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AN ANTIBIOTIC SUBSTANCE PRODUCED BY RHIZOPUS NIGRICANS EHRENBERG

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INTRODUCTION

Plugs of medium were removed from cultures of *Rhizopus nigricans* and placed on freshly-seeded agar plate cultures of *Micrococcus aureus*. When these were incubated, a clear zone in which the bacteria failed to grow appeared surrounding the mold medium. Since previous similar tests did not support this result, the above test was repeated and *Micrococcus aureus* again failed to grow. The failure of a bacterium to grow in the presence of the products of another organism hereafter will be called inhibition or antibiotic activity.

PURPOSE

The experimental work which is outlined here was an effort to explain the cause of these preliminary results.

EXPERIMENTAL METHOD

To justify further investigation of these findings it was desirable to know whether cultures of *Rhizopus nigricans* also would inhibit the growth of other pathogenic bacteria. In an effort to answer this question, plugs of medium again were removed from cultures of the mold and placed on freshly-seeded plate cultures of *Bacillus anthracis*, *Salmonella typhosa*, *Salmonella shottmuelleri*, *Shigella sonnei*, and *Corynebacterium bovis*. Plugs of sterile medium identical to those on which the mold was grown were tested in a like manner to serve as controls. When these test plates were incubated, all of the test organisms were inhibited by the medium upon which the mold had grown, whereas the control medium inhibited none.

Rhizopus nigricans was grown on a broth medium containing proteose-peptone and glucose. Filtrates from these cultures were tested by the cup plate method against *Bacillus anthracis*. The organism was inhibited under these test conditions. The pH of the tested broth filtrate was found to be 3.0; consequently, tests were devised to determine whether this acidity caused the observed inhibitions. Sterile broth and distilled water were adjusted to pH 3.0. A portion of the broth filtrate from the mold culture was adjusted to pH 6.6; an aliquot of this was readjusted to pH 3.0; and a portion of the original culture filtrate was left untreated. These then were tested by the cup plate method against *Micrococcus aureus*. Only the untreated filtrate from a culture of *Rhizopus nigricans* inhibited the test organism. Therefore, it was concluded that the observed inhibitions were not caused by acidity alone, but also by some substance or substances produced from a glucose proteose-peptone solution by the metabolic activities of *Rhizopus nigricans*. Extraction and identification of this substance seemed to be the next logical steps.

Before attempts were made to extract the antibiotic substance from the broth filtrates, tests were devised to determine whether this substance could be concentrated by boiling in air and to determine which pH range would offer the maximum potency and stability. To answer these questions, portions of the broth filtrate were boiled for 5, 10, 15, and 20-minute periods and other portions were adjusted to pH values of 2.3, 3.0, 4.3, 6.0, and 8.0. Each of these portions was tested by the cup plate method against *Bacillus anthracis*. All of the boiled portions inhibited the test organism. Those that were boiled the longest time produced the largest inhibition zones. Therefore, it was shown that the antibiotic substance could be concentrated by boiling broth filtrates in air. None of those portions of the broth filtrates with a pH greater than 4.3 inhibited the test organism.

Preliminary to extracting the antibiotic substance from the crude solution, the broth filtrate was boiled to near dryness. A syrupy brown liquid resulted. This was placed in a 100°C oven to complete the drying process. The resulting dark brown gummy residue was extracted several times with acetone. This solvent

was evaporated and the resulting residue was extracted with boiling ether. In turn, this was evaporated and the resulting residue was extracted with ether cooled to 0°C. The cold ether then was evaporated and the residue was extracted with boiling chloroform. The chloroform extract was evaporated by boiling to dryness. When it cooled, the result was a layer of large amber aggregate crystals interspersed among many small clear crystals. All residues were suspended in distilled water and tested by the cup plate method against *Micrococcus aureus*. The residue from the acetone extraction and the crystalline deposit from the chloroform extract each inhibited the test organism.

An aqueous solution of the chloroform extract and a portion of a broth filtrate from a culture of *Rhizopus nigricans* were tested with ferric chloride for the presence of organic acids. A yellow color resulted. Attempts were made to separate the two crystalline forms found in the chloroform extract by washing in various solvents. Separation was not accomplished and, therefore, additional chemical tests to identify the antibiotic substance as a known compound were not performed. The pH of aqueous solutions of the crystalline deposit showing antibiotic activity was 2.7.

DISCUSSION

These tests have demonstrated clearly that an antibiotic substance is produced as a result of the growth of a local isolate of *Rhizopus nigricans* and that many pathogenic bacteria are inhibited in virto by this substance. The identity of this antibiotic is unknown, although some of its properties are known. Tests have established that this substance is stable to a temperature of 100°C for a period of at least 20 minutes, and that its antibiotic properties are diminished at pH values greater than 4.3. Although there is good reason to suspect that the organic acids produced by the mold are responsible for the observed inhibitions, certain well-known inhibitory acids have been eliminated as possible causes. Kojic acid and phenolic acids were eliminated by the results of the ferric chloride test. Because this antibiotic is soluble in chloroform and is concentrated by boiling, whereas lactic acid is neither, this acid is not considered the cause of the observed inhibitions. Likewise, because of the stability of this antibiotic substance to boiling, all volatile acids are eliminated as possible causes. Since the purity of the extracted material has not been determined, it is not certain whether the acidity of this material is due to the antibiotic substance present in these extracts. Without other evidence to the contrary, however, it can be assumed that this acidity is due in part to the antibiotic substance, and although it has not been proved by test that organic acids are responsible for the observed antibiotic activity, one of these or a similar substance is suspected.

CONCLUSION

The following facts are known as a result of this experiment:

1. An antibiotic substance is produced by a locally isolated strain of *Rhizopus nigricans*.
2. These substances prevent the growth in vitro of the following bacteria:
   a. *Bacillus anthracis*
   b. *Micrococcus aureus*
   c. *Salmonella typhosa*
   d. *Salmonella shottmerelleri*
   e. *Shigella sonnei*
   f. *Corynebacterium bovis*
3. This substance can withstand a temperature of 100°C for a period of at least 20 minutes without appreciable loss in antibiotic activity.
4. In solutions adjusted with sodium hydroxide to pH values greater than 4.3, the ability of this substance to inhibit bacterial growth is greatly diminished.

SUMMARY

A crystalline substance believed to be an organic acid was isolated from broth filtrates of cultures of *Rhizopus nigricans*. This substance prevented the growth in vitro of both gram positive and gram negative pathogenic bacteria.

REFERENCES

1. Wilkins, W. H. and Harris, G. M. C. *British Journal of Experimental Pathology*, 23, p. 166 (1942).