SUMMARY

The effect of thiocyanate on the sedimentation of fumarase has been studied over a range of experimental conditions. It was found that the action of this anion occurred in two distinct phases, an immediate lowering of the sedimentation constant from 9.28 to 8.6 *S*, followed by a slower dissociation into two similar subunits. Under certain conditions aggregation also accompanied dissociation. These physical changes are discussed in the light of fluorescence polarization studies already reported and of changes in the catalytic properties of the enzyme under similar conditions.

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INVESTIGATIONS ON ORGANIC FUNGICIDES

XI. THE ROLE OF METALS AND CHELATING AGENTS IN THE FUNGITOXIC ACTION OF SODIUM DIMETHYLDITHIOCARBAMATE (NaDDC)

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In previous publications[1,2] we reported that apart from histidine various other imidazole derivatives could antagonize on agar media the fungitoxic action of sodium dimethyldithiocarbamate (NaDDC) in the first zone of inhibition. It was suggested that the imidazoles could in some way or other prevent the fungicide from interacting with some essential enzyme system. Since imidazoles are known to form complexes with metals, and metals might well play a role in the action of the strongly metal-binding dithiocarbamates, we investigated whether there is any correlation between antagonistic activity and metal-binding ability of various imidazole derivatives. A close parallel was found for Cu; moreover, other chelating compounds (ethylenediaminetetraacetic acid and mercaptobenzothiazol) were found to have antagonistic activity[3]. Thus there was a strong indication that metals have a function in the fungitoxic action of NaDDC. The role of metals was now further investigated, especially with regard to the work of GOKSØYR.

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In recent publications GOKSØYR[4,5] describes experiments with *Saccharomyces cerevisiae* in which the combined effect of NaDDC and metal salts on growth inhibition as well as on glucose and acetate oxidation was studied. In media containing 25 μmoles copper sulphate (= 6.25 p.p.m. of $CuSO_4 \cdot 5\ H_2O$) a maximum in growth inhibition was seen in the presence of the same NaDDC concentrations (5–8 μmoles; approx. 0.9–1.5 p.p.m. of NaDDC·2 ½ H_2O) that lead to a maximum in inhibition of acetate oxidation. GOKSØYR could show spectrophotometrically that at these NaDDC concentrations the proportion of Cu and NaDDC was such that a maximal amount of the 1:1 complex of dithiocarbamate and Cu was present*. He, therefore, ascribes inhibition of growth and of acetate oxidation to this 1:1 complex. At higher NaDDC concentrations the 1:1 complex was shown to be gradually turned into a 1:2 complex. This stable and insoluble complex is supposed to be non-toxic since there is neither growth inhibition nor inhibition of acetate oxidation when the dithiocarbamate is present in this form.

In a medium without added Cu maximal inhibition of growth (on glucose medium) and of acetate oxidation takes place at lower concentrations of NaDDC (1–2 μmoles, approx. 0.2–0.4 p.p.m.). The shape of the corresponding spectrum was not determined but GOKSØYR assumes that traces of metal that may be carried on the cell surface play a role in the formation of a toxic complex.

Our results with *Aspergillus niger* seem to confirm many of his findings so far as liquid cultures are concerned. We found, moreover, that the accumulation of pyruvic acid by NaDDC[6] as well as the second zone of inhibition are caused by free dithiocarbamate ions.

A preliminary account of these results has already appeared[7].

EXPERIMENTAL

Growth experiments

Aspergillus niger was used as test organism. The basal nutrient medium had the following composition: 1% glucose, 0.5% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4 \cdot 7\ H_2O$, biotin 0.002% γ/ml, aneurin 0.2 γ/ml. The pH was 7.0 after autoclaving. Analytical grade chemicals were used throughout. Agar cultures were seeded with 4000 spores per ml of final medium. For shake cultures in liquid medium 6000 spores were used per ml. Growth was judged after 2, 3 and 4 days of incubation.

Physical measurements

Optical measurements were carried out in a Beckman DU spectrophotometer, using 5 and 10 cm cells. Further details are mentioned below.

RESULTS

The role of metals in the first zone of inhibition and the zone of inversion growth in liquid medium

In order to study the role of metals we used liquid cultures rather than agar media, because of the inevitable presence of traces of heavy metals in the latter.

* Dimethyldithiocarbamic acid can form two complexes with copper, *viz.* CuDDC and $CuDDC_2$. Here and throughout the text DDC stands for the dithiocarbamate anion and charges will be omitted. The first-mentioned complex will be designated the 1:1 complex; it carries one positive charge and is soluble; the second complex is the 1:2 complex and is insoluble in water. This last complex is sometimes referred to as "saturated", because copper here exerts its maximum coordination number.

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A. niger spores were seeded into 150 ml flasks with 20 ml of a glucose mineral salts solution + aneurin and biotin made up with tap water. Estimation showed the total amount of Cu and Fe to be *ca.* 0.02 and < 0.04 p.p.m., respectively. NaDDC concentrations ranged from 0.1 to 50 p.p.m. In one experiment 0, 3, 10 or 30 p.p.m. of $CuSO_4 \cdot 5\ H_2O$ were added respectively to 4 series of flasks with different NaDDC concentrations (Table I). Growth was judged after 4 days shaking at 24°. The first zone of inhibition does not appear unless more than 3 p.p.m. of copper sulphate have been added to the medium. Inversion growth seems to appear in this case with far less delay than on agar media. In another experiment there was inhibition at 0.5 p.p.m. NaDDC + 6 p.p.m. of copper sulphate and at 0.5 and 1 p.p.m. NaDDC + 8 p.p.m. of copper sulphate.

TABLE I

GROWTH OF *Aspergillus niger* IN GLUCOSE MINERAL SALTS SOLUTION CONTAINING DIFFERENT AMOUNTS OF NaDDC AND COPPER SULPHATE

Incubation period 4 days

Added $CuSO_4 \cdot 5\ H_2O$ in p.p.m.	*Concentration of NaDDC in p.p.m.*								
	0	*0.2*	*0.5*	*1*	*2*	*5*	*10*	*20*	*50*
0	+	+	+	+	+	+	+	±	—
1	+	+	+	+	+	+	+	±	—
3	+	+	+	+	+	+	+	+	—
10	+	+	—	—	—	±	+	+	—

$FeSO_4 \cdot 7\ H_2O$, $MnSO_4 \cdot 4\ H_2O$ and $ZnSO_4 \cdot 7\ H_2O$ did not show this effect, even when 30 p.p.m. of the salts had been added. $CoSO_4 \cdot 7\ H_2O$, however, gave a slight or no effect when 10 p.p.m. were added, whereas 20 and 30 p.p.m. of this substance gave a larger zone of inhibition. A quantity of 30 p.p.m. of cobaltous sulphate as such has a slightly toxic effect (Table II).

TABLE II

GROWTH OF *A. niger* IN GLUCOSE MINERAL SALTS SOLUTION CONTAINING DIFFERENT AMOUNTS OF NaDDC AND COBALTOUS SULPHATE

Incubation period 4 days

Added $CoSO_4 \cdot 7\ H_2O$ in p.p.m.	*Concentration of NaDDC in p.p.m.*									
	0	*0.1*	*0.2*	*0.5*	*1*	*2*	*5*	*10*	*20*	*50*
0	+	+	+	+	+	+	+	±	—	—
10	+	+	+	+	±	+	+	+	+	—
20	+	+	+	—	—	—	+	+	+	—
30	±	±	—	—	—	—	—	±	±	+

In view of GOKSØYR's observation that dimethyldithiocarbamate bound with Cu to a 1:1 complex is a growth-inhibiting agent for *Saccharomyces cerevisiae*, whereas the 1:2 complex is not, we compared the amount of 1:1 complex in media with various concentrations of NaDDC in the presence of 10 p.p.m. of $CuSO_4 \cdot 5\ H_2O$.

To this end the solutions were filtered through a sintered glass filter (Jena G5). The 1:2 complex was apparently filtered off quantitatively, since in the ultraviolet

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spectrum only the absorption of the 1:1 complex (λ max = 384 mμ) was found (a dispersion or colloidal solution of the 1:2 complex shows maximal absorption at about 460 mμ).

In Fig. 1 the optical density at 380 mμ is given of filtered solutions containing 10 p.p.m. of copper sulphate and various amounts of NaDDC. It is seen that the maximum amount of CuDDC in water is present at about 2 p.p.m. NaDDC, but in the culture medium this maximum is shifted to 0.5–1 p.p.m. NaDDC (the results were somewhat variable in the latter case). We could show that this shift is due to the presence of phosphate in the medium.

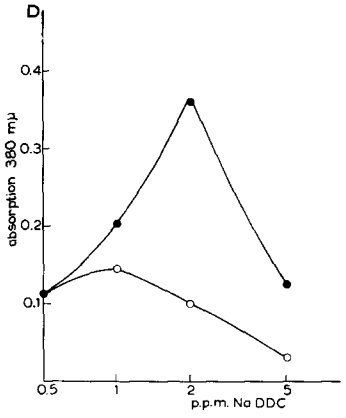

Fig. 1.

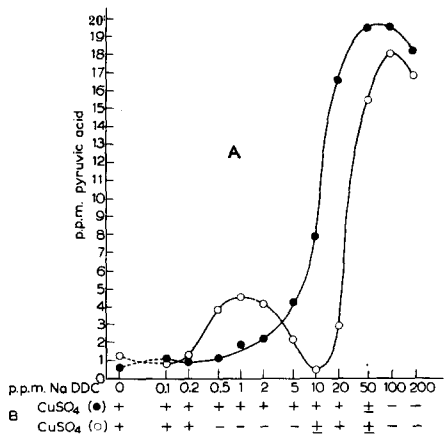

Fig. 2.

Fig. 1. Optical density at 380 mμ of filtered solutions containing 10 p.p.m. of copper sulphate (40 μM) and various amounts of NaDDC; ●——● in distilled water; ○——○ in glucose mineral salts solution.

Fig. 2. **A.** Pyruvic acid accumulation by *A. niger* in glucose mineral salts solution containing various concentrations of NaDDC in absence (●——●) and presence (○——○) of 10 p.p.m. of copper sulfate. Mycelial spheres (dry wt 7 mg); shaken for 4 hours, 20 ml medium, pH 7.0. **B.** Growth response in same cultures after 24 h.

Obviously, the occurrence of the first zone of growth inhibition corresponds to the presence of the 1:1 complex, while in the zone of inversion growth the dithiocarbamate forms a 1:2 complex, which appears to be non-toxic. At concentrations of $CuSO_4$ below 10 p.p.m. a 1:1 complex will certainly be formed as well. It is assumed, however, that the growth-inhibiting concentration is not reached there, so that growth is seen at all NaDDC concentrations tested.

The action of antagonists in the first zone of inhibition

When 300 p.p.m. of L-histidine was added to media containing 10 p.p.m. of copper sulphate and various NaDDC concentrations the first zone of inhibition did not appear, indicating that histidine also antagonizes in liquid medium. It might be suggested that this antagonistic action is due to removal of Cu from the dithiocarbamate complex. However, the same curves as in Fig. 1 were obtained when up to 2% histidine, or 0.05% imidazole pyruvic acid, an even stronger antagonist, were added to the solutions. Thus it is shown that these compounds cannot compete

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effectively with NaDDC in the formation of copper complexes, even in these concentrations, which are well above the concentrations in which they show antagonistic action.

Ethylenediaminetetraacetic acid, however, is able to compete effectively with the above-mentioned amounts of NaDDC for Cu at pH 7 in concentrations above $5 \cdot 10^{-4}$ *M* EDTA (about 200 p.p.m.).

The competition of EDTA and NaDDC for Cu ions can lead to a rough estimate of the stability constant of NaDDC with respect to Cu. The concentration of Cu ions in EDTA solutions in which EDTA is able to compete effectively for Cu with NaDDC (conc. *ca.* 10^{-5} *M*) at pH 7 is about 10^{-16}, as can be calculated from the stability constant for Cu and EDTA[8]. From this figure we can estimate the overall stability constant K of the Cu dithiocarbamate complex:

$$K = \frac{[CuDDC_2]}{[Cu][DDC]^2}$$

The resultant value for log K is about 21. GOKSØR[5] gave a lower limit of 12 for this quantity.

Experiments on agar media

On agar media the effect of Cu appeared to differ from that observed in liquid media. This is illustrated by the results in Table III showing the effect of addition of copper sulphate to a glucose mineral salts medium using unwashed agar and glass-distilled water. The total Cu content of the basal medium was only $\leq$ 0.01 p.p.m. Only 1 p.p.m. of $CuSO_4 \cdot 5\ H_2O$ is required to obtain a first zone of inhibition comparable to that caused by the addition of 10 p.p.m. of this substance to a liquid medium. This result also explains why up till now we obtained a first zone of inhibition on agar media without addition of Cu: the agar was always washed thoroughly with tap water before use. We have found that by this washing procedure the Cu content of the agar medium can easily be raised to 0.25 p.p.m. (eq. to 1 p.p.m. of $CuSO_4 \cdot 5\ H_2O$) by absorption from the tap water used. As appears from Table III this amount is sufficient to cause an inhibition zone on an agar medium.

TABLE III

ACTION OF NaDDC ON THE GROWTH OF *Aspergillus niger* ON GLUCOSE AGAR CONTAINING DIFFERENT CONCENTRATIONS OF $CuSO_4 \cdot 5\ H_2O$

Incubation period 3 days

Added $CuSO_4 \cdot 5\ H_2O$ *in p.p.m.*	*Concentration of NaDDC in p.p.m.*							
	0	*0·5*	*1*	*2*	*5*	*10*	*20*	*50*
0	+	+	+	+	+	+	+	—
0.1	+	+	+	+	+	+	+	—
0.3	+	+	±	+	+	+	+	—
1	+	±	—	—	+	+	+	—
3	+	+	—	—	—	+	+	—

Pyruvic acid accumulation and dimethyldithiocarbamate complexes

In an earlier publication[6] we reported that concentrations of NaDDC of about 10–50 p.p.m. induce mycelium and spores of *A. niger* to accumulate pyruvic acid in liquid cultures without metal addition.

In order to relate pyruvic acid accumulation with growth in liquid medium and with the state in which the dithiocarbamate is present, pyruvic acid accumulation was studied in media with NaDDC that also contained $CuSO_4$.

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Small mycelial spheres of *A. niger* were added to a glucose mineral salts solution of pH 7.0 made up with distilled water, and different amounts of NaDDC were added. A second series contained in addition 10 p.p.m. of $CuSO_4 \cdot 5\ H_2O$. After 4 hours shaking at 24°, 5 ml samples were withdrawn for estimation of pyruvic acid[6]. Growth of the spheres was judged after 24 hours. Fig. 2 gives the results of this experiment.

Growth results were about the same as described above for liquid cultures seeded with spores.

Without addition of copper pyruvic acid accumulation proceeds as described before[6]. In the series with 10 p.p.m. $CuSO_4$, however, two peaks are seen. The lower peak corresponds obviously to the presence of the 1:1 complex (*cf.* Fig. 1) and to the first zone of growth inhibition. The second peak seems to be caused by the action of the free dithiocarbamate, which appears when all Cu is present in the form of the insoluble 1:2 complex. This peak then is comparable to the one in the series without Cu.

DISCUSSION

In liquid medium a close agreement is found between spectrophotometric determinations showing the presence or absence of the 1:1 complex of Cu and dimethyldithiocarbamate and growth results of *A. niger*. The first zone of inhibition appears as soon as the amount of 1:1 complex surpasses a certain value. These facts support those found recently for a yeast, *Saccharomyces cerevisiae*, by GOKSØYR[4,5]. Toxicity of the 1:1 complex must be due to combination of this complex with an essential enzyme. In the zone of inversion growth the dithiocarbamate is present as the apparently non-toxic 1:2 complex with Cu.

Co also appears to form a toxic complex with dithiocarbamate ions. We are, however, at present unable to say which complex this could be, a 1:1 complex with Co^{II} or a 1:1 or 1:2 complex with Co^{III}, corresponding to the saturated complexes $Co^{II}\ DDC_2$ and $Co^{III}\ DDC_3$.

On agar media far less total Cu is required than in liquid media for comparable growth inhibition, a fact yet to be explained. Unfortunately, no spectrophotometric measurements can be carried out in this medium but we assume that here too the 1:1 complex is the toxic agent.

MANTEN, KLÖPPING AND VAN DER KERK[9] described the effect of metal addition on the action of TMTD using *A. niger* on Czapek Dox agar. The basal medium used certainly contained sufficient Cu to explain the high activity of TMTD, because the agar was thoroughly washed beforehand with tap water, which must have introduced certain amounts of Cu; 0.16 p.p.m. of copper sulphate were, moreover, added as a trace element.

We have seen that many chelating agents can antagonize the action of the 1:1 complex. We have earlier suggested that certain imidazole derivatives could antagonize NaDDC by preventing the dithiocarbamate ion from interfering with some essential enzyme system. It seems, however, impossible that their antagonistic action is due to removal of Cu from this toxic complex since even the strongest antagonists amongst the imidazoles, imidazole pyruvic acid and histidine, have a lower Cu-binding ability than dimethyl dithiocarbamate itself[10]. The weaker antagonists even showed still lower Cu-binding ability. It was, moreover, shown above that even high concentrations of histidine and imidazole pyruvic acid appeared unable to

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remove Cu from the 1:1 complex of dimethyl dithiocarbamate. GOKSØYR[5] assumed that the overall stability constant log K of Cu and NaDDC was lower than that of Cu and histidine and for this reason he ascribed the antagonistic action of histidine to removal of Cu from the 1:1 complex. Furthermore, he did not make sufficient allowance for the influence of pH on the metal-binding ability of histidine and derivatives. We are inclined to believe that the imidazoles act by competing with the enzyme for the toxic 1:1 complex, though we cannot rule out the possibility that they inactivate this complex in the medium by formation of a DDC-Cu-Imidazole compound unable to enter the cells.

By reason of its structure EDTA cannot be bound to the 1:1 complex. Therefore, it can only show antagonistic activity in concentrations in which it can withdraw the Cu from the CuDDC complex. In this way we can explain that the antagonistic action of EDTA is inferior to that of imidazole pyruvic acid, in spite of the fact that its metal-binding ability is greater.

Though there is now ample evidence that the first zone of growth inhibition is due to the fact that an essential enzyme is disturbed in its action because it combines with the 1:1 complex, the identity of this enzyme remains unknown. GOKSØYR found that growth-inhibiting concentrations also considerably inhibited the oxidation of added acetate. He remarks, however, that this process is non-essential and that, therefore, its inhibition could not be the cause of growth inhibition. The enzyme inhibition leading to a slight accumulation of pyruvic acid in our experiments presumably does not concern a vital process either.

In an earlier paper we reported that in liquid medium NaDDC causes mycelium and spores of *A. niger* to accumulate pyruvic acid. This medium was essentially free from heavy metals, suggesting that heavy metals are not required for pyruvic acid accumulation. It has now been shown that pyruvic acid accumulation proceeds also in a NaDDC-containing medium in the presence of Cu. In the latter case one peak coincides with the presence of free dithiocarbamate and a second, smaller peak with the presence of the 1:1 complex.

The view expressed in a previous paper that pyruvic acid accumulation might play an important role in bringing about inversion growth in *A. niger* because pyruvic acid is an antagonist in the first zone of inhibition[2], seems untenable in view of the fact that at NaDDC concentrations which lead to inversion growth no toxic agent is present.

It has been reported[6] that the nature of the N-source has some influence on the appearance of inversion growth and on pyruvic acid accumulation. This subject will be treated more extensively in a further communication.

Thus we finally arrive at the following picture of the action of NaDDC on *A. niger* in liquid medium. In a glucose mineral salts medium containing less than about 5 p.p.m. of $CuSO_4$ growth is continuous up to about 20 p.p.m. NaDDC. Larger concentrations of NaDDC are inhibiting, owing to the presence of free dithiocarbamate ions. In the presence of *ca.* 10 p.p.m. of $CuSO_4$, however, the situation is different. At concentrations of about 0.5 to 5 p.p.m. of NaDDC the amount of toxic 1:1 complex formed is sufficient to inhibit growth. Very small amounts of pyruvic acid seem to be accumulated in the presence of this complex. At higher NaDDC concentrations the 1:1 complex is turned into the insoluble 1:2 complex ($CuDDC_2$); this complex causes neither growth inhibition (zone of inversion growth) nor accumulation of pyruvic

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acid. At further increase of the NaDDC concentration free dithiocarbamate appears because all Cu has been bound. It causes pyruvic acid accumulation as in the metal-free medium. Finally the concentration of free dithiocarbamate becomes growth-inhibitory (second zone of inhibition). At approximately this point pyruvic acid accumulation starts to decline.

Thus we may conclude that the toxic agent in the first zone of inhibition is an unsaturated complex of metal and dithiocarbamate, whereas in the second zone of inhibition the free dithiocarbamate acts as such. The zone of inversion growth is the intermediate zone where neither of these compounds is present in toxic concentration.

SUMMARY

1. The role of metals and chelating agents in the fungitoxic action of sodium dimethyldithiocarbamate (NaDDC) has been studied using *Aspergillus niger* as a test mould.

2. In liquid glucose mineral salts medium containing only traces of heavy metals NaDDC does not produce a "first zone of inhibition". Addition of Cu or Co to these media does, however induce this zone. Fe, Zn and Mn are unable to bring about the same effect.

3. On agar media the amount of Cu required to induce a first zone of inhibition for *A. niger* has been found to be far less than in liquid media.

4. Comparison of spectrophotometric measurements and growth results in liquid medium has revealed that the "first zone of inhibition" coincides with the presence of the 1:1 complex of copper and dithiocarbamate.

The zone of inversion growth emerges at higher NaDDC concentrations when the 1:2 complex is present instead of the toxic 1:1 complex. These results support those of GOKSØYR found for *Saccharomyces cerevisiae*.

The second zone of inhibition is related to the presence of free dithiocarbamate.

5. The toxic action of the 1:1 complex must be ascribed to combination with an essential enzyme.

6. The antagonistic activity of imidazole derivatives cannot be due to removal of Cu from the 1:1 complex. These compounds are, however, supposed to protect an essential enzyme by competing with it for the toxic 1:1 complex.

7. The free dithiocarbamate induces the mould to accumulate pyruvic acid; the 1:1 complex seems to do so also, but to a far lesser extent.

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