Inhibition of *Aspergillus niger* Growth by *m*-Dinitrobenzene and its Reversal by Amino Acids.* (24408)

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The toxic effect of 2,4-dinitrophenol on living systems appears to result at least in part from uncoupling of oxidative phosphorylation with consequential depression of energy utilization for biosynthetic reactions. However, the mechanism of toxicity has not been elucidated. It has recently been reported that growth inhibiting effect of 2,4-dinitrophenol on *Escherichia coli* could be counteracted by an amino acid mixture similar in composition to that obtained from an acid hydrolyzate of cow's liver(1). It was suggested that this antagonism was similar to that existing between a protein-derived "antithyrotoxic factor of liver" and the thyroid stress produced by 2,4-dinitrophenol, desiccated thyroid powder, or iodinated protein. In the present study on effect of various organic nitro compounds upon microorganisms we have found that *m*-dinitrobenzene (DNB) is an effective growth inhibitor of molds and that this inhibition can be reversed by various amino acid mixtures. Further, we found that under appropriate conditions nitroaryl compounds may be reduced by *Aspergillus niger* to corresponding arylamines which appear to be relatively nontoxic to the mold. Certain properties and requirements of the reductive enzyme system are described.

Methods. For growth studies, surface cultures of *A. niger* (Mulder strain)(2) were grown in 250 or 500 ml Erlenmeyer flasks containing 50 ml of a medium composed of (per liter): 50 g glucose, 2.65 g NH_4Cl, 2.5 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeCl_3 \cdot 6H_2O$, 20 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnSO_4 \cdot 4H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, and HCl to pH 2. After inoculating with *A. niger* spores and incubating at 27-30°C for 5 days, the mycelial felts were harvested, washed, dried at 80°C overnight and weighed. *In vitro* studies on enzymic reduction of nitro compounds by *A. niger* preparations were conducted as described previously(3). The side arm of the Thunberg tube contained 0.5 μmole reduced diphosphopyridine nucleotide (DPNH) and the body of the tube contained 1.5 μmoles of nitroaryl substrate, 0.1 mmole tris(hydroxymethyl)aminomethane buffer, pH 7.8 and 0.5 ml enzyme in final volume of 2.5 ml. Reaction time was 30 minutes at 37.5°C.

Results. A series of 25 compounds representing nitrobenzoic acids, nitrophenols, nitroanilines, nitrobenzimidazoles, nitro analogs of pyrimidines, aminobenzoic acids, *p*-nitrobenzenesulfonamide, and DNB were all tested for their toxicity against microorganisms. The sensitivities of 14 common bacteria, yeasts and molds to nitro compounds were assayed by means of the disc type sensitivity test on Trypticase soy agar. Only DNB was effective, and this only on molds (3 strains of *Aspergillus* and unidentified strains of *Penicillium, Alternaria* and *Hormodendron*). There was no growth inhibition of any of the 6 strains of bacteria (*Aerobacter aerogenes, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and the spore former *Bacillus globigii*). Likewise, there was no inhibition of yeasts, represented by unidentified species of *Saccharomyces* and *Oidium*. Thus inhibition by DNB is apparently selective and may be restricted to molds. All subsequent studies were conducted with *A. niger* as test organism.

Growth of *A. niger* in liquid culture medium was prevented completely by concentrations of DNB as low as 2.5×10^{-5} M, but at levels of 2.5×10^{-6} M or lower, mycelial dry weights matched those of controls. 2,4-Dinitrophenol was about as effective as DNB as a growth inhibitor in the liquid medium. At levels as high as 2.5×10^{-3} M only the nitrobenzoic acids, *m*-nitroaniline, and 2,6-dinitrophenol showed inhibitions of the order of 90% or more. All other compounds tested

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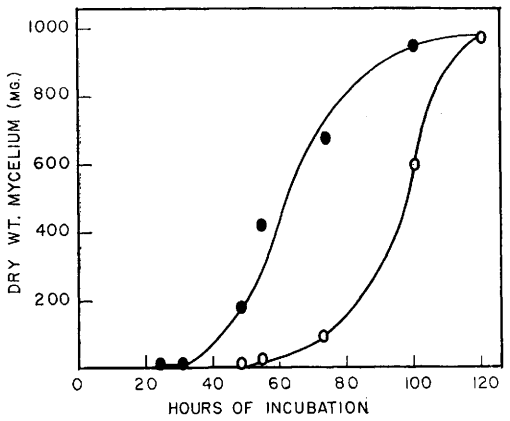

FIG. 1. Growth curves of *A. niger*. ○, Growth normally occurring in DNB-free cultures at hours indicated. ●, Growth obtained after 120 hr when DNB (2.5×10^{-5} M) is added to growing cultures at hours indicated.

were without effect at this latter level.

Although growth was prevented by 2.5×10^{-5} M DNB in the culture medium, if DNB was added at any time after 30 hours' incubation, inhibition was not complete and further growth occurred (Fig. 1). The later DNB was added in the growth period, the less effective it was as an inhibitor. Occasional mycelial growths of about 1-2 mm size which developed when DNB was added before 30 hours, were viable and grew out normally when transferred to sterile DNB-free medium. Thus DNB has static rather than cidal properties. Preparations from *A. niger* are capable of reducing organic nitro compounds to the corresponding amines(3) and a similar reduction appeared to occur *in vivo*. When DNB was added to growing cultures after 30 hours, up to 35% of the administered dose could be measured as the nontoxic *m*-nitroaniline by diazotization and coupling assay (4).

Enzymic reduction of nitroaryl compounds to arylamines might represent a method of detoxication for the mold and therefore the nature of the reducing system was investigated further. *A. niger* was grown and a K_2HPO_4 extract of the mycelium was prepared as described previously(3) except that NH_4Cl (2.65 g/l) was substituted as the sole nitrogen source in the culture medium. The $20,000 \times g$ supernatant of this extract was subjected to ammonium sulfate fractionation, and the protein collected between 30 and 60% saturation with ammonium sulfate was dialyzed against 0.1 M $KHCO_3$ which contained 10^{-3} M cysteine. This preparation represented 30% of the DNB-reducing activity of the original homogenate at a 3-fold purification (based on protein). About 2% of activity was lost after 2 weeks at −20°C. Inactivation was rapid at 57°C.

Rate of DNB reduction at the pH optimum of 7.8 was linear over the first 30 minutes with protein concentrations up to 10 mg/Thunberg tube. The 2 pyridine nucleotides were equally effective as electron donors but neither succinate, xanthine, cysteine nor glutathione would serve in this capacity.

The 12 to 85% inhibition of DNB reduction produced by 5×10^{-6} to 5×10^{-4} M *p*-chloromercuribenzoate was reversed completely by 10^{-3} M cysteine. Cysteine, glutathione, diethylaminoethanethiol and thiomalic acid all increased the rate of nitro reduction by some way other than by nonspecific chemical reduction of nitroso or hydroxylamino intermediates. The system was strongly inhibited by a series of 8 common metal binding agents and by atabrine. Although ferrous ions and flavin nucleotides were previously implicated in the reduction of nitro groups by *A. niger*(3), the purity of these enzyme preparations was inadequate to establish cofactor requirements with certainty.

Attempts were made to reverse the growth inhibition produced by DNB *in vivo* by utilizing information obtained on the nitro-reducing enzyme system. The culture medium was supplemented with: (a) cysteine and other thiols, (b) mixtures of the known vitamins, and (c) all the trace minerals suspected of being essential to *A. niger*(5). None of these factors, alone or in combination, were capable of counteracting DNB inhibition. However, it was possible to reverse the growth depression produced by various levels of DNB by adding an appropriate amount of any of several commercial casein hydrolyzates to the culture flasks (Table I). Additional hydrolysis of the commercial preparations with HCl or H_2SO_4 produced no decrement in their ability to reverse the DNB inhibition, and isonitrogenous amounts of amino acid mix-

TABLE I. Inhibition of *A. niger* Growth by DNB and Its Reversal by Casein Hydrolyzate.*

DNB content	Casein hydrolyzate added (mg)† 0	100	200	300
0	857	948	970	1071
1.0×10^{-5} M	683	911	967	1001
2.0 "	22	613	958	1045
3.0 "	0	32	653	968
4.0 "	0	0	27	316

* mg dry wt of mycelium.
† Pancreatic digest of casein, U.S.P., Difco.

tures were equally effective in this regard (Table II). In each case, the inhibition produced by DNB in the basal medium was reversed by the added amino acid mixtures (column 3). It was not necessary that the mixtures be constituted to approximate the relative amino acid composition of casein(6). Levels of DNB which essentially prevented growth in the basal medium also depressed growth somewhat in the various amino acid-containing media. Growth of *A. niger* was also prevented by DNB when KNO_3 served as the nitrogen source. (The ammonium medium supports better growth than the nitrate medium(2).) Levels of NH_4Cl as high as 20-fold that in the basal medium (the highest level tested) were incapable of affecting DNB inhibition; reversal appeared to be dependent on amino acid nitrogen.

Discussion. The nature of the DNB-reducing system in *A. niger* remains obscure. It does not involve an adaptive enzyme as is the case in reduction and utilization of inorganic nitrate, nitrite and hydroxylamine (7). Also, DNB-reducing preparations have been found to be free of pyridine nucleotide-linked cytochrome *c* reductase activity, and they show no other activity which would readily relate them to known metalloflavoproteins. It is problematical whether reduction of organic nitro groups occurs primarily as a protective mechanism or if detoxication is merely incidental to the nitroaryl compounds serving as nonspecific electron acceptors for certain pyridine nucleotide-coupled enzymes. It is an interesting coincidence that the nitro compound most readily reduced by *A. niger*—DNB—is also the most toxic *in vivo*. The observation is reminiscent of a similar relationship between *E. coli* and chloramphenicol since the nitro group of the latter compound is also readily reduced by a metalloflavoprotein from the sensitive organism(8). Several strains of bacteria, all sensitive to chloramphenicol, are able to reduce the drug to the arylamino form and thus destroy its bacteriostatic properties(9).

It seems possible that addition of DNB to *A. niger* cultures may result in interference with oxidative phosphorylations by a mechanism similar to the known action of 2,4-dinitrophenol. Hence if the inhibitor were added to new cultures growth would be prevented, but in older cultures the very low level of DNB used might only partially depress growth (Fig. 1). It could be postulated that in the older cultures amino acids are being synthesized at a rate adequate for formation of detoxifying (*i.e.* nitro-reducing) enzymes, while in the new cultures preformed amino acids are capable of counteracting the DNB effect by the same mechanism. A second explanation, based on the *in vivo* experiments, would require that DNB in some manner interferes with incorporation of ammonium nitrogen in amino acid synthesis and such a block is readily overcome by supplying

TABLE II. Effect of DNB on *A. niger* Growth in Various Media.*

Additions§		Basal medium	+DNB‡	NH_4^+-free medium†	+DNB‡
None		961	<20		
Casein hydrol.	A	1252	1096	1080	816
	B	1216	1005	1050	850
	C	1221	1009	1058	856
Amino acid mixture	I	1266	1123	1114	901
	II	1186	1155	1096	855
KNO_3				610	0

* mg dry wt of mycelium.
† Same as basal medium *(Methods)* except NH_4Cl omitted.
‡ 2.5×10^{-5} M final concentration.
§ 34.7 mg N of each. This is equivalent to N content of basal medium.

Casein hydrolyzates: A, pancreatic digest of casein, U.S.P.; B, same as A but treated with 6 N H_2SO_4 at 100°C for 8 hr. H_2SO_4 removed with $Ba(OH)_2$. Neutral solution concentrated under reduced pressure; C, same as A but treated with 6 N HCl at 100°C for 8 hr. HCl removed by vacuum distillation. Solution neutralized with KOH.

Amino acid mixtures: I, same as relative amino acid composition of casein(6); II, equal weights of all amino acids occurring in casein.

preformed amino acids. These postulations are being investigated.

Summary. 1) *A. niger* growth was prevented when DNB at a final concentration of 2.5×10^{-5} M was added to the culture medium at any time up to 30 hours' incubation. When DNB was added at any time later, some additional growth occurred. The DNB effect appeared to be limited to molds and was static rather than cidal. 2) Growth inhibition by DNB was overcome by synthetic mixtures of amino acids, but not by ammonium nitrogen, vitamins or minerals. 3) Nitroaryl compounds can be reduced to relatively nontoxic arylamines by a sulfhydryl-dependent, pyridine nucleotide-linked enzyme from *A. niger*. 4) The possible significance of these findings is discussed.

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Purification of Fungal Phenolsulfatase.* (24409)

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The female sex hormones occur in urine not only in free form but also as glucuronides and sulfates which are hydrolyzed to the free compounds before quantitative measurement. Due to production of artifacts by acid hydrolysis, milder methods of enzymatic hydrolysis have been introduced. The sulfates are hydrolyzed by phenolsulfatase which was discovered by Neuberg and Kurono(1). Distribution and properties of the enzyme have been reviewed by Fromageot(2,3). After Abbott(4) pointed out that the most active fungal source of phenolsulfatase is Mylase P,‡ Cohen and Bates(5,6) used this commercial product for hydrolysis of urinary estrogen sulfates. Subsequently Katzman and associates (7) found that this preparation would hydrolyze estrone sulfate quantitatively. Since Mylase P is a mixture of fungal enzymes and no intensive purification of fungal phenolsulfatase has been reported, we have undertaken the preparation of this enzyme from Mylase P. In these studies fractionations with ethanol, zone electrophoresis on cellulose, and treatment with aluminum hydroxide gel were used to purify the enzyme.

Materials and methods. Starting material. The same lot of Mylase P was used during these studies. Its specific activity remained unchanged upon storage for one year at 5°C. Analysis for protein (Lowry *et al.*, 8) gave 12%, reducing sugars expressed as lactose(9, 10) 42%. *Determination of phenolsulfatase.* The procedure of Huggins and Smith(11) was used for determination of the activity of the enzyme. Potassium p-nitrophenylsulfate was synthesized by a modification of the procedure of Burkhardt and Wood(12). The substrate (0.005 M) in a solution of 0.1 M sodium acetate to which a few drops of $CHCl_3$

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