A STUDY OF THE FUNGISTATIC AND FUNGICIDAL PROPERTIES AND OF THE TOXICITY FOR MICE OF SODIUM AZIDE

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The inhibitory action of sodium azide (NaN₃) on the growth of bacteria was first reported by Schattenfroh (1896). Apparently no further work of this nature was published until 1937. In the past few years several contributions have appeared dealing with the effects of NaN₃ on microorganisms, but, so far as the writers can determine, these studies have not included the filamentous fungi. A familiarity with the influence of NaN₃ on several species of bacteria, together with the chance observation of its pronounced inhibitory effect on Penicillium notatum, led to an investigation of this compound as possible fungicide, and as a chemotherapeutic agent in fungous infections. Early experiments gave sufficiently striking results to warrant the publication of a preliminary report by Kempf and Nungester (in press).

The principal purpose of this article is to present additional experimental data relative to the fungistic and fungicidal properties and the toxicity for mice of NaN₃.

In order to obtain data of broad mycological significance, the following group of organisms was studied. These species represent a wide taxonomic range as well as a great diversity of normal habitats. Mucor rhiizopodiformis is a saprophytic phycomycete; Aspergillus niger and Penicillium notatum are common saprophytic ascomycetous molds; Stereum gausapatum, a basidiomycete, is the cause of a heart rot in hardwood trees. Alternaria solani, an imperfect fungus, is the etiological agent of a common blight of solanaceous plants. The remaining organisms, namely, Trichophyton gypseum, Blastomyces dermatitidis, Histoplasma capsulatum, and the yeast-like Monilia albicans, are all animal pathogens of the class Fungi Imperfecti.

FUNISTIC ACTION

The ability of NaN₃ to inhibit the growth of fungi was determined by growing the test organisms in the presence of graded concentrations of the chemical. In certain preliminary experiments the NaN₃ was incorporated in agar culture media and the organisms grown in petri dishes. Such a procedure was employed because of its simplicity. Two serious faults were soon appreciated and the method therefore abandoned. The most obvious difficulty was the lack of a simple criterion for determining the amount of growth. When cultured on solid media, it is commonly observed with fungi that various environmental factors, such as nutritive materials, hydrogen ion concentration and oxygen tension, may greatly

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¹ Culture No. 601, Trichophyton mentagrophytes (T. gypseum), of the U. S. Public Health Service, obtained through the courtesy of C. W. Emmons.
alter the amount of mycelium produced, without a corresponding variation in the diameter of the colony. Very low concentrations of NaN₃ have been observed to act in a like manner. Another criticism of the agar plate method is the problem of controlling the composition of the medium, since the exact chemical composition of commercial agar is unknown. Day (1942) has recently shown, for example, that agar may contain significant amounts of thiamin. The method finally adopted involved growing the fungi on synthetic liquid media containing various concentrations of NaN₃, removing the mycelia by filtration, and determining their dry weights.

A suitable amount of a liquid medium* was prepared. To a portion of this, sufficient NaN₃ was added to make a one per cent solution. By dilution, a series of culture media was prepared having NaN₃ concentrations of the following percentages, respectively: 0.01, 0.001, 0.0001, 0.00001 and 0.000001. The same medium, with no NaN₃, served as a control. These media were dispensed into 250-ml erlenmeyer flasks, 50 ml per flask, and sterilized in the autoclave at

*Constituents: NaNO₃ 2.0 g, KH₂PO₄ 1.0 g, KCl 1.0 g, MgSO₄ 0.5 g, MnSO₄ 0.01 g, glucose 20.0 g, peptone 5.0 g, and distilled water to make 1 liter.

### TABLE 1

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>INOCULUM</th>
<th>DAYS INCUBATED</th>
<th>CONCENTRATION OF NaN₃</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td>About 1,600,000 washed conidia from a 7-day agar culture</td>
<td>12 0*</td>
<td>100 587 614 595 590</td>
</tr>
<tr>
<td><em>Penicillium notatum</em></td>
<td>About 2,500,000 washed conidia from a 7-day agar culture</td>
<td>12 0 17 424 646 462 470</td>
<td></td>
</tr>
<tr>
<td><em>Mucor rhizopodiformis</em></td>
<td>About 2,500,000 washed sporangiospores from a 7-day agar culture</td>
<td>12 0 14 215 283 286 278</td>
<td></td>
</tr>
<tr>
<td><em>Trichophyton gypseum</em></td>
<td>About 2,500,000 washed spores from a 7-day agar culture</td>
<td>12 0 17 322 278 369 311</td>
<td></td>
</tr>
<tr>
<td><em>Alternaria solani</em></td>
<td>0.1 ml of a washed mycelial suspension scraped from an agar culture</td>
<td>12 0 8 713 604 565 611</td>
<td></td>
</tr>
<tr>
<td><em>Stereum gausapatum</em></td>
<td>One 8-mm disk cut from a young agar petri plate culture</td>
<td>12 0 8 77 84 103 91</td>
<td></td>
</tr>
<tr>
<td><em>Blastomyces dermatitidis</em></td>
<td>One 8-mm disk cut from a young agar petri plate culture</td>
<td>25 0 12 169 422 214 206</td>
<td></td>
</tr>
<tr>
<td><em>Histoplasma capsulatum</em></td>
<td>One 8-mm disk cut from a young agar petri plate culture</td>
<td>25 0 4 216 229 314 233</td>
<td></td>
</tr>
<tr>
<td><em>Monilia albicans</em></td>
<td>About 5,000,000 washed cells from a 2-day agar culture</td>
<td>12 0 63 268 245 241 281</td>
<td></td>
</tr>
</tbody>
</table>

* Dry weight in mg of growth in two flasks.
121 C for 20 minutes. Two flasks of each medium were inoculated with each of the nine fungi.

The method of inoculation could not be uniform because of differences in the morphology of the several species. For the common molds, it was found satisfactory to use washed spore suspensions. These were prepared by flooding agar slant cultures with sterile water and suspending the spores by rubbing the culture with a sterile glass rod. Since M. albicans is yeast-like, the vegetative cells were handled in the same manner as the mold spores. Cultures of A. solani produced a relatively small number of conidia which could not be washed free of hyphae. Suspensions of spores, together with fragments of mycelium, were prepared by scraping a water-covered agar culture with a bent inoculating wire. Except for the latter species, the suspensions were passed through glass wool to remove fragments of agar and mycelium. All were washed by centrifugation. Cultures of those strains which produced few or no spores were prepared by inoculating the flasks with 8-mm disks cut from young petri dish agar cultures.

With the exception of two relatively slow-growing organisms, H. capsulatum and B. dermatitidis, the cultures were incubated for a period of 12 days, at room temperature (22-25 C). In order to allow a comparable development of these two fungi, incubation was extended for an additional 18 days. The growths were then filtered through weighed filter papers, dried and the weights determined. This technique was modified for M. albicans because the yeast-like cells could not be easily collected on filter paper. Cultures of this organism were centrifuged, the cells washed in distilled water and subsequently reduced to dryness in evaporating dishes.

The results obtained in two, and in certain instances three, experiments were not significantly different. The data for one typical experiment are presented in the accompanying table (table 1). No growth occurred in the presence of 0.01% NaN₃ and a concentration of 0.001% was distinctly inhibitory. The appearance of the mycelia, as well as the dry weights, indicated that concentrations of 0.0001% or less had no toxic effect. In fact, in several instances the results with 0.0001% concentration were suggestive of the well-known phenomenon of stimulation of organisms by minute amounts of chemicals ordinarily toxic.

FUNGICIDAL STUDIES

To determine the fungicidal properties of NaN₃, vegetative cells, spores or mycelium, depending upon the morphology of the species, were exposed to several concentrations of the chemical for various periods of time. Following such exposures the fungi were transferred to a liquid medium and incubated to determine viability.

The test materials, except as subsequently described, were prepared in the same fashion as those for the fungistatic experiments. Three of the fungi, S. gausapatum, B. dermatitidis, and H. capsulatum, when grown on agar, produced a dense mycelial mat with few or no spores. In order to test these strains, broth cultures containing small submerged mycelia were used. Mycelia from such cultures were handled in a manner similar to that employed with the other or-
organisms. Portions of each of these preparations were then placed in 1%, 2.5% and 5% aqueous solutions of NaN₃. After exposure for periods of 4, 8 and 24 hours, a portion of each was taken and tested for viability. The samples were centrifuged, the supernatant NaN₃ solutions decanted, the fungi re-suspended in sterile water, again centrifuged, drained and finally mixed with a small amount of water. A large loopful of this material was used to inoculate 25 ml of liquid medium. This volume was sufficient to dilute adequately the trace of NaN₃ which undoubtedly accompanied the inoculum. The appearance of the characteristic growth was accepted as proof of the presence of viable organisms.

In our earlier work the "proposed standard method for the evaluation of fungicides" as outlined by McCrea (1931) was employed. The results of such experiments indicated that NaN₃ had a high degree of fungicidal power. It was found, however, that transferring a loop of spore suspension in a NaN₃ solution, in some cases as dilute as 0.1%, carried sufficient NaN₃ to the agar slant to produce a pronounced fungistatic effect. Such fungistatic action obviously interfered with the recognition of any fungicidal action which may have occurred.

Eight of the nine organisms studied were viable after 24 hours in 1.0%, 2.5% or 5.0% NaN₃ solutions. The remaining strain, H. capsulatum, was not killed by the lower two concentrations in 24 hours, but the 5% solution was found to be weakly fungicidal for the mycelium of this species. In four experiments, a 5% solution was fungicidal in 2 of 4 exposures for 4 hours; in 3 of 4 exposures for 8 hours; and in all 4 of 4 exposures for 24 hours.

As shown above, eight of the nine fungi were not killed by 24 hours of contact with a 5% NaN₃ solution. H. capsulatum, even though killed by this treatment, was able to survive, in solutions of this concentration, for several hours. It is therefore obvious that NaN₃ has very little fungicidal value, and that the pronounced influence of this chemical is a fungistatic phenomenon.

ANIMAL TOXICITY

As previously suggested, it seemed desirable to investigate the therapeutic possibilities of NaN₃. Fungal infections are not readily produced in laboratory animals. Attention was therefore directed towards the more fundamental problems of toxicity and the effects of blood on the action of the chemical.

In order to determine the toxicity of NaN₃ for mice (Peromyscus maniculatus), various amounts in aqueous solution were injected intraperitoneally. Each animal was given a single injection of NaN₃, the doses employed being 0.032 mg, 0.027 mg and 0.023 mg per gram of body weight. The animals were observed for a ten-day period. All of six mice which were injected with 0.032 mg of NaN₃ per gram of body weight died during the observation period. A single dose of 0.027 mg per gram of body weight proved fatal to 9 of 14 animals, and quantities of 0.023 mg per gram of body weight killed 2 of 17 mice.

To investigate further the action of this substance, an experiment was performed to determine its cumulative toxic effects. Fifteen mice were injected intraperitoneally with 0.005 mg per gram of body weight twice daily for a period of 10 days. At the end of this time 4 mice had died, a mortality rate of 25%.
In a final experiment, seven mice were given intraperitoneally 0.005 mg of NaN₃ per gram of body weight, every two hours for a period of three days. Of the seven animals, only two were alive at the end of the experiment.

It has already been demonstrated that the action of NaN₃ is one of fungistasis. To determine the effect of blood on this phenomenon, experiments were performed in the presence of rabbit blood. The mold, *P. notatum*, whose reaction to NaN₃ is typical of the species studied, was selected as a test organism. A series of tenfold dilutions of NaN₃, ranging from 0.1% to 0.0001% was prepared. The basic medium was the aforementioned culture solution containing 50% defibrinated blood. For a control, a similar series was prepared with physiological saline solution substituted for the blood. One tube of each medium was inoculated with a loop of a spore suspension and incubated for a period of 10 days, at room temperature. The experiment was repeated, and the results confirmed in general those of the first experiment. The findings are presented in table 2. As was noted earlier (table 1), 0.001% NaN₃ produced considerable inhibition, while 0.01% completely prevented growth of *P. notatum*. In the presence of 50% blood it was necessary to increase the concentration tenfold to obtain the same results.

Because of the high degree of toxicity and the neutralizing effect of blood, it is concluded that NaN₃ holds no promise as a systemic agent for the treatment of fungous diseases.

**SUMMARY**

The fungistatic and fungicidal properties of NaN₃ have been investigated. Nine species of fungi were studied; the growth of all was completely inhibited by 0.01% NaN₃. A concentration of 0.001% caused pronounced inhibition in all of the species, while 0.0001% had no apparent inhibitory action.

With the exception of *Histoplasma capsulatum*, none of the fungi was killed by exposures of 24 hours to 5% NaN₃ solutions. It is therefore concluded that the pronounced ability of NaN₃ to suppress the growth of fungi is due to its fungistatic action, and that the chemical has no practical fungicidal power.

### TABLE 2

*The effect of 50% rabbit's blood on the fungistatic action of sodium azide*

<table>
<thead>
<tr>
<th>CONCENTRATION OF NaN₃</th>
<th>GROWTH OF PEPCILLIUM NOTATUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% blood</td>
</tr>
<tr>
<td>0.1</td>
<td>- (no growth)</td>
</tr>
<tr>
<td>0.01</td>
<td>+ (growth)</td>
</tr>
<tr>
<td>0.001</td>
<td>+</td>
</tr>
<tr>
<td>0.0001</td>
<td>+</td>
</tr>
</tbody>
</table>

*Results of two experiments.
† Trace of growth on second experiment.
The animal toxicity was studied by intraperitoneal injections of NaN₃ into mice. Single doses of 0.023 mg per gram of body weight, or of 0.005 mg per gram of body weight in repeated doses, were highly toxic, and the fungistatic action was reduced tenfold by the presence of blood.

REFERENCES