AN ANTIPHAGE AGENT ISOLATED FROM ASPERGILLUS SP.

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The ability of some known antibiotics such as streptothricin, streptomycin, and clavacin to prevent bacteriophage action has been demonstrated (Jones, 1945). Schatz and Plager (1948), working with an actinomycete that demonstrated antiphage activity, extracted a material that was capable of weakly inhibiting a rodent-paralyzing virus (MM), but it appeared to be too toxic for further study. We have isolated a substance from *Aspergillus* sp. that was capable of inhibiting *Staphylococcus aureus* 209 bacteriophage, but exhibited no antiviral activity *in vivo* when tested against poliomyelitis (MM) and influenza (PR-8-A) infections in mice. This report is submitted for its intrinsic value as a method of testing and isolating an antiphage agent.

The isolation and testing of the antiphage-producing organisms were accomplished by techniques similar to those described by Jones and Schatz (1946). Various soil samples, used as a source of antiphage-producing organisms, were cultured on nutrient agar plates for 10 days at room temperature. The plates were then sprayed with semisolid nutrient agar (0.7 per cent agar), seeded with 1 per cent of a 24-hour *S. aureus* 209 culture and a 0.1 per cent *S. aureus* phage suspension (6×10^8 phage particles per ml), and incubated at 37 C for 16 hours. The presence of *S. aureus* growth in the sprayed layer surrounding a soil colony indicated inhibition of the bacteriophage.

Several molds, actinomycetes, and bacteria antagonistic to *S. aureus* phage were isolated. An *Aspergillus*, designated culture H-3, demonstrated greater phage-suppressing ability than other isolates and was selected for further study.

Culture H-3 produced the antagonistic factor in several liquid media. A dextrin corn steep medium gave the highest yield in shake flasks and has been used successfully in 10-gallon and 100-gallon fermenters. Potencies ranging from 20 to 200 units per ml of beer have been obtained.

To test quantitatively the activities of fermentation beers and subsequent extraction samples a plate assay was developed in which zones of phage inhibition were measured. One-fourth-inch paper disks were dipped into the solutions to be tested and placed on nutrient agar plates composed of a 10-ml base layer plus a 5-ml semisolid surface layer seeded with 1 per cent 24-hour *S. aureus* culture and 0.1 per cent *S. aureus* phage suspension. Plates were incubated for 16 hours at 37 C to allow for zone development. A solution containing enough antibiotic H-3 to give a zone of *S. aureus* growth 8 mm in diameter was defined as having a potency of 1 unit per ml. A preparation containing 40 units per mg

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was subsequently prepared as a standard. This preparation was diluted daily to afford solutions of 1.25 to 10 units per ml. This range in concentration represented a total zone diameter difference of about 10 mm on the standard curve.

Extraction of the neutral filtered beer with chloroform gave a brownish-yellow gum which was dried to a powder under vacuum. It was soluble in most organic solvents with the exception of the saturated hydrocarbons. The solubility of antibiotic H-3 in water decreased as it was purified. It was fairly stable to heat and acid but was quickly destroyed by alkali.

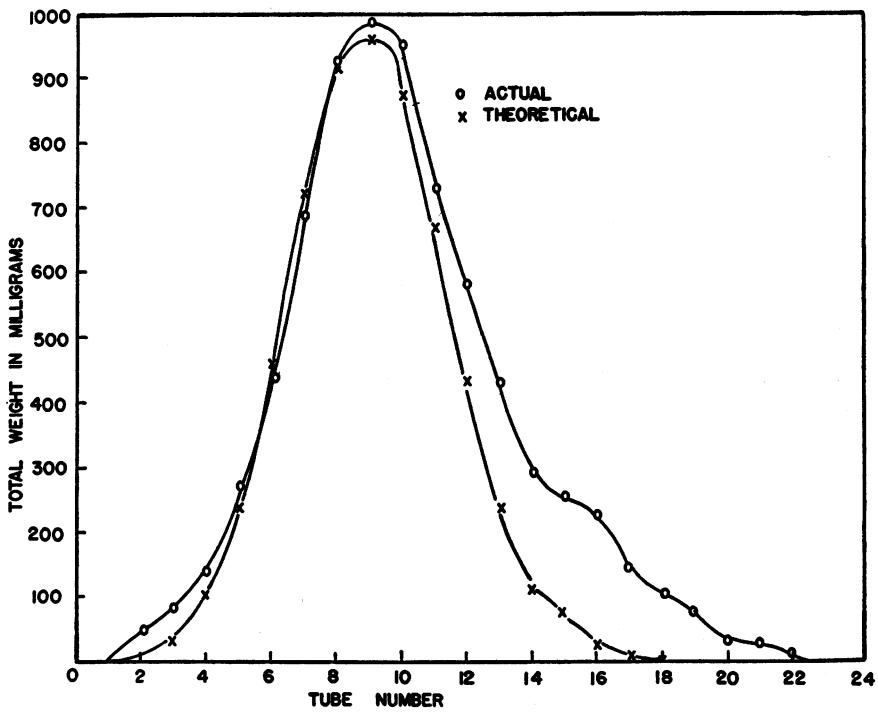

Figure 1. Countercurrent distribution of a sample of H-3.

Although ultimate purity has not been achieved at this time, a 400-fold purification from the beer solids has been obtained by means of countercurrent distribution. The dried material from the neutral chloroform extraction of the beer was dissolved in ethyl ether, and the resulting solution was shaken several times with a 30 per cent methanol and 70 per cent water mixture. The ethereal phases, when dried under vacuum, afforded a powder the activity of which was from one to five times greater than that of the starting material.

A sample of antibiotic H-3 prepared by this procedure contained 270 units per mg and demonstrated little antibacterial or antifungal activity. In a broth dilution test, *Streptococcus faecalis* 6057 was inhibited at 300 μg per ml, *Bacillus subtilis* at 100 μg per ml, and *S. aureus* at 30 μg per ml. A concentration of 300 μg per ml did not inhibit *Escherichia coli* 26, *Salmonella schottmuelleri* 9149,

Salmonella typhosa 167, *Klebsiella pneumoniae* PCI-602, *Brucella bronchiseptica* B-140, and *Mycobacterium tuberculosis* var. *hominis* 607. The fungal pathogens of man were not inhibited by the preparation in concentrations of less than 100 μg per ml when tested by an agar dilution method.

This preparation had a subcutaneous LD_{50} of about 9.6 mg per mouse. However, doses of 3.0 mg per mouse given subcutaneously twice daily for $3\frac{1}{2}$ days to mice infected with either poliomyelitis (MM) or influenza (PR-8-A) hastened death. One-mg doses gave no protection.

The limiting factor in the further purification of antibiotic H-3 through solvent distribution was its insolubility in water. A solvent system, analogous to that of Marshak *et al.* (1947), prepared by mixing equal volumes of 20 per cent cyclohexane in benzene and 10 per cent water in methanol, was used to obtain a preparation of antibiotic H-3 having a potency of 800 to 1,000 units per mg. Figure 1 illustrates a 24-tube distribution of 7 grams of a preparation of antibiotic H-3, having a potency of 450 units per mg, using 95 ml of each solvent phase. The material in tube 9 had an activity of about 1,000 units per mg. Conformity to the theoretical curve was good, and a subsequent distribution of 200 mg of fraction 9, using 10 ml of each phase, through 24 tubes gave a curve similar to that in figure 1. This would indicate that the material was close to maximum purity; however, all attempts at crystallization of the material in fraction 9 have so far failed. Hydrolysis of a portion of this fraction by the method of Sanford and Humoller (1947) followed by paper strip chromatography of the hydrolyzate indicated the presence of three ninhydrin-positive zones. In view of the limited amount of material available these zones were not identified.

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