INVESTIGATIONS ON ORGANIC FUNGICIDES

VIII. THE BIOCHEMICAL MODE OF ACTION OF BISDITHIOCARBAMATES AND DIISOTHIOCYANATES*

by

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INTRODUCTION

It has been suggested in a previous communication that the antifungal action of the bisdithiocarbamates is primarily ascribable to their transformation *in situ* into the corresponding diisothiocyanates (KLÖPPING AND VAN DER KERK[1]) *e.g.:*

$$^{(-)}S-\underset{\underset{S}{\|}}{C}-N-(CH_2)_2-N-\underset{\underset{S}{\|}}{C}-S^{(-)} \longrightarrow S=C=N-(CH_2)_2-N=C=S + 2\,HS^{(-)}$$

ethylene bisdithiocarbamate ion — ethylene diisothiocyanate

The biochemical mode of action of bisdithiocarbamates and diisothiocyanates remained, however, still obscure. Interference with the respiration of the moulds could not account for growth inhibition, since the concentration of fungicide required for inhibition of respiration is considerably higher than that required to prevent growth (VAN DER KERK AND KLÖPPING[2]). It has been found, moreover, that the mode of action differs from that of a chemically closely related group of fungicides, the lower dialkyl dithiocarbamates and their oxidation products the thiuram sulphides; while representatives of this latter group were found to be strongly antagonized by histidine, the action of bisdithiocarbamates and diisothiocyanates is not influenced by this amino acid (SIJPESTEIJN AND VAN DER KERK[3]).

A search for antagonists of the latter two groups has revealed that thiol compounds can abolish their fungitoxity almost completely. It seems highly probable that this inactivation is brought about by a direct chemical reaction of fungicide and –SH compound, without interference of the cell constituents. This reactivity towards added thiol compounds suggests that the antifungal action of the bisdithiocarbamates and the diisothiocyanates is due to a reaction between the fungicide and essential –SH compounds in the cells.

MATERIALS AND METHODS

Disodium ethylene bisdithiocarbamate (nabam**) and tetramethylene diisothiocyanate (TMDI) were used as a representative of the bisdithiocarbamates and diisothiocyanates, respectively.

The experiments were carried out with a strain of *Penicillium italicum* and of *Aspergillus niger*

* Previous communication: *Rec. trav. chim.*, 71 (1952) 1179.

** Name approved by the American Phytopathological Society.

References p. 552.

using the following medium: 1% glucose, 0.5% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4.7\ H_2O$, 2% washed agar, tap water; 0.002 γ biotin and 0.2 γ aneurin were added per ml medium to obtain optimal growth. The pH values were always checked electrometrically after autoclaving.

Fungitoxicity was assessed by the roll culture method (MANTEN, KLÖPPING AND VAN DER KERK[4]) in the following way: Small standard bottles were autoclaved with 1.5 ml agar medium; a graded solution of the fungicide (0.1 ml nabam in water or 0.03 ml TMDI in acetone), glucose (0.05 ml 30%), the growth factors + the spore suspension (0.05 ml containing 4.000 spores) and, if required, the antagonist (0.05 ml of a solution of proper pH) were added from vertically fixed 1 ml pipettes to the agar when still at *ca.* 50°. Subsequently the bottles were cooled while being mechanically turned along their longitudinal axis. The fungicidal concentrations used were 0; 0.01; 0.02; 0.05; 0.1; 0.2 p.p.m. etc.

After 2, 3, 4 and 5 days of incubation at 24° the minimal concentration of nabam or TMDI at which no visible growth had occurred was read.

RESULTS

Fungitoxity on glucose agar. The toxicity of nabam appeared to be slightly influenced by the pH of the medium, in so far as it was somewhat higher at low pH values. In contrast hereto the action of TMDI proved to be independent of pH (see figures).

Antagonistic activity of different compounds. When 1% of a casein hydrolysate (casamino acids, Difco) was incorporated in the medium the toxicity of the fungicides remained unchanged at all pH values. This result seemed to rule out the possibility that amino acids antagonize these fungicides. Yet, as certain amino acids are known to be badly represented in casein hydrolysate, these were tested as well, at a concentration equivalent to 0.2% of the L-isomer. Thus glycine, L-alanine, DL-methionine, DL-tryptophan, DL-threonine and L-cysteine were tested separately as possible antagonists for nabam. Whereas the first five compounds were inactive, L-cysteine proved to have exceptional activity in counteracting nabam (Fig. 1)*. The same was found for TMDI though the effect of pH on the antagonistic activity is different, an optimum occurring between pH 5 and 6 (Fig. 2).

Cysteine is not specific in counteracting these fungicides, however, as was shown by experiments in which thioglycollic acid was used instead (Figs. 2 and 3). It is striking that with this compound the effect of the pH on the antagonistic action differs from that found with cysteine.

The antagonistic effect of thiol compounds on both fungicides is of a competitive nature. As an example, the result obtained with the combination of nabam and cysteine is given in Fig. 4. Some experiments were carried out with 2,3-bismercaptopropanol (BAL) as an antagonist. This thiol compound was, however, too fungitoxic to obtain any reliable results.

Contrary to thioglycollic acid, its oxidation product, dithiodiglycollic acid, has no antagonistic activity at all towards TMDI and only slight activity towards nabam (Fig. 3). While these results seemed to suggest the importance of reducing compounds as antagonists, ascorbic acid (0.1%) had only slight activity towards nabam and none towards TMDI. Although this result might be due to the fact that the reducing capacity of ascorbic acid is less than that of cysteine or thioglycollic acid, a more plausible explanation may be found in the assumption that only -SH compounds can inactivate the fungicides used. This inactivation of the fungicides can be pictured as the formation of a stable compound of much reduced toxicity in the following way:

* Since essentially the same results were obtained with *P. italicum* and *A. niger* figures are only given for experiments with the former species.

References p. 552.

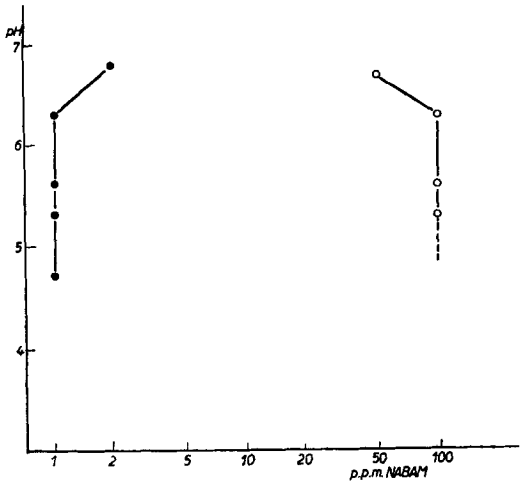


Fig. 1. Effect of the addition of cysteine on the toxicity of nabam at different pH values. *P. italicum:* incubation period 4 days.

-●-●-●- nabam
-O-O-O- nabam + 0.05 % cysteine

Note: pH values below 5.5 could not be maintained at nabam concentrations higher than 20 p.p.m. At these concentrations complete growth occurred.

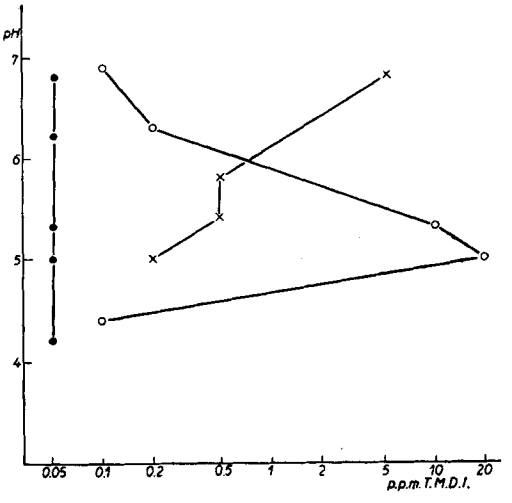


Fig. 2. Effect of the addition of cysteine and of thioglycollic acid on the toxicity of tetramethylene diisothiocyanate (TMDI) at different pH values.
P. italicum: incubation period 4 days.

-●-●-●- TMDI
-O-O-O- TMDI + 0.05 % cysteine
-×-×-×- TMDI + 0.05 % thioglycollic acid

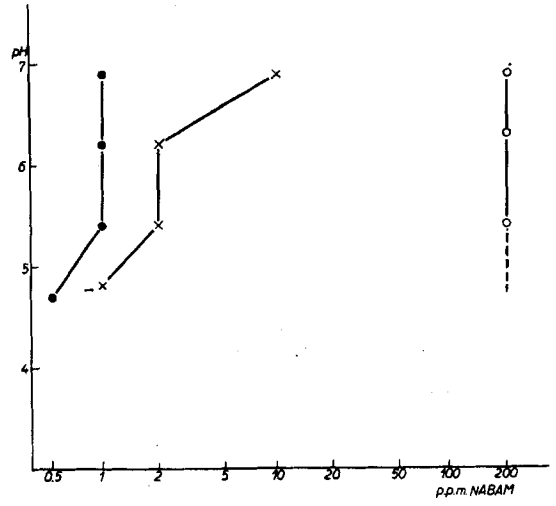


Fig. 3. Effect of the addition of thioglycollic acid and of dithioglycollic acid on the toxicity of nabam at different pH values. *P. italicum:* incubation period 4 days.

-●-●-●- nabam
-O-O-O- nabam + 0.05 % thioglycollic acid
-×-×-×- nabam + 0.05 % dithioglycollic acid

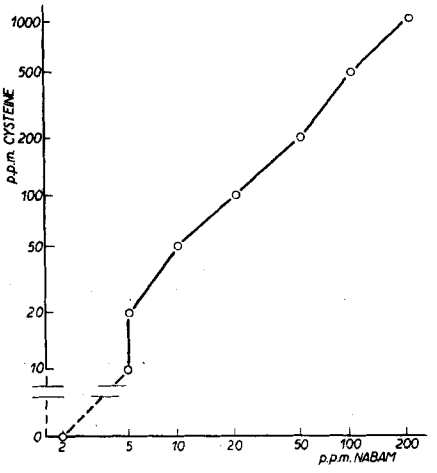
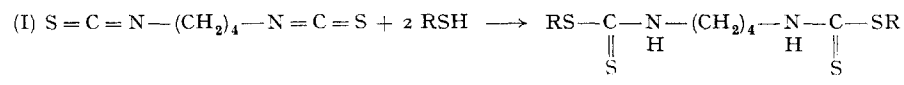
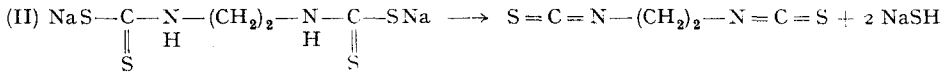


Fig. 4. The competitive nature of the antagonistic effect of cysteine on the toxicity of nabam. *P. italicum:* incubation period 4 days; pH 5.5.

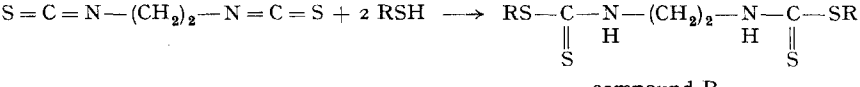
References p. 552.



TMDI compound A



nabam ethylene diisothiocyanate



compound B

Fungitoxicity of the reaction products from diisothiocyanates and thioglycollic acid. To lend support to this hypothesis the compounds A and B were synthesized from the corresponding diisothiocyanates with thioglycollic acid for RSH and their fungitoxicity was assessed at different pH values.

The toxicity of these compounds proved to be indeed very much less than that of the fungicides from which they are derived. Compound B was still harmless for both moulds at a concentration of 200 p.p.m. Compound A was found equally inactive towards *A. niger* while *ca.* 50 p.p.m. of this compound inhibited growth of *P. italicum* at the pH values used, ranging from 4.3 to 6.7.

DISCUSSION

The fact that the *in vitro* reaction product of TMDI with the –SH compound thioglycollic acid (compound A) shows a greatly reduced fungitoxicity, suggests strongly that the observed antagonistic activity of thioglycollic acid is attributable to a direct chemical reaction with the fungicide. The rate of this reaction under the experimental conditions appears to be dependent on the pH value of the medium (see Fig. 2). From Fig. 2 it becomes likely, moreover, that this reaction rate is also dependent on the chemical nature of the –SH compound used. In the same way it may be understood that different toxicity curves are found with the preformed reaction product of TMDI and thioglycollic acid and with the combination of the latter two compounds when simultaneously added to the nutrient medium.

The assumed reactivity of TMDI and thiol compounds meanwhile leads to the view that the antifungal action of TMDI and other isothiocyanates is due to their chemical combination with cell constituents carrying essential –SH groups.

For the bisdithiocarbamate nabam a very similar mode of action must be accepted. As put forward in previous communications the compound is supposed to owe its antifungal activity to its transformation *in situ* into the corresponding diisothiocyanate. At first sight the different behaviour of cysteine and thioglycollic acid on the antifungal action of TMDI and of nabam seems to rule out this assumption (compare Fig. 2 with Figs. 1 and 3). It must be realized, however, that with nabam a still more complex situation exists than is depicted for TMDI, since in the former case a decomposition reaction has to precede which is pH-dependent as well.

In the next section a more general discussion on the mode of action of bisdithiocarbamates and related compounds will be given.

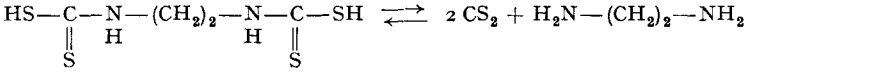
References p. 552.

GENERAL REMARKS ON THE ANTIFUNGAL ACTION OF BISDITHIOCARBAMATES AND RELATED COMPOUNDS

There have been several attempts to correlate the antifungal activity of the bisdithiocarbamates with the well-known chemical instability of these compounds and thus to make certain decomposition products responsible for the observed antifungal action. When considering such attempts two remarks must be made. Firstly, holding responsible a chemical decomposition or conversion product for the toxic effect merely means a shifting of the problem unless the biochemical mode of action of such a product can be made plausible in its turn. Secondly, the antifungal activity of the decomposition or conversion product should at least equal the activity of the parent compound.

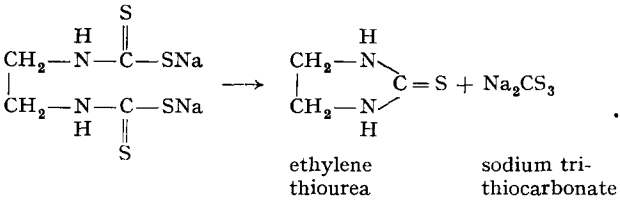
Under physiological conditions the salts of ethylene bisdithiocarbamic acid are decomposed in several ways.

1. Free ethylene bisdithiocarbamic acid, always being present in small amounts in the solutions of its salts owing to hydrolysis, is split as follows:



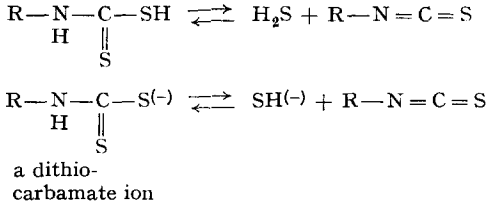
It has been suggested (PARKER-RHODES[5]) that the activity of the dithiocarbamates in general depends on the toxicity of these two decomposition products.

2. From solutions of disodium ethylene bisdithiocarbamate the compound ethylene thiourea is formed:



ethylene thiourea — sodium trithiocarbonate

3. It is a well-known fact that dithiocarbamic acid derivatives bearing free hydrogen at the nitrogen atom can be decomposed to isothiocyanates:



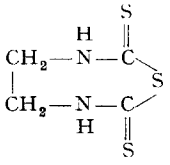
a dithiocarbamate ion

This decomposition occurs very easily in the presence of the ions of heavy metals, since in this case insoluble metal sulphides are formed and the equilibrium is forced to the right.

Whereas the decomposition products mentioned under 1 and 2 are of low antifungal activity it was found that alkylene diisothiocyanates show exceptional high activity, which considerably surpasses that of the alkylene bisdithiocarbamates themselves. This combined with the observation that the latter two groups of compounds possess similar antifungal spectra led us to the assumption that the antifungal activity of the bisdithiocarbamates is due to their transformation into the corresponding diisothiocyanates[1,2].

References p. 552.

In a recent preliminary communication LUDWIG AND THORN[6] reported an interesting new decomposition product of disodium ethylene bisdithiocarbamate which they related to the mode of action of this compound. After aerating an aqueous solution of this salt during some days a compound could be isolated which was identified as ethylenethiuram monosulphide. Although no structural formula was given the reported analytical data as well as the estimated molecular weight in all probability point to the following structure, containing a seven-membered ring:



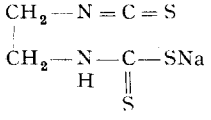
ethylenethiuram monosulphide

When repeating these experiments the same compound was obtained and additional proof for its structure could be given (to be published).

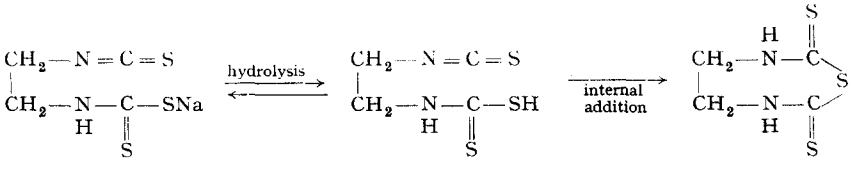
LUDWIG AND THORN found for ethylenethiuram monosulphide a high antifungal activity and thus made the suggestion that the formation of this compound might explain the activity of disodium ethylene bisdithiocarbamate. They argued, moreover, that this compound in fact had been isolated from solutions of disodium ethylene bisdithiocarbamate whereas the active isothiocyanates postulated by us had not.

The following considerations in our opinion, however, bring these apparent discrepancies into line. Chemically the formation of ethylenethiuram monosulphide from nabam is coupled with the loss of one S atom from the molecule, evidently in the form of the SH ion or of H_2S.

In accordance with the decomposition mechanism mentioned under 3, the following primary decomposition product can be expected from nabam by the loss of one S atom:



The formation of ethylenethiuram monosulphide from this decomposition product can very easily be understood:



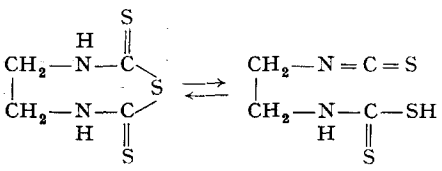
Thus, ultimately, ethylenethiuram monosulphide is formed from nabam as the logical consequence of two circumstances, *viz.*:

a. the initial formation by loss of H_2S of HS^- of a reactive isothiocyanate group;

b. the simultaneous presence in one molecule of an isothiocyanate and a dithiocarbamic acid residue in a favourable spatial position.

In solution an equilibrium will exist between the seven-membered ring structure and the open form:

References p. 552.



In conclusion it can be said that the observed formation of ethylenethiuram monosulphide from nabam solutions may serve as a direct indication for the at least intermediate appearance of isothiocyanate groups.

With a view to the high chemical reactivity of the isothiocyanates one need not be surprised that the presence of these compounds in solutions of bisdithiocarbamates has not been demonstrated under the experimental conditions. Instead of isothiocyanates more stable decomposition or reaction products such as substituted thioureas and ethylenethiuram monosulphide were isolated.

From the similar antifungal spectra of bisdithiocarbamates and diisothiocyanates and from their corresponding behaviour towards the presence of thiol compounds in the medium it must be concluded that these groups of compounds act biochemically much in the same way. Table I, containing some test results with four mould species, shows that ethylenethiuram monosulphide fits entirely in the pattern found for nabam and the corresponding ethylene diisothiocyanate.

TABLE I

FUNGITOXICITY OF NABAM AND RELATED COMPOUNDS IN GLUCOSE AGAR; pH 6.5. INCUBATION PERIOD 2 DAYS

Compound	*Minimum concentration in p.p.m. causing complete growth inhibition*			
	Botrytis allii	*Penicillium italicum*	*Aspergillus niger*	*Rhizopus nigricans*
Nabam	1	0.5	2	10
Ethylene diisothiocyanate	0.05	0.02	0.05	10
Ethylenethiuram monosulphide	0.5	0.5	1	5

Moreover, the action of ethylenethiuram monosulphide could be antagonized by the addition of thiol compounds in the same way as was demonstrated for nabam and TMDI.

All these facts point strongly to a common biochemical mode of action for the compounds in question. From the present results it follows that the isothiocyanate group is able to combine chemically with compounds containing reactive –SH groups with the formation of stable, inactive reaction products. The same reactivity towards essential –SH groups within the living cell would offer the most logical explanation for the observed antifungal action of the compounds mentioned in Table I.

With a view to the high chemical reactivity of isothiocyanate groups it is not easy to give direct evidence, *e.g.* by isolation, for the formation of isothiocyanates from bisdithiocarbamates. The isolation of ethylenethiuram monosulphide from nabam solutions constitutes, however, an indirect argument in favour of this view.

As a matter of fact only such bisdithiocarbamates or derivatives therefrom are highly active fungicides that bear in their molecular structure the potential possibility for an easy transformation into isothiocyanates. Thus the most reasonable conclusion

References p. 552.

seems that the conversion of the bisdithiocarbamates into the corresponding isothiocyanates must be seen as the basis of their antifungal action and that the latter compounds act by chemically inactivating essential –SH systems within the living cell.

SUMMARY

The inhibitory action of tetramethylene diisothiocyanate (TMDI) and of disodium ethylene bisdithiocarbamate (nabam) on the spore germination of *Penicillium italicum* and of *Aspergillus niger* is strongly antagonized by the thiol compounds, thioglycollic acid and cysteine. This antagonism is supposed to be due to a chemical reaction between fungicide and –SH compound. The observation that the preformed reaction products of either of the fungicides with thioglycollic acid are almost non-toxic is strong evidence for this hypothesis.

The observed reactivity of thiol compounds towards TMDI and nabam is an indication that the antifungal action of the latter compounds is due to their combination with cell constituents carrying essential –SH groups.

In a final section the mode of antifungal action of the bisdithiocarbamates and related compounds is discussed more generally.

RÉSUMÉ

L'action du tétraméthylène-diisothiocyanate (TMDI) et de l'éthylènebisdithiocarbamate de sodium (nabam) sur la germination des spores de *Penicillium italicum* et d'*Aspergillus niger* est fortement contrecarrée par les substances sulfhydriques: l'acide thioglycolique et la cystéine. Cette inactivation est supposée être causée par une réaction chimique entre le fongicide et la substance sulfhydrique. L'observation que les produits préformés de la réaction entre chacun des fongicides et l'acide thioglycolique ne sont presque pas toxiques constitue un argument puissant en faveur de cette hypothèse.

La réactivité des substances sulfhydriques vis-à-vis du TMDI et du nabam indique que l'action fongicide de ces substances serait causée par leur combinaison avec des constituants cellulaires portant des groupes sulfhydriques essentiels.

Dans une section finale le mode d'action fongicide des bisdithiocarbamates et substances alliées est plus amplement discuté.

ZUSAMMENFASSUNG

Die hemmende Wirkung von Tetramethylen-diisothiocyanat (TMDI) und von Dinatrium-aethylen-bisdithiocarbamat ("Nabam") auf die Keimung der Sporen von *Penicillium italicum* und *Aspergillus niger* wird von den Thiolverbindungen Thioglykolsäure und Cystein kräftig antagonistisch beeinflusst. Es ist anzunehmen, dass dieser Antagonismus auf einer chemischen Reaktion zwischen Fungicid und –SH Verbindung beruht. Die Umsetzungsprodukte von TMDI und von Äthylen-diisothiocyanat, dem das "Nabam" zugrunde liegt, mit Thioglycolsäure zeigen nur eine sehr geringe Wirksamkeit; dies ist in Übereinstimmung mit der obengenannten Annahme.

Die beobachtete Reaktionsfähigkeit von Thiolverbindungen mit TMDI und "Nabam" macht es wahrscheinlich, dass die fungitoxische Wirkung dieser Stoffe auf der Inaktivierung von unentbehrlichen –SH Gruppen in der Zelle beruht.

Im letzten Teil dieser Mitteilung wird der Wirkungsmechanismus der Bisdithiocarbamate und verwandter Stoffe ausführlich diskutiert.

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