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Chemical and Fungicidal Reactions of 3,5-Dimethyltetrahydro-1,3,5-thiadiazine-2-thione (3,5-D). A Comparison with Sodium N-Methyl Dithiocarbamate and Methyl Isothiocyanate

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Methyl isothiocyanate inhibits the acetate respiration in baker's yeast at pH values above 6, but not at pH below 5. At the pH values where the inhibitory action is shown, methyl isothiocyanate reacts with cysteine to form a compound with a dithiocarbamate spectrum. The same kind of reaction could also be demonstrated in intact yeast cells.

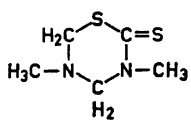
Sodium N-methyl dithiocarbamate acts inhibitory on the acetate respiration at lower pH values, but not at higher. At higher pH values, cupric ions act strongly synergistic. Spectrophotometric studies did not show any reaction between sodium N-methyl dithiocarbamate and cysteine. The spectra showed, however, that the compound is very unstable in dilute aqueous solutions.

3,5-D in aqueous solution is rapidly hydrolyzed to form a N-methyl dithiocarbamate ester, and probably more slowly to form free N-methyl dithiocarbamate ion. In aged solutions (24–48 h old), methyl isothiocyanate is present. This could be spectrophotometrically detected by the reaction with cysteine. Freshly prepared 3,5-D solutions showed less toxicity than 24 h old ones. In both cases cupric ions acted synergistic at pH 6.5. This effect was most pronounced in the fresh solution. In the old solution, the effect of pH was not pronounced. The inhibitory effect at pH 6.5 was, however, variable from one experiment to the other. In both kinds of solution, ether extraction reduced the toxicity in the absence of cupric sulfate at pH 6.5 to practically zero.

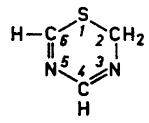
It is concluded that 3,5-D acts as a mixture of N-methyl dithiocarbamate and its decomposition product, methyl isothiocyanate. In practical use, the effect of formaldehyde must not be overlooked, but this compound and other possible decomposition products, such as carbon disulfide and dithiocarbamate, had no effect of the acetate respiration of yeast.

In the later years, 3,5-derivatives of tetrahydro-1,3,5-thiadiazine-2-thione have aroused interest as fungicides with special use, particularly in the agriculture as soil fungicides.

This class of compounds has been known since the first synthesis by Mulder [1] in 1873, but the correct structure was given in 1944 by Ainley *et al.*[2] (formula I). The rational name is based on the yet not isolated cyclic 1,3,5-thiadiazine (II).



I



II

Davies and Sexton [3] studied the insecticidal action of 1,3,5-thiadiazine-2-thione derivatives, and found 3,5-dimethyl, 3-phenyl-5-propyl, 3-phenyl-5-methyl, 3-phenyl-5-hydroxyethyl and 3-*p*-tolyl-5-methyl derivatives to be highly toxic for grasshoppers. They indicated that the chemically active group might be the sequence —SCN—, and that the substances act by being converted to aryl thiocarbimides (isothiocyanates) which they parallel in insecticidal activity.

Herschler [4] reported on the use of the 3,5-dimethyl derivative as an effective slimicide (acting against slime-forming fungi and bacteria) for industrial use, particularly in paper mills. The fungicidal activity was, according to him, comparable to that of organic mercury compounds. Freyschuss [5] found the 3,5-dimethyl derivative to be less effective than phenyl mercury acetate; about 10 times weaker in fungicidal activity. It was, however, comparable to that of pentachlorophenol.

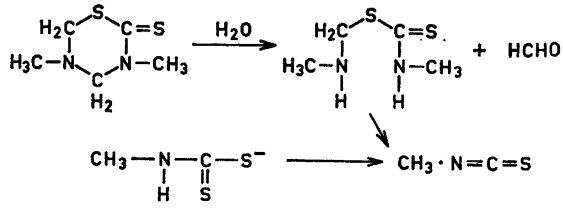
Under various trade and test names (Mylone, Crag Mylone, N 521, Crag fungicide 974), the 3,5-dimethyl derivative has been tried as soil fungicide and nematodicide. [6–10] For sake of brevity, the neutral abbreviation 3,5-D for the 3,5-dimethyl derivative, introduced by Herschler [4] will be used in this report.

Regarding the chemical behaviour of 3,5-D, Mulder [1] found that it was rather unstable, especially in aqueous solutions. By addition of heavy metal salts, the corresponding metal dithiocarbamates, *e. g.* $[H_2N.C(:S)S]_2Pb$ were formed. Levi and Gimignani [11] claimed, however, that the reaction is more complex. They described the formation both of dimethyl dithiocarbamate and methylene dithiocarbamate silver complexes. These findings will be commented upon later. Ainley *et al.*[2] also stated that 3,5-D is unstable and is completely decomposed under mild conditions by dilute acids or by mercuric oxide.

On the other hand, the fungi-toxicity of the compound is shown in dilute aqueous solutions, and is quite persistent. Thus, Burgis and Overman [7] found the compound to remain in soil for 6 days, and to give a high degree of root-knot-nematode control for approximately 15 weeks after treatment. Herschler [4] also mentions that in aqueous solution of 3,5-D which are left standing for

a couple of days, a precipitate is formed, without seemingly to affect the fungicidal or bactericidal activity.

These facts must naturally lead to the conclusion that it is the decomposition products of 3,5-D which are, wholly or in part, responsible for the fungicidal and other biocidal activities. This line of reasoning has been further extended by van der Kerk,[12] who compared the fungicidal activities of 3,5-D and sodium N-methyl dithiocarbamate, and concluded (without furnishing chemical evidence) that 3,5-D in aqueous solution is hydrolyzed to form formaldehyde and a N-methyl dithiocarbamate ester, which is then further decomposed to form methyl isothiocyanate. Decomposition to methyl isothiocyanate was also thought to occur with sodium N-methyl dithiocarbamate (III).



III

The similarity between 3,5-D, sodium N-methyl dithiocarbamate and methyl isothiocyanate is, according to van der Kerk [12] among other things also based on the fact that the fungicidal effect of all these compounds is counteracted by cysteine.

The question whether mono-N-substituted dithiocarbamates act as such or only after decomposition to the corresponding isothiocyanates has been much disputed in the bisdithiocarbamate series, where the diisothiocyanates have been believed to be the toxic agent (see Rich and Horsfall [13]).

As to sodium N-methyl dithiocarbamate, Wedding and Kendrick [14] could show that it has an inhibitory action of itself, not dependent on conversion to methyl isothiocyanate.

According to this, it must still be regarded as an open question which of the possible decomposition products of 3,5-D that are responsible for its toxicity.

The aim of the present investigation has been to clarify this, by means of spectrophotometric studies and a biological model system. As such a model system has been used the respiration of acetate by yeast, which previously has been shown to be as sensitive towards dialkyldithiocarbamates as the total growth process.[15]

EXPERIMENTAL

The 3,5-D used in these experiments was received several years ago as a gift from Crown Zellerbach Corp., U.S.A. (they do not produce the compound). It was purified by recrystallization from acetone. The purified product was kept in a refrigerator. Stock solutions were made in acetone and diluted with distilled water before use. What are termed fresh solutions were diluted not more than a couple of hours before use. The 24 h old solutions were diluted and kept in room temperature for approximately 24 h before use.

Methylene isothiocyanate was a commercial product from A/G Fluka, Switzerland. Sodium N-methyl dithiocarbamate was prepared as described by Pluijgers.[16] The product was kept in a refrigerator. In order to avoid contamination with methylisothiocyanate formed by decomposition, the crystals were washed with ether before weighing out to make stock solutions. These were never prepared more than a couple of hours before use.

As test organism has been used Baker's yeast (*Saccharomyces cerevisiae*), suspended in 0.05 M phosphate buffer of the desired pH in an amount of 1 g fresh weight per 100 ml. The respiratory studies have been carried out using ordinary Warburg technique, with alkali in the center cup (only oxygen uptake was determined). The inhibitor was mixed with the yeast suspension in the main compartment (eventually together with cupric sulfate), while sodium acetate, adjusted to the same pH as the yeast suspension, was added from the side arm after 30 min from the start of the experiment, and to give a final concentration of 0.05 M. The experiments lasted normally 3 h. The degree of inhibition was calculated for the period 90–150 min (the second hour after addition of acetate). During this period the respiratory intensity was fairly constant, so that the mean respiratory values could be estimated with reasonable accuracy. But it should be stressed that the degree of inhibition varies considerably during the experimental period, probably reaching a maximum shortly after the addition of acetate, and then diminishing. As in the present studies only a formal value for the degree of inhibition was found to be necessary, the kinetics of the inhibitory reaction have not been further investigated. Due to the same reasons, the respiratory values have not been corrected for endogenous respira-

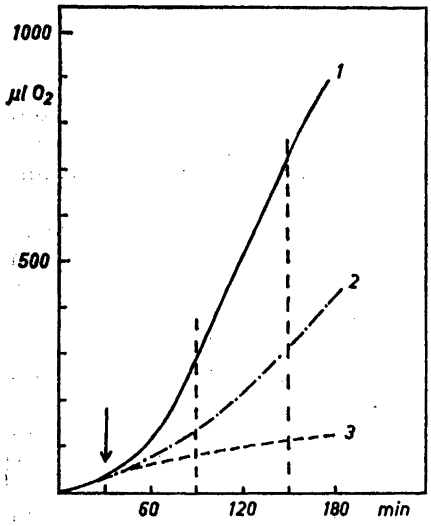


Fig. 1. Oxygen uptake curves for baker's yeast at pH 6.5, measured with Warburg technique. Curve 1 and 2: Sodium acetate to a final concentration of 0.05 M added after 30 min. Curve 2: 10^{-4} M methyl isothiocyanate from the start. Curve 1: No inhibitor added. Curve 3: Endogenous respiration (no respiratory substrate added) The vertical lines at 90 and 150 min indicate the interval for which the degree of inhibition was determined.

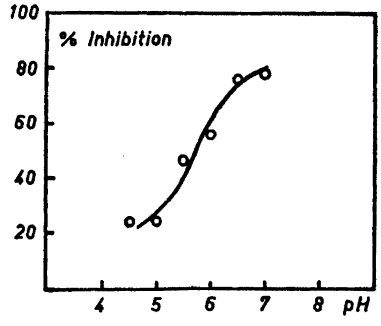


Fig. 2. The degree of inhibition caused by 10^{-4} M methyl isothiocyanate on acetate respiration at different pH values.

tion, although there are indications that the endogenous respiration is litte affected by these compounds.[15]

For the spectrophotometric studies, a Beckman model DU spectrophotometer with photomultiplier was used.

RESULTS

A. Methyl isothiocyanate. Preliminary investigations showed that methyl isothiocyanate in a concentration of 10^{-4} M did not inhibit the respiration of yeast with glucose as the external respiratory source. With sodium acetate as respiratory source, an inhibition was, however, found at pH 6.5, but practically no inhibition at pH 4.5. The degree of inhibition at different pH values was therefore studied more closely. The results obtained are shown in Fig. 2. In Fig. 1 are shown typical respiration curves at pH 6.5, without and with methyl isothiocyanate added.

From Fig. 2 one is led to the suggestion that the inhibitory action of methyl isothiocyanate must be due to a reaction that is pH-dependent. Most probable is the reaction between isothiocyanate esters and SH-compounds, whereby dithiocarbamate esters (dithio-urethans) are formed.

Methyl isothiocyanate has a relatively low UV-absorption (λ_{max} at 2370 Å, molar extinction value *ca.* 550). When 10^{-4} M methyl isothiocyanate was mixed with 10^{-3} M cysteine at pH 6.5 and incubated for a couple of hours,

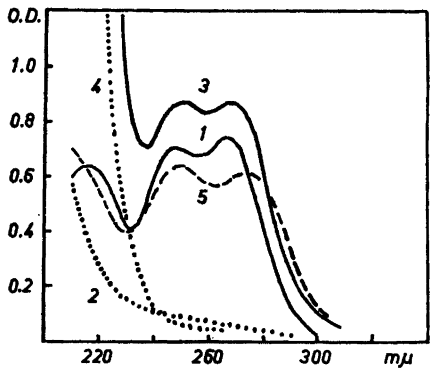


Fig. 3. Curve 1: The spectrum of the reaction mixture of 10^{-4} M methyl isothiocyanate and 10^{-3} M cysteine incubated at room temperature and pH 6.5 for 2 h. Curve 2: The spectrum of 10^{-3} M cysteine alone. (The spectrum of 10^{-4} M methyl isothiocyanate will not exceed an O.D. of 0.05). Curve 3: The spectrum of a similar reaction mixture between 10^{-3} M glutathione and 10^{-4} M methyl isothiocyanate. Curve 4: 10^{-3} M glutathione alone. Curve 5: The spectrum of the reaction mixture between a 72 hours old solution of 3.5-D (10^{-4} M) and cysteine.

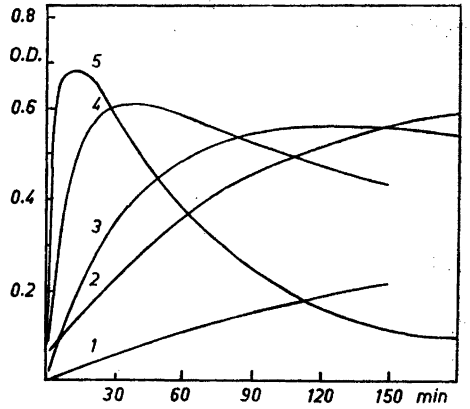


Fig. 4. The changes in optical density at 265 mμ with time in a mixture of 10^{-3} M cysteine and 10^{-4} M methyl isothiocyanate, at different pH values in room temperature. Curves 1—5 are for the following pH values 5.6; 6.1; 6.5; 6.8; 7.7.

the UV absorbancy of the mixture was found to have increased considerably, and the spectrum of the mixture showed two peaks at 2480 and 2660 Å, a spectrum characteristic of dithiocarbamate compounds (Fig. 3).

A kinetic study of the reaction was undertaken, and the results are shown in Fig. 4. It is seen that the reaction proceeds at a rapid rate at the pH values where methyl isothiocyanate is inhibitory on the acetate respiration. At higher pH values, the curves show a typical consecutive reaction sequence; a very rapid first reaction whereby the UV-absorbing compound is formed, followed by a relatively slow second reaction resulting in reduction in the UV absorbancy. We have not carried out any detailed investigations to identify the reaction products.

Results similar to these have been obtained with tetramethylene isothiocyanate by the van der Kerk group.[17]

Control experiments with alanine gave additional evidence that it was the SH-group in cysteine which was responsible for the reaction with isothiocyanate. Glutathione reacted essentially similar to cysteine (Fig. 3), and by analogy, one is led to the conclusion that methyl isothiocyanate must react with available SH-compounds in the cell.

In fact, it was also possible to demonstrate the reaction *in vivo*, in a yeast cell suspension which was incubated with methyl isothiocyanate. The results are shown in Fig. 5.

Owing to the very high absorbancy of the yeast in this spectral region (nucleic acids and proteins), compared to the absorbancy of the UV-absorbing

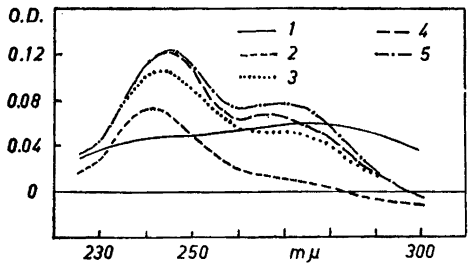


Fig. 5. The spectra obtained when a yeast suspension (5 g fresh weight/100 ml) at pH 6.5 was incubated with 10^{-4} M methyl isothiocyanate. The blank cuvette contained the same amount of yeast. The photomultiplier was set at maximum. Curve 1: The two yeast suspensions measured against each other (a check on the accuracy of the measurements). Curve 2: Immediately after addition of methyl isothiocyanate, a typical spectrum for this compound is obtained. Curves 3—5: The spectra after 1, 2 and 3 h of incubation.

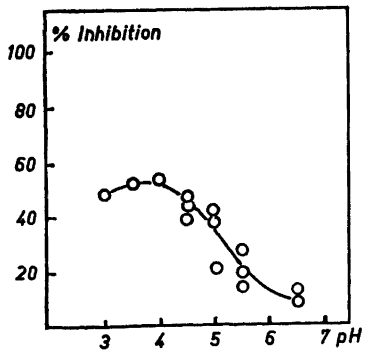


Fig. 6. The degree of inhibition of 10^{-4} M sodium N-methyl dithiocarbamate on the acetate respiration of yeast, at different pH values. Experimental conditions and calculations as indicated for Fig. 1.

compound formed when methyl isothiocyanate is added, the spectra obtained are not very accurate, but their resemblance to those obtained with cysteine are obvious.

B. Sodium N-methyl dithiocarbamate. As with methyl isothiocyanate, sodium N-methyl dithiocarbamate in the concentration of 10^{-4} M inhibited the acetate respiration in yeast.

In this case, however, the effect of varying the pH was the opposite of that with methyl isothiocyanate. The highest degree of inhibition was found at low pH values while at pH 6.5, very little inhibition occurred. Results from a number of experiments are shown in Fig. 6. These results give a strong support to the findings of Wedding and Kendrick [14] that sodium N-methyl dithiocarbamate has an action of its own. A further substantiation of this was found by adding cupric sulfate together with the dithiocarbamate. This was only done at pH 6.5, since cupric sulfate alone at lower pH values was found to act strongly inhibitory. Addition of cupric sulfate together with sodium N-methyl dithiocarbamate at pH 6.5 resulted in a strong inhibition. This can be seen from Fig. 7. Even as low cupric sulfate concentration as 10^{-5} M has caused a 70 % inhibition of the respiration. The cupric complex formed (probably a 1:2 complex, since the dithiocarbamate ions are in excess)

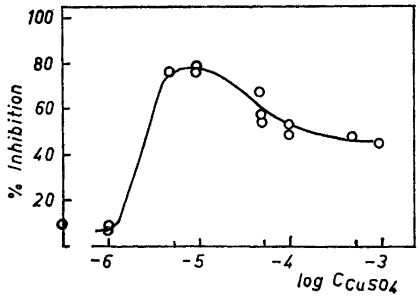


Fig. 7. The effect of cupric sulfate and sodium N-methyl dithiocarbamate (10^{-4} M) on the acetate respiration of yeast, at pH 6.5.

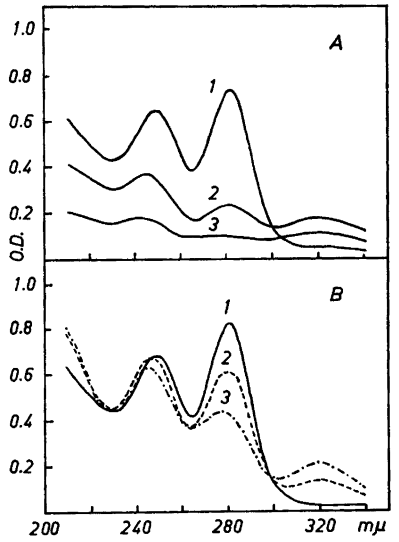


Fig. 8. Spectra of a 10^{-4} M aqueous solution of sodium N-methyl dithiocarbamate. Part A of the figure, Curve 1: Freshly prepared. Curve 2: After 2 h. Curve 3: After 24 h. Part B: The same concentration of the dithiocarbamate, but 10^{-3} M cysteine, adjusted to pH 6.5 added. Curve 1: Freshly prepared. Curve 2: After 1 h. Curve 3: After 2 h.

must thus be a powerful toxicant. It is not probable that under these conditions the complex should spontaneously decompose to form methyl isothiocyanate and cupric sulfide. No inversion effect could, however, be observed in these experiments. The cupric complex has a very featureless spectrum.

Sodium N-methyl dithiocarbamate did not seem to react with cysteine at pH 6.5. However, both in the presence and absence of cysteine, the spectrum of a 10^{-4} M aqueous solution was already after two hours quite different from that of a quite freshly prepared solution. This can be seen in Fig. 8. After 24 h, the absorbancy was much reduced, and only one maximum at 2400 Å was discernible. Cysteine seems to stabilize the compound to some extent.

These spectra will be discussed in connection with those of 3,5-D.

C. 3,5-D. Spectrophotometric studies did in part support van der Kerk's [12] suggestion that 3,5-D in aqueous solution is hydrolysed, so that a dithiocarbamate ester is formed. Fig. 9 shows the spectra of 3,5-D in carbon tetrachloride and water. The very much lower absorbancy in water indicates that the ring structure has been split and the spectrum of the aqueous solution is characteristic for dithiocarbamete derivatives. The spectra of different dithiocarbamate compounds [18] are, however, so similar that a further identification is impossible.

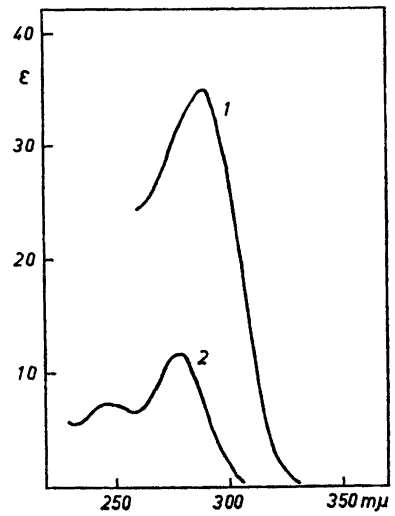


Fig. 9. Absorbtion spectra of 3,5-D. Curve 1: in carbon tetrachloride. Curve 2: in water. Ordinate: Molar extinction values.

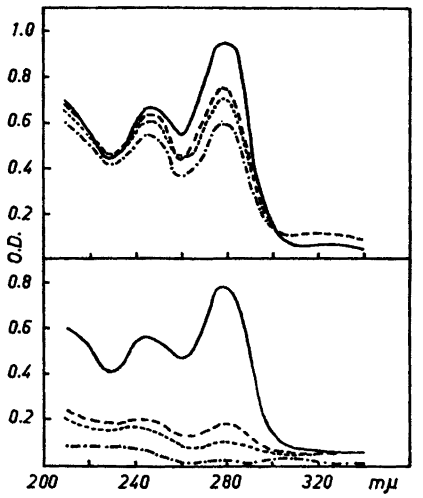
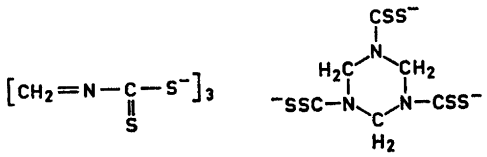


Fig. 10. Absorbtion spectra of fresh and aged solutions of 3,5-D. In both sets of curves, the fully drawn give the spectrum of a freshly prepared solution. Curves with decreasing degree of absorbancy are after 1, 2 and 3 days of storage. Note the great difference in stability between the solutions made in similar manner, but on two different occasions.

On standing, the spectrum of an aqueous 3,5-D solution changes very much in the same way as was found for sodium N-methyl dithiocarbamate. This can be seen from Fig. 10, where the spectra of a number of aged 3,5-D solutions are shown. These spectra were never very reproducible, and the relative ratios of the heights of the peaks can also be seen to be different, indicating that the solution consists of a mixture of UV-absorbing compounds.

With cupric sulfate, 3,5-D formed a yellow precipitate, not extractable with carbon tetrachloride. Thus, no dimethyl dithiocarbamate can be present. (In more concentrated solutions, however, a small fraction of the cupric complex is extractable, and shows the same spectrum as cupric dimethyl dithiocarbamate (1:2)). The spectrum of the copper complex was somewhat different in the UV region from the spectra of the copper complexes of dithiocarbamate and N-methyl dithiocarbamate. However, also in this case, the possibility exists that the reaction mixture contains several UV-absorbing compounds.

As already mentioned, Levi and Gimignani[11] described the formation of methylene dithiocarbamate as a decomposition product of 3,5-D. A verification of this would be highly interesting. In another publication,[19] the same authors have described the synthesis of methylene dithiocarbamate (ammonium salt), and some of its properties. It was stated to occur in a trimeric form, possibly as in (IV).



IV

Several attempts were made to synthesize the compound according to the methods described by Levi and Gimignani, but none were successful. It is worth while to note that methylene dithiocarbamate has not been seen to be further mentioned in the literature, although hundreds of dithiocarbamate derivatives have later been made and tried for fungicidal activity.[13, 16, 20] The alleged structure of this compound would make it highly interesting to test for fungicidal activity. The present author is therefore led to the conclusion that Levi and Gimignani must have made some mistake, and that methylene dithiocarbamate probably does not exist. Supporting this conclusion is also the fact that they had set their task at elucidating the correct structure of 3,5-D, but in this they evidently failed completely.

Aged solutions of 3,5-D reacted with cysteine to form a UV-absorbing compound with much the same spectrum as when methyl isothiocyanate reacted with cysteine (Fig. 3). Methyl isothiocyanate will not be distinguishable as such in the spectra of 3,5-D solutions, owing to its relatively low UV-absorbancy. But the reaction with cysteine in aged solutions indicate its presence. In fresh solution, no reaction could be detected.

Table 1. The inhibition caused by ether-extracted and not extracted solutions of 3,5-D in the presence and absence of cupric sulfate. Final concentration of 3,5-D 10^{-4} M, and of cupric sulfate 10^{-5} M. In all experiments the pH was 6.5. See the text for further details.

	No Cu^{2+} added		10^{-5} M $CuSO_4$	
	Not extr.	Ether extr.	Not extr.	Ether extr.
Fresh 3.5-D	18 %	0 %	78 %	66 %
24 h old 3.5-D	60 %	15 %	70 %	61 %

For the respiratory studies, both fresh and 24 h old solutions of 3,5-D were used. In the absence of cupric sulfate, the inhibitory effect of the fresh solution was considerably less than of the 24 h old one. This can be seen from the data in Table 1. With 24 h old solutions, the effect of pH was studied, and the values are given in Fig. 11. It is seen that in this case, the inhibitory action is depen-

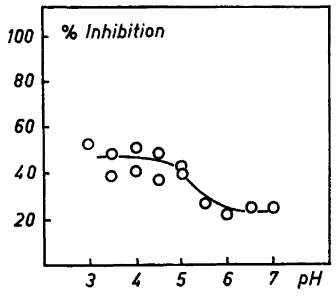


Fig. 11. The degree of inhibition caused by 10^{-4} M 3,5-D (24 h old) on the acetate respiration of yeast, at different pH values. Experimental conditions and calculations as indicated for Fig. 1.

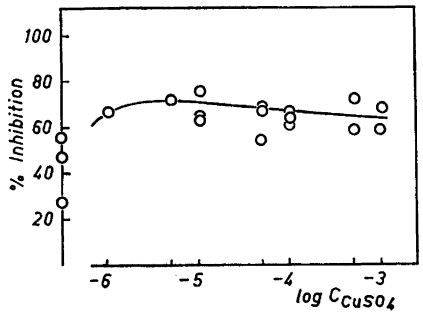


Fig. 12. The effect of cupric sulfate and 3,5-D (10^{-4} M; 24 h old) on the acetate respiration of yeast, at pH 6.5.

dent of pH, but to a lesser degree than with methyl isothiocyanate or sodium N-methyl dithiocarbamate. At higher pH values, the results varied a good deal from one experiment to the other. Cupric sulfate acted synergistically also with 3,5-D at pH 6.5, as can be seen from Fig. 12.

A natural explanation of these results would be that in the aqueous solutions of 3,5-D, there is a mixture of N-methyl dithiocarbamate ester, free N-methyl dithiocarbamate ions, and methyl isothiocyanate.

To check this, some experiments were carried out, in which fresh or 24 h old solutions of 3,5-D were extracted with ether three times in order to remove methyl isothiocyanate, before they were assayed without and with addition of cupric sulfate. The results are shown in Table 1. It is seen that ether-extraction has reduced the toxicity to practically zero in the absence

of cupric sulfate, while with addition of cupric sulfate, the ether extraction only causes a minor reduction in toxicity. These results are entirely consistent with what would be expected if the 24 h old 3,5-D solution contained a mixture of N-methyl dithiocarbamate and methyl isothiocyanate, while the fresh solution contained little or no isothiocyanate.

D. Other decomposition products. Experiments were also carried out to check whether the other possible decomposition products would affect the acetate respiration in yeast. Neither formaldehyde, carbon disulfide nor sodium dithiocarbamate did show any inhibitory effects at 10^{-4} M concentrations. These compounds would thus not interfere with the results obtained above.

DISCUSSION

The finding that methyl isothiocyanate reacts with SH-compounds at pH values above 5.5—6.0 both *in vitro* and *in vivo*, strongly suggests that this compound acts by blocking reactive SH-groups in the cytoplasm. Further studies on representative enzyme systems would, however, be necessary to show if it inhibits all SH-containing enzymes, or if its activity is more restricted.

For dialkyldithiocarbamates, the van der Kerk group [21] has advocated the opinion that they act by combining with dithiols, a view that is not far from that of the present author. Regarding sodium N-methyldithiocarbamate, Wedding and Kendrick [14] have suggested that it acts in much the same way as proposed by Goksøyr [15] for dialkyldithiocarbamates. Supporting this is the present finding that copper acts strongly synergistic also on N-methyl dithiocarbamate. The chemical reactions leading to the blocking of metabolic pathways must, however, be expected to be somewhat different. It should also be stressed that, owing to the instability of N-methyl dithiocarbamate, in growth experiments lasting several days, and even in practical use, it must be expected to behave very much like methyl isothiocyanate.

3,5-D is unstable in aqueous solution, and is most probably hydrolyzed as proposed by van der Kerk. The spectrophotometric studies support this, and further that the hydrolysis may go on so that free N-methyl dithiocarbamate ions are formed. In accordance with the low toxicity of dithiocarbamate esters in general, a freshly prepared solution shows less toxicity than a 24 h old one; but in this latter solution, a substantial amount of the dithiocarbamate has been converted to methyl isothiocyanate. In the presence of cupric ions, also the freshly prepared solution shows a high degree of toxicity, indicating that the ester bond is then broken and an active copper chelate of N-methyl dithiocarbamate is formed.

In practical use, the formation of formaldehyde in these reactions should not be overlooked. Formaldehyde is an effective soil fumigant, and may be responsible for a part of the effects of 3,5-D as a soil fungicide.

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