.048	0.23	0.17	1.42	31.0
DSE + water-glass	0.031	......	0.63	4.71	31.0
DSE + aluminum hydroxide	0.043	0.24	0.15	4.31	31.0
DSE + $ZnSO_4$ + $Ca(OH)_2$	0.170	0.26	0.13	0.26	1.88

Table 13. Effect of Filtering on the Stability of DSE + $ZnSO_4$ + Lime

	Micrograms of DSE for 90 per cent inhibition of germination after		
Material	0 days	3 days	7 days
Unfiltered	0.21	0.33	0.33
Filtered	0.28	1.95	3.91

of various molar ratios of zinc sulfate plus lime to DSE, (3) of the mixture with lime, (4) of the mixture with zinc sulfate, and (5) of premade zinc ethylene bisdithiocarbamate.

Table 13 shows the comparative stabilities of filtered and unfiltered DSE plus zinc sulfate and lime on sprayed slides. From the instability of the filtered material, which earlier had been shown to contain the fungicidal fraction (see page 35), one concludes that the zinc sulfate and lime must act in a spray deposit to form a new and stable compound.

Table 14. Effect of Individual Components Involved in "Heuberger Effect" on Stability of DSE

Material	Micrograms/cm^2 to give 90 per cent inhibition of spores				
	0 day	1 day	2 days	4 days	14 days
DSE + $Ca(OH)_2$	0.056	0.17	0.40	5.4[1]	11.6
DSE + $ZnSO_4$	0.203	0.25	0.26	0.26	2.15
DSE + $ZnSO_4$ + $Ca(OH)_2$	0.175	0.20	0.16	0.26	3.30

[1] Value obtained by extrapolation of dosage-response curve.

Table 15. Comparison of Stability of DSE Plus Zinc Sulfate and Lime with Zinc Ethylene Bisdithiocarbamate

Material	Micrograms/cm^2 to give 90 per cent inhibition of spores						
	0 day	1 day	2 days	7 days	9 days	28 days	36 days
DSE (Dithane A 10)	.041	0.27	1.00	3.16	5.48[1]	....	....
DSE (Dithane D-14)	.045	0.30	2.64	3.30	5.48	....	....
DSE + $ZnSO_4$ + $Ca(OH)_2$	.25	0.25	0.42	0.91	1.48[1]	....	....
Zinc ethylene bisdithiocarbamate	1.16	1.26	1.23	0.70	0.38	22.0[1]	12.9[1]

[1] Value obtained by extrapolation of dosage-response curve.

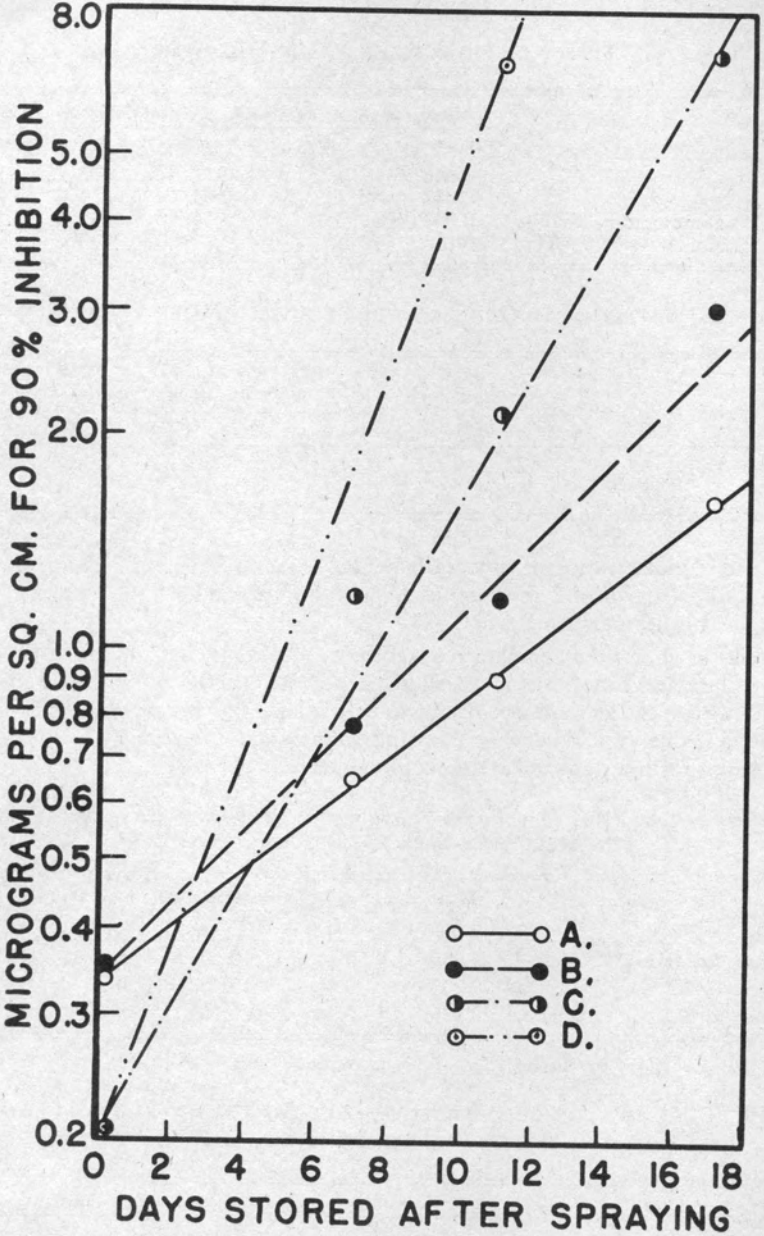


Figure 14. Effect of varying molar ratios of DSE to zinc sulfate to lime on stability of DSE on a sprayed surface.

A. 1 to 2 to 2.
B. 1 to 1 to 1.
C. 1 to 0.25 to 0.25.
D. 1 to 0.0625 to 0.0625.

The conclusion may be checked by testing the stability of different ratios of zinc sulfate plus lime to DSE. The decomposition curves of four molar ratios are charted in Figure 14. They show that as the ratio of zinc sulfate plus lime to DSE is decreased, the stability falls. In Figure 15, which summarizes this

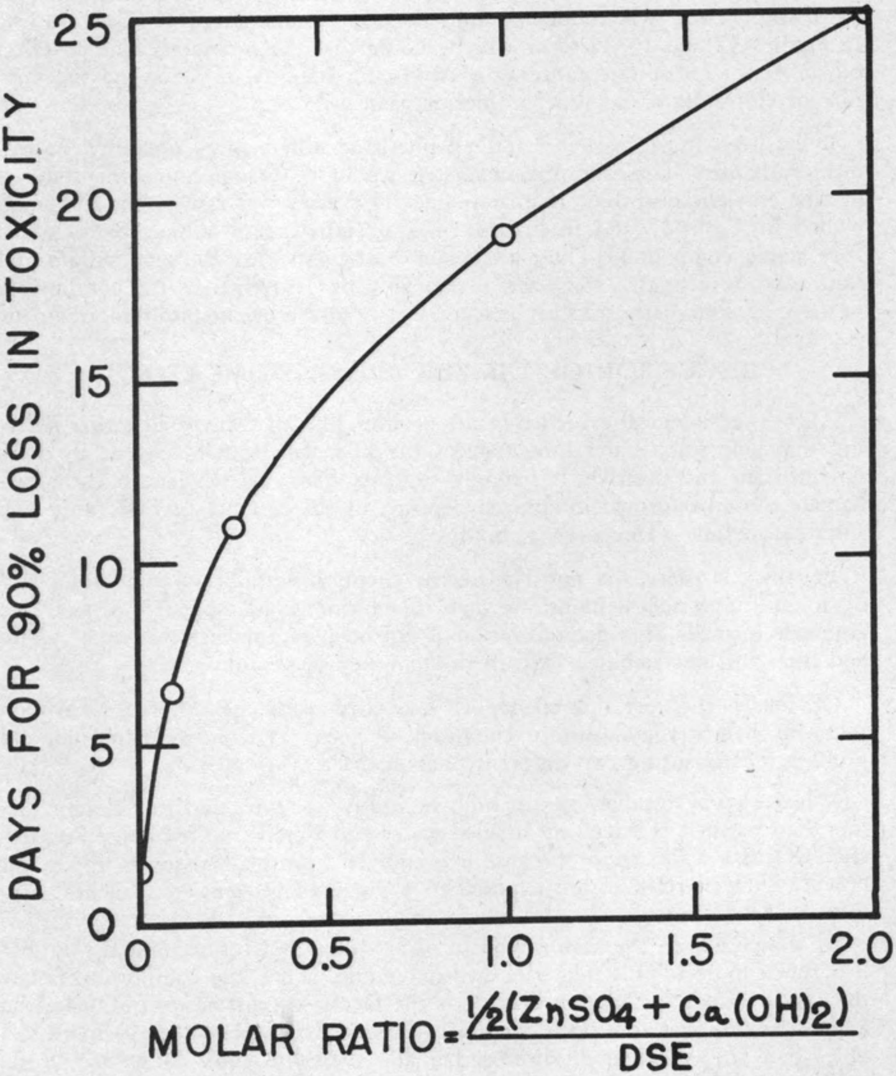


Figure 15. Summary of effect of molar ratios of DSE, zinc sulfate, and lime on stability. The data behind the zero molar ratio were taken from a different experiment.

effect, stability is expressed as number of days required for the mixture to lose 90 per cent of its potency. From this curve, one can conclude that the zinc sulfate and calcium hydroxide exert a quantitative effect on stability which is probably due to the amount of zinc salt of ethylene bisdithiocarbamic acid formed.

Checking next the stabilizing action of zinc sulfate alone and lime alone on DSE, another test was conducted and the results summarized in Table 14 and in Figures 12 and 13. Here, it will be noted that the lime alone has no effect on stability; zinc sulfate confers the maximum stability on DSE, and the mixture of zinc sulfate and lime is intermediate.

It has been shown earlier that DSE plus zinc sulfate gives zinc ethylene bisdithiocarbamate. Logically, the next step would be to determine the stability of zinc ethylene bisdithiocarbamate alone. This assay was carried out and is included in Table 15 and in Figure 13. Zinc ethylene bisdithiocarbamate is a very stable compound.[1] Thus, all evidence suggests that the zinc sulfate and lime mixture acts as a stabilizer, presumably by a reversal of the equilibrium in the spray film that results in the formation of zinc ethylene bisdithiocarbamate.

DISCUSSION OF THE ZINC SULFATE-LIME EFFECT

All of the biological evidence in this section, plus that discussed earlier showing that zinc sulfate and lime reduces the hydrogen sulfide released by DSE is consistent and positive. It strongly suggests that zinc ethylene bisdithiocarbamate must be formed in sprayed deposits of the mixture of DSE with zinc sulfate and lime (Heuberger's mixture).

Equally consistent and positive are the chemical equilibria (page 34) based on solubilities which demonstrate that, in solution, it is impossible to have the zinc salt formed. This demonstration is fortified by the fact that zinc sulfate and lime will not stabilize DSE in dilute water suspension.

Obviously, the real discrepancy is associated with the difference between solution chemistry and surface chemistry. It looks as if it were the old, old problem of measuring two different phenomena.

When a spray residue ages in high humidity, it is exposed to different factors than when it is mixed in distilled water and stored in a test tube. Presumably, CO_2 is a major factor, because it is evolved from plant tissue at night, and because sprayed residues are exposed to a greater CO_2 tension than are mixtures in a test tube.

As shown above, the reason why the zinc salt is not formed initially is that it is much more soluble than zinc hydroxide and, hence, the equilibrium favors the zinc hydroxide. If carbon dioxide is the factor, it must act to carbonate the zinc hydroxide just as it does calcium hydroxide. If zinc carbonate is more soluble than zinc ethylene bisdithiocarbamate, then the equilibrium should be shifted toward the latter.

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[1] The potency of zinc ethylene bisdithiocarbamate increases with age. This has been observed in all the stability assays on the compound. As yet this interesting phenomenon is unsolved. Presumably, it is due to the gradual formation in the spray deposit of a more toxic derivative.

Mr. D. R. Conlon of Rohm and Haas Co. kindly determined the solubility of zinc ethylene bisdithiocarbamate for us. It is of the order of 0.001 gm/100 gm of water. According to Haehnel (12) the solubility of zinc carbonate in the presence of one atmosphere of CO_2 is 0.07 gm/100 gm of water. The solubility is, perhaps, one-third this value in pure water. Hence, zinc carbonate is about 23 times as soluble as zinc ethylene bisdithiocarbamate and, thus, the equilibrium should be shifted toward zinc ethylene bisdithiocarbamate and away from zinc hydroxide, thus yielding a stable compound.

Could zinc ethylene bisdithiocarbamate be identified in Heuberger's mixture if CO_2 were added? Dr. James Stroupe of Rohm and Haas Co. kindly made some X-ray analyses for us and showed that it could be so identified. He confirmed the other data that it could not be formed if CO_2 is absent.

All this being true, it can be concluded that the "Heuberger effect" is due to the formation of a different compound, zinc ethylene bisdithiocarbamate, when zinc sulfate and lime are permitted to react with disodium ethylene bisdithiocarbamate in the presence of CO_2. Simple as that conclusion looks now, the keystone was not put in until April, 1947, when Conlon and Stroupe made their analyses. This was more than three years after Heuberger and Manns (17) made their first report.

SUMMARY

1. Disodium ethylene bisdithiocarbamate, abbreviated herein to DSE, was first synthesized in 1935, first tested as a fungicide in 1940.
2. It gave erratic results in the field, and almost certainly would never have reached its present stage of commercial adoption had Heuberger and Manns (17) not discovered the salutary effect of zinc sulfate and lime on its field performance.
3. The research described herein was conducted wholly in the laboratory, using the spore inhibition technique. The test fungus was *Stemphylium sarcinaeforme.* The spores were germinated either on cellulose nitrate-coated glass slides or in the depressions in so-called culture slides.
4. The toxicity of some 36 analogues and homologues of DSE were investigated.
5. The dosage-response curves for the open chain salts were flatter than those for the polymerized or closed chain materials. This suggested different mechanisms of fungicidal action.
6. The number of sulfur atoms in the polysulfide linkage seemed to have no effect on potency. The length of the central aliphatic chain had no effect on potency except insofar as the size of the molecule was changed. If the secondary amine in DSE was converted by any device whatever into a tertiary amine, the potency of the compound was sharply reduced.
7. The performance of the analogues from the standpoint of both slope and position of the dosage-response curve was very useful in appraising the mechanism of fungicidal action on the spore.

8. In general, it was concluded that these dithiocarbamate analogues inhibit spores through two reactions: (1) by liberating H_2S and (2) by forming metal salts of the compound.

9. It is proposed on the basis of these two reactions that there are two mechanisms by which DSE poisons spores: (1) the proteins of the spore may be altered by the addition of sulfhydryl groups, (2) the trace metals which the fungus needs in its metabolism are made unavailable either through the formation of metallic sulfides or metallic salts of ethylene bisdithiocarbamic acid. These mechanisms could operate singly or simultaneously.

10. In an attempt to elucidate the "Heuberger effect", the effect of various electrolytes in solution including zinc sulfate and lime was investigated. There are no clear cut accelerative or depressive effects, but rather both. In some ranges the toxicity rises, in others it falls, thus generating a polymodal curve. In the case of some compounds, the electrolyte seems merely to exaggerate a tendency already present to produce polymodal curves. Different cations produce different types of curves. Cations appear to be more important in producing polymodal curves than anions. There appears to be a lyotropic series of cations with respect to total kill. In some cases, at least, the existence of polymodal curves is independent of, but may be confused with, molecular proportions of the ingredients.

11. The polymodal curves for the effect of the electrolytes suggested further discussion of the typical "TMTD" curve, named because it was first observed with tetramethylthiuram disulfide. The TMTD curve is a polymodal curve for concentration. It was observed in this study for several analogues of DSE, including ferric dimethyl dithiocarbamate, sodium diethyl dithiocarbamate, and cupric ethyl xanthate. An explanation of the polymodal curve for TMTD is advanced. It is assumed that the peak at high concentration is due to the liberation of H_2S by hydrolysis of the $=C=S$ group, and that the peak in the lower concentration is due to the splitting of the molecule into two dithiocarbamate ions that could combine with metals in the spore to form direct salts. Other polymodal curves are discussed on the theory that they are generated by the interaction of two or more factors acting at different rates or in opposite directions.

12. The second phase of the studies of the Heuberger effect was to investigate the mixture and its ingredients in fresh spray deposits. Conclusive evidence was obtained that there is no chemical reaction between the ingredients when all three are present, although the zinc salt could be formed when lime is absent. The explanation is that zinc hydroxide is very much more insoluble than zinc ethylene bisdithiocarbamate and, hence, the equilibrium favors zinc hydroxide, not zinc ethylene bisdithiocarbamate.

13. The clarity of this conclusion is clouded by the fact that zinc sulfate and lime greatly reduce the ability of DSE to liberate hydrogen sulfide. This suggested a chemical reaction.

14. It could be contended that the colloidal precipitate formed by zinc sulfate and lime improves the tenacity of deposits. In fact, that was the original objective of Heuberger and Manns when they prepared the mixture. No

data could ever be adduced to indicate any effect whatsoever on the tenacity of DSE, either by the zinc-lime mixture or by other colloids.

15. When the research turned to the study of aged mixtures, the secret of the Heuberger effect appeared. Zinc sulfate and lime very greatly increase the stability of DSE. Almost certainly this is the effect that causes potent residues to be maintained for longer periods on foliage.

16. The data indicated that the real effect is due to the formation of zinc ethylene bisdithiocarbamate in the weathered spray residues. Obviously, the spray residues must be exposed to some factor that does not appear in the fresh mixtures. The easiest factor to suspect is carbon dioxide. A study of relative solubilities and X-ray analysis showed clearly that in the presence of CO_2, the Heuberger mixture forms zinc ethylene bisdithiocarbamate, but it does not do so in the absence of CO_2.

17. The mystery was solved. As a result the trade is rapidly shifting to factory-made zinc ethylene bisdithiocarbamate.

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