INVESTIGATIONS ON ORGANIC FUNGICIDES

X. PYRUVIC ACID ACCUMULATION AND ITS RELATION TO THE PHENOMENON OF INVERSION GROWTH AS EFFECTED BY SODIUM DIMETHYLDITHIOCARBAMATE*

by

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In previous papers of this series[1,2] a description was given of the peculiar growth response which arises when spores of *Penicillium italicum* and especially of *Aspergillus niger* are sown on glucose agar containing various concentrations of sodium dimethyldithiocarbamate (NaDDC). This growth response is characterized by a first zone of growth inhibition at low concentrations of NaDDC, followed at higher NaDDC concentrations by a zone of somewhat delayed growth and at further increase of NaDDC concentration by a second zone of inhibition (Table I). This phenomenon of inversion of the toxicity of dithiocarbamates, leading to a zone of "inversion growth", obviously corresponds to that described for spore-germination tests with various dithiocarbamates (DIMOND, HORSFALL, HEUBERGER AND STODDARD[3]; MONTGOMERY AND SHAW[4]).

In the first zone of inhibition (*cf.* Table I) the action of NaDDC on spore germination of 4 moulds tested could be completely antagonized by the addition of histidine or certain other imidazole derivatives to the glucose-mineral salts medium[1,2]. Imidazole pyruvic acid proved to be ten times as active as histidine. For *A. niger*, moreover, also α-keto acids were found to act as antagonists; an action not noticed for *P. italicum* and the other two test moulds. Mycelial growth is only slightly affected by these concentrations of NaDDC[1].

In the second zone of inhibition the antagonists mentioned are all completely ineffective which suggests that at these high NaDDC concentrations another effect of NaDDC is superimposed on the first effect, with the result that the addition of the mentioned antagonists alone is insufficient to restore growth. This is in accordance with the general experience that with increase of the concentration of a toxic agent gradually more enzyme systems become inhibited.

Neither for the occurrence of the peculiar zone of inversion growth nor for the anomality in spore germination tests has a satisfactory explanation in our opinion been offered as yet. It seemed, however, possible to us that inversion growth might quite generally be due to the accumulation of an antagonist of the fungicide caused by an action of certain concentrations of the fungicide itself on mould metabolism[2,5].

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This action of NaDDC on the mould should itself be harmless and should result in the accumulation of a substance capable of antagonizing the first effect of NaDDC. The necessity for accumulation of a compound would also explain the fact that inversion growth becomes visible with some delay.

The three effects mentioned can be tentatively pictured as shown in Table I.

TABLE I

			First zone of inhibition				*Zone of inversion growth*			*Second zone of inhibition*		
NaDDC-concentration	0	0.2	0.5	1	2	5	10	20	50	100	200	p.p.m.
Growth response (4 days)	+	+	—	—	—	—	+	+	—	—	—	
First effect of NaDDC (inhibition of spore germination; antagonized by certain imidazole derivatives and in the case of *A. niger* by α-keto acids			———	———	———	———	———	———	———	———	——→	
Second effect of NaDDC (accumulation of antagonist of first effect)							———	———	———	———	——→	
Third effect of NaDDC (inhibiting effect on spore germination and mycelium growth; not antagonized by imidazoles *etc.*)									———	———	——→	

In order to check this hypothesis we had to search for an antagonist in the culture medium of moulds exposed to such NaDDC concentrations as lead to inversion growth.

Most experiments were carried out with *A. niger*, since this organism shows very pronounced inversion growth. Since α-keto acids are effective antagonists for this mould it was first investigated whether such compounds possibly accumulate in the medium.

This proved indeed to be the case. Pyruvic acid was found to accumulate at those NaDDC concentrations that lead to inversion growth, but not, or only in small amounts, at lower concentrations of NaDDC. This result suggests that pyruvic acid may play a role as a causative agent of inversion growth, at least for *A. niger*[5].

The phenomenon of inversion growth exhibited by *A. niger* and other moulds is discussed in view of these new findings.

MATERIALS AND METHODS

Shake cultures

Small mycelial spheres of *Aspergillus niger* and *Penicillium italicum* were grown in flasks containing the following medium: glucose 1 or 2 %, K_2HPO_4 0.5 %, $(NH_4)_2SO_4$ 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, NaCl 0.05 %, tap water. pH 6.8; biotine (0.002 μg/ml) and aneurine (0.2 μg/ml) were added to allow optimal growth. After 2 or 3 days incubation at 24° in a shaking machine the spheres were washed several times in a measuring cylinder with a 0.5 % phosphate buffer of pH 7.0 for *A. niger* and pH 6.7 for *P. italicum*. Of the washed suspension thus obtained 10 ml were pipetted into 150 ml flasks containing 10 ml of a glucose mineral salt solution to give together 20 ml medium of the composition mentioned above. The amount of mycelium was taken to be *ca.* 5–12 mg dry weight per flask and the glucose used was always sterilized by Seitz filtration. Different amounts of NaDDC were added to the flasks and these were subsequently shaken at 24° for a few hours.

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Then the liquid was filtered off, acidified with 4 N H_2SO_4 to pH 1 and stored in the refrigerator until used for estimation of α-keto acids.

Some experiments were carried out with shake cultures of fungal spores. These were obtained from cultures of *A. niger* and *P. italicum* grown on glucose mineral salts agar for 4–10 days. The spores were washed from the plates with a sterile salt solution (see above) and filtered through cotton wool. The spore suspension was then diluted with the salt solution to obtain the required concentration of spores. Finally 1 % glucose and the growth factors were added. 20 ml portions of the spore suspension were pipetted into sterile 150 ml flasks and different amounts of NaDDC were added. The flasks were then shaken at 24° for a few hours and further treated as mentioned above.

Agar cultures

The mould spores were suspended in glucose mineral salts agar medium of the same composition as the nutrient solution mentioned above. For *A. niger* the agar was adjusted to pH 7.0 and seeded with 4,000 spores per ml medium.

For *P. italicum* the pH was 6.8 and a tenfold inoculum was used.

NaDDC concentrations employed were 0, 0.05; 0.1; 0.2; 0.5 p.p.m. *etc.*

Identification and estimation of α-keto acids

Paper chromatograms were made according to CAVALLINI, FRONTALI AND TOSCHI[6] to examine the filtrates for the presence of α-keto acids. Quantitative estimations of pyruvic acid were made by the spectrophotometric method of FRIEDEMANN AND HAUGEN[7].

Determination of glucose content

Glucose was determined by HASSIDS modification[8] of the method of HAGENDORN AND JENSEN.

RESULTS

Accumulation of pyruvic acid by A. niger in the presence of NaDDC

As it was supposed that α-keto acid production, if occurring, would only be small, we used mycelial spheres rather than spores in the preliminary experiments. Washed mycelial spheres of *A. niger* were shaken for 4 hours in flasks with nutrient medium containing in addition different amounts of NaDDC (0; 0.1–100 p.p.m.), growth being negligible. Paper chromatograms were made of the liquid to check if any α-keto acids had been produced. An α-keto acid spot was detected at several of the NaDDC concentrations used and this compound could be identified as pyruvic acid. No other α-keto acids appeared to be present.

A more quantitative assay was then made in a similar experiment in which the amount of pyruvic acid produced was estimated spectrophotometrically*.

The results (Fig. 1A) reveal that there is a definite relation between NaDDC concentration applied and the amount of pyruvic caid accumulated.

Moreover, comparison with growth

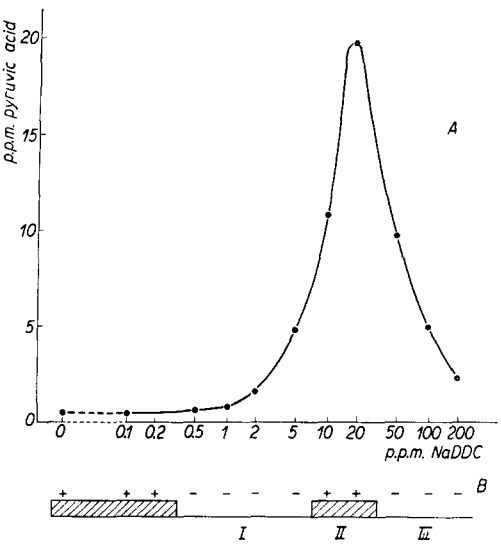

Fig. 1A. Pyruvic acid accumulation by *A. niger* in glucose mineral salt solution containing various concentrations of NaDDC. Mycelial spheres (dry wt 8 mg); 4 h; 20 ml medium pH 7.0. Fig. 1B. Growth response of spores of *A. niger* on glucose mineral salts agar with various concentrations of NaDDC. I = First zone of inhibition; II = Zone of inversion growth; III = Second zone of inhibition.

* We gratefully ackowledge the assistance of Mr H. C. VAN Os who carried out chromatographic and spectrophotometric estimations.

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experiments on agar shows (Fig. 1B) that pyruvic acid accumulation is inconspicuous at those concentrations of NaDDC which in growth experiments are not yet harmful or lead to the first zone of inhibition. A distinct accumulation is perceptible, however, just about at those concentrations which in growth experiments on agar give rise to inversion growth.

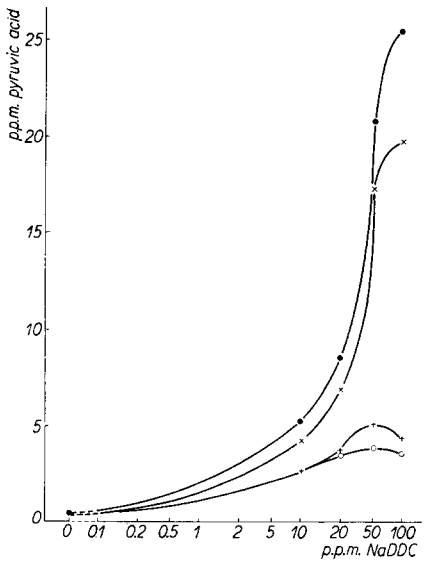

Fig. 2. Pyruvic acid accumulation by *A. niger* in glucose mineral salts solution containing various concentrations of NaDDC. Effect of composition of medium. Mycelial spheres (dry wt 16 mg); 3 hr; 20 ml medium pH 7.0. ● = K_2HPO_4 (0.5%) + $(NH_4)_2SO_4$ (0.1%) + $MgSO_4 \cdot 7\,H_2O$ (0.05%) + NaCl (0.05%). × = K_2HPO_4 (0.5%) + $(NH_4)_2SO_4$ (0.1%); + = K_2HPO_4 (0.5%) + $MgSO_4 \cdot 7\,H_2O$ (0.05%) + NaCl (0.05%); ○ = K_2HPO_4 (0.5%).

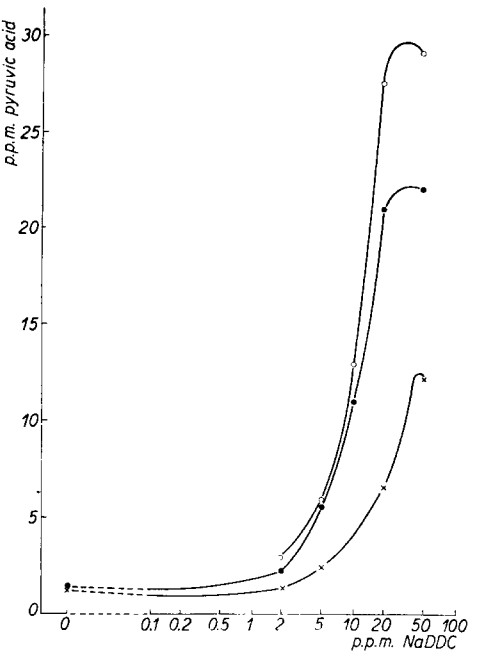

Fig. 3. Pyruvic acid accumulation by *A. niger* in glucose mineral salts solution containing diverse concentrations of NaDDC. Effect of concentration of $(NH_4)_2SO_4$. Mycelial spheres (dry wt 11 mg); 4 h; 20 ml medium pH 7.0. ○ = 0.3% $(NH_4)_2SO_4$; ● = 0.1% $(NH_4)_2SO_4$; × 0.01% $(NH_4)_2SO_4$.

When a similar experiment with mycelial spheres was put up in phosphate buffer (0.5%) + glucose, instead of in the complete nutrient medium, pyruvic acid accumulation was found to be almost negligible. This appeared to be largely due to the lack of $(NH_4)_2SO_4$ as was shown by the results of an experiment in which the salts were omitted in turn (Fig. 2). Fig. 3 shows, moreover, that the rate of pyruvic acid

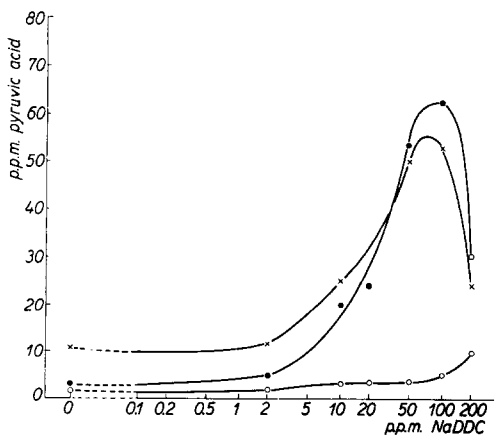

Fig. 4. Pyruvic acid accumulation by *A. niger* in glucose mineral salts solution containing diverse concentrations of NaDDC. Effect of N-source. Mycelial spheres (dry wt 11 mg); 3 h; 20 ml medium pH 7.0. ● = 0.1% $(NH_4)_2SO_4$; ○ = 0.15% KNO_3; × = 0.1% $(NH_4)_2SO_4$ + 0.15% KNO_3.

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accumulation is dependent on the ammonium sulphate concentration. NH_4Cl proved to be about equally active as $(NH_4)_2SO_4$ when the same N-content was used. KNO_3 did not stimulate pyruvic acid accumulation as was shown by an experiment in which media were compared which contained either KNO_3 or $(NH_4)_2SO_4$ in amounts with corresponding N-contents (Fig. 4). The mixture of the two salts gave about the same picture as $(NH_4)_2SO_4$ alone. The final pH was 6.5 irrespective of the N-source used in these experiments.

Thus one may conclude that NH_4-ions are required for pyruvic acid accumulation caused by NaDDC.

Glucose consumption was determined in several experiments. It appeared not to be influenced by the NaDDC concentrations used in the present investigations. In the experiment shown in Fig. 1A glucose consumption was *ca.* 30 mg per flask.

Pyruvic acid accumulation by spores of A. niger

We have now seen that the concentrations of NaDDC which in growth experiments with spores give rise to inversion growth, correspond with those which induce mycelial spheres to accumulate the antagonist pyruvic acid; at lower concentrations of the fungicide spore germination is inhibited and mycelial spheres accumulate little or no pyruvic acid. Though it seems tempting to relate inversion growth to accumulation of this antagonist of NaDDC, we know from earlier experiments that low concentrations of NaDDC inhibit especially spore germination (first zone of inhibition) while mycelial growth is only slightly affected by these concentrations. Thus, pyruvic acid accumulation can only be considered as a cause of inversion growth if it can be proved that the spores before their germination accumulate pyruvic acid in sufficient amounts to antagonize NaDDC.

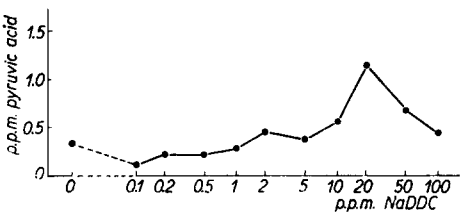

Fig. 5. Pyruvic acid accumulation by spores of *A. niger* in glucose mineral salts solution containing diverse concentrations of NaDDC. 400,000 spores/ml; 6 h in 20 ml medium pH 7.0; $(NH_4)_2SO_4$ content 0.5%

To gain information on this point spores of *A. niger* were shaken in the glucose mineral salts medium ($(NH_4)_2SO_4$ content 0.5%) to which various concentrations of NaDDC had been added. The concentration of spores was taken 100 × larger than normally used in agar cultures. After 6 h the spores were neither swollen nor had they germinated. Yet, pyruvic acid accumulation was undeniable even at this stage, with a peak occurring at *ca.* 20 p.p.m. of NaDDC (Fig. 5). This result could be confirmed in later experiments. The amount of pyruvic acid accumulated is certainly small in comparison with the amount which must be added to antagonize NaDDC in the first zone of inhibition (10–30 p.p.m.). One must, however, realize that in the first case the concentration in the medium is caused by excretion by the spores and, therefore, will undoubtedly be significantly lower than the concentration inside the spores.

Effect of N-source on occurrence of zone of inversion growth

As we were looking for a correlation between antagonist accumulation and occurrence of inversion growth, it was also of interest to study the effect of the N-source on inversion growth. It was then found that 0.5% $(NH_4)_2SO_4$ in the medium gives a

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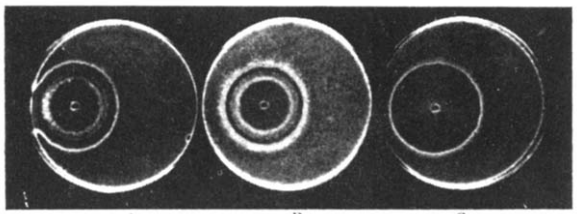

Fig. 6. *Aspergillus niger* on glucose mineral salts agar with A = 0.1 % $(NH_4)_2SO_4$; B = 0.5 % $(NH_4)_2SO_4$; C = 0.2 % KNO_3. The cup contains 0.1 % NaDDC. A and B show around the cup the second zone of inhibition, the zone of inversion growth and the first zone of inhibition (black ring). C does not show a zone of inversion growth, but one large zone of inhibition.

more pronounced and more extended zone of inversion growth (5, 10, 20 p.p.m.) than 0.1 % of this salt. When, however, KNO_3 (0.2 %) was substituted for $(NH_4)_2SO_4$ inversion growth was only very faint or absent, though on control plates not containing NaDDC *A. niger* grew equally well with KNO_3 or with $(NH_4)_2SO_4$. The same effect of the N-source on inversion growth was obtained when *A. niger* spores were sown on various media on which a cup had been placed with 1.000 p.p.m. of NaDDC (Fig. 6).

Thus there appears to be a very close correlation between the factors leading to occurrence of inversion growth and to accumulation of pyruvic acid, an antagonist of NaDDC in the first zone of inhibition for *A. niger*. In fact the conclusion is almost inescapable that pyruvic acid accumulation plays a role in establishing inversion growth for this mould.

Meanwhile, as already pointed out above, the behaviour of *A. niger* towards NaDDC is exceptional both in its very pronounced inversion growth and in the fact that pyruvic acid is an antagonist.

For this reason some of the experiments described above were repeated with *P. italicum* as a test mould with little pronounced inversion growth and lacking the ability to use pyruvic acid as an antagonist for NaDDC.

Experiments with Penicillium italicum

a. *Pyruvic acid accumulation by* P. italicum. Experiments were carried out in the same way as with *A. niger*, with the exception, however, that the pH value of the medium was adjusted to 6.7. Here, too, there is a decided accumulation of pyruvic acid by mycelial spheres (Fig. 7) but this seemed to proceed far slower than with the former mould. After 6 hours shaking hardly any pyruvic acid is present, but after 24 hours the accumulation is quite considerable at a certain range of NaDDC concentrations. The top usually is at a lower NaDDC-concentration than with *A. niger*. It is noteworthy that inversion growth too occurs at a somewhat lower concentration of NaDDC for *P. italicum* than for *A. niger* (5 and 10

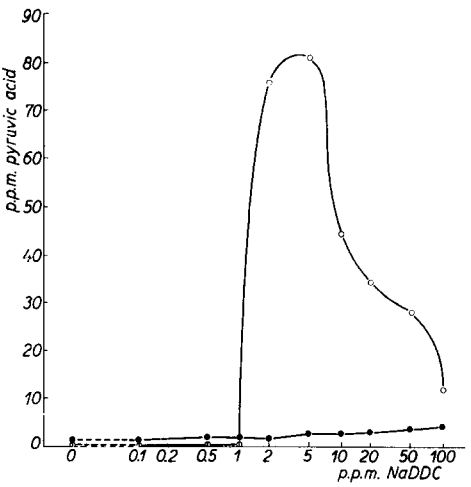

Fig. 7. Pyruvic acid accumulation by *Penicillium italicum* in glucose mineral salts solution containing diverse concentrations of NaDDC. Mycelial spheres (dry wt 10 mg); 20 ml medium pH 6.7 containing aneurine and biotine. ● = incubation period 6 h; ○ = incubation period 24 h.

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p.p.m. instead of 10 and 20 p.p.m.). Also the spores of this mould accumulate pyruvic acid.

Accumulation was found to increase with increasing ammonium sulphate concentration of the medium.

b. *Influence of N-source on occurrence of inversion growth.* Experiments with solid media showed that the degree of inversion growth depends on the composition of the N-source used in the same way as with *A. niger*.

DISCUSSION

A survey of the observations dealt with above, leads to the following picture.

In liquid medium pyruvic acid is accumulated by spores as well as by mycelium of both test moulds and this accumulation reaches a peak just about at those NaDDC concentrations that in agar media lead to inversion growth. Both pyruvic acid accumulation and inversion growth are favoured by high NH_4-concentrations.

Assuming that the results obtained on both media are directly comparable, there seems little doubt that in the exceptional case of *A. niger*, for which organism pyruvic acid is an antagonist of NaDDC, the accumulation of this compound plays a role in establishing inversion growth. For *P. italicum*, however, the situation is different. Pyruvic acid, though it is also accumulated, is not an antagonist of NaDDC as we have seen. Therefore, pyruvic acid accumulation cannot play a direct role in the establishment of inversion growth.

The fact that mycelial growth is not sensitive to NaDDC in the first zone of inhibition is supposed to be due to a more rapid accumulation of the antagonist by mycelium than by spores.

Though the cause of inversion growth has not been fully elucidated by the observations described above, they have made clear that the metabolism of the mould does play a definite role in the establishment of inversion growth. A purely physico-chemical explanation has been given recently in a preliminary communication by GOKSØYR[9]. This author suggests that the inversion phenomenon is due to the fact that at concentrations of NaDDC leading to inversion growth the dithiocarbamate ion combines with a metal ion to an insoluble 1:2 complex. Lower NaDDC concentrations give rise to an 1:1 complex which is supposed to give strong growth inhibition.

Though in the presence of metals metal complexes will certainly be formed*, we feel that, within the scope of our present paper, the following facts are not reconcilable with a purely physico-chemical explanation of the inversion phenomenon, but show that mould metabolism plays a definite role in the establishment of inversion growth.

1. NH_4-ions are required for inversion growth.

2. There are many moulds that do not show inversion growth, as for instance *Botrytis allii*[2].

3. Inversion growth appears with a delay of 1–2 days.

4. At NaDDC concentrations leading to inversion growth the dithiocarbamate ion cannot be present as an insoluble and hence inactive complex, since at these concentrations the same anget causes inhibition of an enzyme, with the result that pyruvic acid accumulates.

A few remarks may still be made about the accumulation of pyruvic acid. We

* The role of metal chelates in the action of NaDDC will be the subject of a future paper.

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have to deal with an enzyme inhibition (leading to pyruvic acid accumulation) which does not seem to be harmful for the organisms but which quite to the contrary counteracts the primary inhibition seen at low NaDDC concentration. Assuming that pyruvic acid accumulation results from an inhibition of the pyruvic oxydase system, a vital system is concerned. From a comparison of glucose consumption and maximal pyruvic acid accumulation by *A. niger* we can, however, calculate that on a molecular base the latter did not exceed 3% of the former if carried out in a medium with 0.1% $(NH_4)_2SO_4$ and usually is even lower. Thus it can be understood that this inhibition is not harmful and also that Warburg experiments did not reveal a decrease of O_2-consumption at this level of NaDDC[10].

The peculiar drop of pyruvic acid accumulation with increase of NaDDC concentration is not properly understood at present. Unpublished experiments with other mould species have, however, revealed that in many instances the rate of pyruvate accumulation increases regularly with increasing NaDDC concentration.

With regard to the role of NH_4-ions in pyruvic acid accumulation it seems noteworthy that we have found that the inhibition of the pyruvic oxidase system caused by K-arsenite is also favoured by a high NH_4-ion concentration, although there is a marked accumulation in the absence of ammonium ions as well (unpublished results). These experiments were carried out with mycelial spheres of *A. niger*.

Though not proved by the present experiments it seems likely that the accumulation of pyruvic acid caused by NaDDC is due to interference with the pyruvic oxidase system. Little is known at present about the enzymes and coenzymes that play a role in the oxidative decomposition of pyruvic acid by moulds. If the same coenzymes operate as in animal tissues and in certain bacteria, one might think of interference with α-lipoic acid or with coenzyme A.

The closely related compound sodium diethyldithiocarbamate which is the reduced form of the well-known "antabuse" (T.E.T.D.) was found by us to give the very same pyruvic acid accumulation as NaDDC. It seems possible that this phenomenon plays a role in the not yet completely understood reaction to ethyl alcohol of patients treated with antabuse.

SUMMARY

1. Sodium dimethyldithiocarbamate (NaDDC) induces spores and mycelium of *A. niger* and *P. italicum* to accumulate pyruvic acid in the medium. This accumulation shows a maximum at those NaDDC concentrations which in growth experiments on agar lead to the zone of inversion growth, and decreases rapidly at higher concentrations.

2. With mycelial spheres this phenomenon can be studied more easily than with spores.

3. There is a close correlation between factors leading to pyruvic acid accumulation and to inversion growth in so far as NH_4-ions are required for both.

4. Pyruvic acid accumulation is supposed to play an important role in bringing about inversion growth in *A. niger*, because α-keto acids are strong antagonists of NaDDC for this mould. Thus inversion growth would occur because the fungicide induces the mould spores to accumulate an antagonist.

5. Various arguments are put forward to show that mould metabolism plays a definite role in the establishment of inversion growth and that a purely physico-chemical explanation of this phenomenon is improbable.

6. It is suggested that pyruvic acid accumulation by NaDDC is due to interference of this fungicide with one of the coenzymes functioning in pyruvate oxidation.

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RÉSUMÉ

1. Le diméthyldithiocarbamate de sodium (NaDDC) induit les spores et le mycélium d'*Aspergillus niger* et de *Penicillium italicum* à accumuler dans le milieu l'acide pyruvique. Cette accumulation atteint un maximum aux concentrations de NaDDC qui dans les expériences de croissance donnent lieu à la zone d'"inversion growth"; elle décroît rapidement aux concentrations plus hautes.

2. Ce phénomène peut être étudié plus facilement avec des sphères mycéliques qu'en utilisant des spores.

3. Une forte corrélation existe entre les facteurs favorisant l'accumulation d'acide pyruvique et l'"inversion growth" en tant que des ions NH_4 sont essentiels pour tous les deux.

4. Il est supposé que l'accumulation d'acide pyruvique joue un rôle important dans la provocation de l'"inversion growth" chez *A. niger*, parce que les acides α-cétoniques sont de forts antagonistes de NaDDC pour cette moisissure. L'"inversion growth" aurait donc lieu parce que le fongicide induit les spores à accumuler un antagoniste.

5. Divers arguments sont avancés pour démontrer que le métabolisme des moisissures joue une rôle définitif dans l'origine de l'"inversion growth" et qu'il n'est pas possible de donner une explication complètement physico-chimique de ce phénomène.

6. Il est suggéré que l'accumulation d'acide pyruvique est causée par l'interférence de NaDDC avec un des coenzymes impliqués dans l'oxydation de cet acide.

ZUSAMMENFASSUNG

1. Natriumdimethyldithiocarbamat (NaDDC) veranlasst Sporen und Myzelium von *Aspergillus niger* und *Penicillium italicum* zur Anhäufung von Brenztraubensäure im Medium. Diese Anhäufung erreicht ein Maximum bei den NaDDC-Konzentrationen, die in Wachtumsversuchen zu der Zone von "Inversionswachstum" Anlass geben. Bei höheren NaDDC-Konzentrationen fällt die Brenztraubensäureanhäufung rasch ab.

2. Dieses Phänomen kann leichter an Myzelsuspensionen als an Sporen studiert werden.

3. Es wurde eine enge Korrelation gefunden zwischen Faktoren, die zur Brenztraubensäureanhäufung führen und Inversionswachstum hervorrufen insofern NH_4-Ionen für beide nötig sind

4. Die Brenztraubensäureanhäufung spielt vermutlich eine wichtige Rolle bei der Verursachung von Inversionswachstum bei *A. niger*, weil α-Ketosäuren für diesen Pilz sehr kräftige NaDDC-Antagonisten sind. Inversionswachstum würde somit auftreten weil das Fungizid die Pilzsporen anregt einen Antagonist anzuhäufen.

5. Es werden verschiedene Gründe angeführt um zu zeigen, dass der Pilzstoffwechsel eine entscheidende Rolle in der Verursachung von Inversionswachstum spielt und dass eine rein physico-chemische Erklärung dieses Phänomens weniger wahrscheinlich ist.

6. Die von NaDDC hervorgerufene Brenztraubensäureanhäufung beruht vermutlich auf einer Störung der Funktion eines der Koenzyme, die an der Brenztraubensäureoxydation beteiligt sind.

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