Effect of Lathyrogenic Aminonitriles, Related Amines, and Copper-Complexing Agents on Conidial Germination of Molds.* (29159)

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Dasler *et al*(1,2) have postulated that diverse chemical substances inducing spinal curvatures and other lathyritic symptoms in experimental animals may exert their effects by means of formation of coordination complexes or chelates. Mager *et al*(3) have reported that low levels of copper ions are necessary for the toxic action of aminoacetonitrile on fibroblasts *in vitro*. The well-known medial lesions in aortas of lathyritic animals bear a marked resemblance to those produced by copper deficiency in chicks(4) or pigs(5).

Common molds are reported to be affected by trace elements and ion-complexing agents. *Aspergillus niger* requires Cu^{++} for sporulation(6). The germinations of *Neurospora tetrasperma* conidia and activated ascospores are inhibited by ethylenediaminetetracetic acid (0.01 M), and at least partial reversal of these inhibitions can be obtained with Cu^{++}(7). We have found conidial germination of *A. niger* to be inhibited by Beta-aminopropionitrile (BAPN)(8). Therefore, a comparison of the effect of aminonitriles and Cu^{++}-complexing agents on spore germination was undertaken using *A. niger* and *N. crassa,* a species closely related to *N. tetrasperma.*

Materials. A. niger (strain AN-6275) and *N. crassa* (strains NC-11943, histidine requiring; NC-10334, proline requiring; NC-9279, wild type) were obtained from American Type Culture Collection. BAPN acid fumarate (BAPN·Fu) was obtained from Abbott Laboratories; BAPN free base from Calbiochem; propionitrile, ethylenediamine dihydrochloride and ethylamine hydrochloride from Distillation Products, Inc. (Eastman); D-glucosamine hydrochloride, L-histidine monohydrochloride, and L-proline from Nutritional Biochemicals Corp.; imidazole from General Biochemicals; α,α'-dipyridyl, 8-hydroxyquinoline, reagent grade inorganic salts, and sucrose from Fisher Scientific Co. Aminoacetonitrile hydrochloride (AAN) was synthesized in our laboratory.

Methods. Molds were grown on *Neurospora* culture agar (Difco 0321-15) used at 2/3 strength. It was supplemented with 0.005 M histidine or 0.02 M proline when growing NC-11943 or NC-10334, respectively. Deionized water, made by passing distilled water thru a Crystalab water demineralizer, was used for preparation of all solutions.

A. niger spores were obtained from an agar slant culture grown at 26°C† for 2½ to 3 days. They were harvested by suspension in potassium phosphate buffer at concentrations ranging from 0.025 M to 0.1 M. The spore suspensions were dispersed by means of a Vortex Jr. mixer and diluted with buffer to an optical density of 0.5 at 560 mμ (Coleman Universal Spectrophotometer). After a further 1:20 dilution with spore germination media, the suspensions were inoculated onto slides (0.1 ml of suspension per slide). The slides and cover slips had been previously washed with detergent, soaked in 0.6 N HCl for at least a day, and rinsed 4 times with deionized water.

N. crassa spores were treated in a like manner except that they were filtered thru cheesecloth to remove poorly germinating macroconidia, and the initial dilutions were made to an optical density of 0.2 at 635 mμ. In order that the number of spores per slide be comparable to that of *A. niger* (ca. 15,000), two slants were used for each run.

The medium for studies of conidial germ-

* This study was supported by research grants from Nat. Inst. of Arthritis and Metab. Dis., U.S.P.H.S.

† Intermittent illumination from incandescent lamps was used to keep the incubator at 26 ± 2°C and to provide light for growth of all cultures and for spore germination studies. The *N. crassa* strains appeared to require light at some time during their growth in order for the spores to show good germination.

ination consisted of 0.5% agar (Difco 0140-01), potassium phosphate buffer (0.025 to 0.1 M as needed to control the pH), sucrose (0.01%), and the compounds under study. The medium was not sterilized but was used fresh or stored at 0 to 4°C for periods not exceeding 2 weeks. Routine examinations indicated that bacterial growth was not a problem at the incubation temperature. All molds were found to germinate satisfactorily in buffer concentrations up to 0.1 M in the absence of test compounds.

Inoculated slides were incubated at 26°C† for 20 hours over 20 ml water in 4-section, plastic petri dishes, after which they were rapidly scanned at 430 ×. The conidia in fields representative of the entire slides were counted. Only spores showing definite germ tubes were considered as germinants. Consistent results were found to be obtainable counting as few as 100 spores as indicated by Sharvelle(9), but generally 200 to 300 were counted.

Results and discussion. 1. Preliminary trials (pH 5.0 to 7.7). BAPN-Fu (0.01 M to 0.05 M, pH 6.1) inhibited conidial germination of *A. niger* when the spores had been harvested from slants over 2½ to 3 days old. It had no effect on any of the *N. crassa* strains even when the spores were obtained from slants up to 5 days old. Identical molar concentrations of potassium acid fumarate either had no effect or were stimulatory to *A. niger* conidia.

BAPN (0.001 M to 0.1 M) had no effect on *A. niger* conidial germination except at the higher concentrations (above 0.01 M) and at more alkaline pH levels (above pH 7.4) when the spores had been harvested from slants grown for 2½ to 3 days. At pH 7.7, 0.05 M BAPN was clearly inhibitory to both *A. niger* and *N. crassa*; 0.05 M AAN appeared to be inhibitory at pH 7 or higher.

Cupric chloride (1.5×10^{-4} M Cu^{++}) tended to inhibit germination of *A. niger* conidia, but the severity of inhibition dropped and Cu^{++} seemed to precipitate out of the media as the pH was increased. At pH 7.7, even 1×10^{-3} M Cu^{++} did not inhibit *A. niger* or *N. crassa* conidial germination. However, both concentrations of Cu^{++} appeared to potentiate the inhibition due to 0.05 M BAPN at this pH.

2. BAPN ± equimolar Cu^{++} vs certain ion-complexing agents ± equimolar Cu^{++} (Table I). Conidial germination of *N. crassa* was affected similarly by BAPN and by ethylenediamine. Both compounds inhibited conidial germination and both had their inhibitory effect potentiated by Cu^{++}.

Conidial germination of *A. niger* was inhibited by BAPN, by complexing agents known to form chelate rings (8-hydroxyquinoline, dipyridyl, and ethylenediamine) and by the complexing agent, imidazole, which would not be expected to form a chelate ring. All of the chelate-ring-forming agents appeared to be more toxic than BAPN. The toxicity of BAPN appeared to be potentiated by Cu^{++} while the inhibitory effects of dipyridyl and ethylenediamine were at least partially reversed by Cu^{++}. The toxicity of 8-hydroxyquinoline appeared unaffected by Cu^{++} although the latter is reported to be more toxic to fungi in the presence of Cu^{++}(10). The inhibitory effect of imidazole appeared to be potentiated by Cu^{++}. Of the agents tried, imidazole seemed to resemble BAPN in its effects more than any of the others.

Judging from changes in the medium (pH, color, precipitate formation), all of these ion-complexing agents formed a complex with Cu^{++}, whereas BAPN may not have done so (no pH drop). At most, BAPN appeared to behave as a weak complexer similar to imidazole (color and nature of precipitate). Therefore, spore germination was abandoned as a technique for studying ion-BAPN interactions in favor of more sensitive chemical procedures. Such studies might be of value since ion-complexing cannot be completely excluded from a possible role in lathyrism at this time. Blackwood and Naber(11) have shown that certain divalent metals, including Cu^{++}, can either protect chick embryos against BAPN • Fu or potentiate its lathyrogenicity depending on the levels of salts used and the time of their administration in relation to the administration of BAPN • Fu. We have observed that strongly colored products appear in solutions containing arginine (or

TABLE I. Effect of BAPN, Certain Ion-Complexing Agents, and Cu^{++} on Germination of *N. crassa* and *A. niger* Conidia.

Compound (concentration)	Cu^{++} concentration	Media characteristics		% Germination†		
		pH*	Colors and precipitates	AN 6275	NC 10334	NC 11943
None	0	7.7	None	100	100	100
BAPN (0.05 M)	0	7.8	"	17.4	52.7	44.1
Idem	.05 M	8.0	Light blue‡	0	25.6	4.72
Ethylenediamine (0.01 M)	0	7.7	None	<.67	86.1	83.2
Idem	.01 M	6.3	Intense blue	80.4	18.8	<2.01
Ethylenediamine (0.005 M)	0	7.7	None	2.50	—	—
Idem	.005 M	6.4	Intense blue	120.8	—	—
α, α′-Dipyridyl (0.005 M)	0	7.4	Light pink	0	—	—
Idem	.005 M	6.0	Blue-green	39.5	—	—
8-Hydroxyquinoline (0.005 M)	0	7.8	Yellow‡	0	—	—
Idem	.005 M	7.1	Yellow-green‡	0	—	—
Imidazole (0.005 M)	0	7.7	None	93.4	—	—
Idem	.005 M	7.1	Blue-green‡	77.5	—	—

* Media buffered at pH 7.7; pH drops due to ion-complex formation; spores germinate readily at these lower pH's.

† % of controls.

‡ Media turbid due to precipitation; particles (crystals, etc.) dissolved in 1 drop of .6 N HCl before counting germinants.

gelatin), Cu^{++}, and BAPN (or AAN) which do not form in the absence of any one of these 3 substances. It is therefore possible that Cu^{++} can interact with aminonitriles to form complexes with other substances occurring naturally in the cells, *e.g.*, arginine or protein.

3. Trials with compounds related to aminonitriles (Table II). During this work it was observed that BAPN and AAN induced the production of swollen, yeastlike cells in the germinants of *N. crassa* and *A. niger*. It seemed of interest to investigate structurally related compounds for these effects. Since both species belong to a group of fungi reported to possess chitinous cell walls(12), *A. niger* was selected for study of these compounds and glucosamine was included as a possible protectant.

All amino compounds tested, with the exception of ethylenediamine but including glucosamine, tended to produce a germinant possessing a less mycelial morphology as well as to inhibit conidial germination. Propionitrile did not have these properties. Glucosamine showed no protective activity against BAPN or AAN. On the other hand, glyceraldehyde and certain hexoses which possess a free carbonyl group but no amine group protected *A. niger* conidia against BAPN (13).

The amino group is therefore probably involved in the effects of the aminonitriles on both molds. This is reminiscent of observations by others(14,15) which indicate the unsubstituted amino group to be essential for lathyrogenic activity in the rat.

Cochrane(16) has recently surveyed the literature for compounds capable of inducing abnormalities in fungi. Apparently amino compounds have not received much study for their morphogenetic effects on fungal growth. The principal exception appears to be cysteine, which is reported to favor the nonmycelial growth phase over the mycelial growth phase in certain dimorphic yeasts. This effect has been attributed to the reduced sulfhydryl group(17) rather than to the amino group. In view of the results reported here and the importance of mycelial morphology in the taxonomy of fungi, it should be of value to study the effect of amino compounds on the production of yeastlike cells in much more detail.

Summary. Lathyrogenic aminonitriles were found to be inhibitory to conidial germination of *N. crassa* (3 strains) and *A. niger* (1 strain). AAN was intensely inhibitory and induced yeastlike forms in the germinants at neutral and at alkaline pH values. BAPN, a somewhat weaker lathyrogen, showed strong inhibition and produced similar yeastlike tendencies with conidia only at alkaline pH

TABLE II. Effect of Lathyrogenic Aminonitriles and Related Compounds on Germination of *A. niger** Conidia at pH 7.7.

Compound	Concentration	Morphology of germinant	% germination†
None	—	Thin, tapering mycelium	100
Beta-amino-propionitrile	1×10^{-2} M	Some swollen cells.	48.6
	5×10^{-2} M	Swollen, yeast-like cells and occasional short, thick mycelium.	17.4
Propionitrile	1×10^{-2} M	Thin, tapering mycelium at both molarities.	109
	5×10^{-2} M		108
Aminoacetonitrile hydrochloride	5×10^{-2} M	Swollen cells and occasional blunt, short, thick mycelium.	2.80
D-glucosamine hydrochloride‡	5×10^{-2} M	Swollen cells.	20.7
Ethylamine hydrochloride	5×10^{-2} M	Some swollen cells and much short, thick dichotomously branched mycelium.	66.3
Beta-aminopropionitrile + D-glucosamine hydrochloride‡	5×10^{-2} M for each compound	Swollen cells.	7.3
Aminoacetonitrile hydrochloride + D-glucosamine hydrochloride‡	5×10^{-2} M for each compound	" "	0

* *N. crassa* affected similarly by both BAPN and AAN; other compounds not tried.
† Reported as % of controls.
‡ pH 7.0; spores germinate readily at this pH; AAN, but not BAPN, shows same effects as at pH 7.7.

values. Cu^{++} tended to inhibit conidial germination at lower pH's but appeared relatively nontoxic in alkaline media. Cu^{++} seemed to potentiate the inhibition due to BAPN at pH 7.7. It appeared to be either synergistic or protective towards certain ion-complexing agents, depending on the agent and the species of mold. Those agents which form chelate rings appeared to be more toxic than BAPN. All amino compounds tested, including glucosamine, inhibited conidial germination of *A. niger* at pH 7.7, and except for ethylenediamine, they induced a tendency towards a yeastlike morphology in the germinants. Glucosamine showed no protection against BAPN or AAN. Amino compounds appeared to differ from BAPN in their effects only in degree, while propionitrile did not show these effects. Therefore, the amino group probably was involved in the effects produced by aminonitriles in both molds. It is concluded that the spore germination technique is not specific enough to differentiate lathyrogens from other inhibitors containing an amino group, and that it probably does not lend itself to the study of ion-BAPN interactions. However, further study of the role of amino compounds in morphogenesis of fungi would appear to be of value.

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Received January 15, 1964. P.S.E.B.M., 1964, v116.

Effect of Necrosis on Hemoglobin, Serum Protein Profile and Erythroagglutination Reaction in Golden Hamsters.* (29160)

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The results of our previous studies have emphasized the importance of tissue necrosis in the pathogenesis of the anemia of malignancy and the anemia accompanying chronic inflammation(1-4). It was further noted that in hamsters a positive erythroagglutination reaction (EAR) developed before onset of anemia(5,6). This abnormal agglutinability of red cells by rabbit antihamster serum *in vitro* is attributed to a protein "coating" on the test erythrocytes. Since the rabbit antihamster serum is prepared with normal hamster serum as the antigen, presumably such a "coating" represents a protein normally present in the hamster serum. It was therefore decided to explore further the effect of tissue necrosis on development of the erythroagglutination reaction, on the serum protein electrophoretic pattern, and on the development of anemia.

Materials and methods. Forty-seven mature golden hamsters were divided into 7 groups, 5 experimental and 2 smaller control groups. They were all maintained on Purina chow and water *ad lib.* Blood samples were obtained by cardiac puncture immediately prior to the experimental procedure, and on days 3, 6 and 8, or as otherwise indicated in Fig. 1, during the experiment. Animals were anesthetized with 0.15 cc of intraperitoneal sodium pentobarbital (Nembutal, Abbott, 50 mg per ml) before cardiac puncture. The following determinations were made on the samples: hemoglobin, hematocrit, serum electrophoretic pattern and erythroagglutination reaction.

Hemoglobins were measured by the cyanohemoglobin technique (normal = 16.3 ± 1.3 g%). Hematocrit was determined by micro-method (normal = 53-56%). Serum electrophoresis was carried out in the Shandon Electrophoresis apparatus on cellulose acetate (Oxoid) strips, 2.5 × 0.2 cm in Barbitone acetate buffer pH 8.6, at 125 v-0.4 ma per cm width. Strips were stained with Ponceau S 0.2% in 3% tricholoracetic acid. Bands were then eluted with chloroform and absolute ethanol (90%/.10%) and the percent transmission of the eluate was read in a Coleman Jr. spectrophotometer. Hamster normals: albumin 65-71%, alpha$_1$ globulin 8.0-10.5%, alpha$_2$ globulin 5.0-7.0%, gamma globulin 9.5-12.5%. Erythroagglutination reactions were performed by the technique reported previously. The rabbit antihamster serum was absorbed with normal hamster red cells before being used. Surgery was performed under aseptic technique after intraperitoneal sodium pentobarbital anesthesia. Animals were weighed daily.

* This investigation was supported by U. S. Public Health Service grants and by U. S. Atomic Energy Commission Contract with New England Deaconess Hospital.