SOME PROPERTIES OF A BACTERIAL-INHIBITORY SUBSTANCE PRODUCED BY A MOLD[1]

ROGER D. REID

Division of Bacteriology, The Pennsylvania State College, State College, Pennsylvania

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In 1929, Fleming observed that a mold which he identified as *Penicillium rubrum* (Biourge), but which more closely resembles *Penicillium notatum* (Westling) and belongs to the group of which *Penicillium chrysogenum* is the type species, (Thom, 1930) was able to produce a substance which inhibited the growth of certain bacteria. During 1930 a large number of molds[2] were studied in this laboratory in an attempt to find others than the one isolated by Fleming which would produce a similar inhibitory substance. Since none were found, the following experiments were conducted on the filtrate from Fleming's culture when grown on veal infusion broth or on a modified Czapek-Dox synthetic medium having the composition described by Clutterbuck, Lovell and Raistrick (1932) with the hope of gaining some information with regard to the nature of the inhibitory substance.

The mold was permitted to grow at 20°C. for a period varying from seven to ten days on veal medium or twenty to twenty-five days on the synthetic medium. After the growth period, the

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[2] The molds studied were collected from various sources and identified by Dr. Chas. Thom, U. S. Department of Agriculture. They include the following: *Mucor mucedo, Mucor piriformis, Rhizopus nigricans, Aspergillus glaucus, A. nidulons, A. niger, A. flavus, Penicillium expansum, P. roqueforti, P. digitatum, P. illaticum, P. rubrum* (Biorge) according to Fleming, *P. cephatotherium roseum, P. cladosporum herbarum, P. hormondendrum cladosporoides, P. notatum* (Westling), *P. rubens, P. chrysogenum, Oidium lactis, Fusarium oxysporum* and three species of Actinomycetes.

mold was separated from the substrate by filtration through a Berkefeld filter, leaving a sterile filtrate. The filtrate containing the inhibitory principle had a bright yellow color. This pigmentation regularly occurred soon after fructification and became deeper, approaching a red orange, if allowed to incubate for a long period of time. The filtrate was tested for inhibitory substance in the following manner: Into one-half of a Petri dish was poured nutrient agar without the filtrate; into the other, the same agar plus filtrate. The plates were then streaked with the cultures of bacteria. Growth on the half of the plate containing the nutrient agar only was evidence of no error in streaking. Absence of growth on the other half indicated the presence of the inhibitory substance.

Among the organisms that are inhibited by the filtrate are *Staphyloccocus aureus* (which became the test organism), *Staphylococcus citreus*, *Staphylococcus epidermidis*, a pneumococcus, a hemolytic streptococcus, *Bacillus anthracis*, *Corynebacterium diphtheriae* and *Streptococcus pyogenes*. The organisms not inhibited include *Bacillus subtilis*, *Escherichia coli*, the typhoid and paratyphoid organisms, *Pseudomonas fluorescens*, *Bacillus influenzae* and *Bacillus proteus*. An investigation of the Gram's reaction, physiological and cultural characteristics of the inhibited organisms reveals no characteristics consistently common to this group, nor was there any characteristic which differed uniformly from those of the organisms not inhibited. The potency of the inhibitory substance is indicated by the fact that a 1:100 dilution of filtrate in nutrient medium was found sufficient to inhibit the sensitive organisms. In some instances dilutions of 1:800 brought about complete inhibition.

Effect of inhibitory substance on bacteria. In order to determine if inhibition is due to a lysing of the organism, as stated by Fleming (1929), tubes containing 10 ml. of nutrient broth and varying amounts of the filtrate were inoculated with *Staphylococcus aureus* and incubated at 37°C. Streaks were made from tubes showing no apparent growth at the end of twenty-four hours. Normal growth was obtained on all plates. From this it is concluded that the organism is not destroyed but merely inhibited.

Effect of light. That light has a deleterious effect upon the production of the inhibitory material was shown by subjecting the actively growing culture to the rays from a carbon arc lamp with a quartz filter, giving rays including the longer ultra violet, infra red and other light rays. The cultures were grown in Pyrex glass flasks[3] and exposed to these rays for a period of fifteen minutes daily for the period of incubation. Caution was exercised to prevent contamination during exposure and to prevent the action of heat from the lamp upon the culture. Filtrates from cultures so treated contained no inhibitory substance, showing that the inhibitory substance was either not produced or if produced, was destroyed by the action of the light rays.

Effects of oxygen, hydrogen and carbon dioxide. Since oxygen plays an important rôle in the production of toxins, it was decided to increase the concentration of that gas in the substrate upon, and in the atmosphere in which, the molds were growing. This was accomplished by bubbling oxygen through the medium at the rate of about 100 bubbles per minute for fifteen minutes each day. The flasks were stoppered with 2-holed rubber stoppers through which passed glass tubes equipped with rubber tubes and pinchcocks. In this way oxygen was passed through the substrate and an atmosphere of oxygen maintained above the medium at all times. The gases used in this work were obtained in tanks from the Ohio Chemical Company. The presence of oxygen produced no noticeable effect on the amount of growth or pigment formation although no inhibitory substance was produced under these conditions. This was contrary to expectations and would indicate a dissimilarity between the inhibitory substance and a true soluble toxin because it was either not produced, or if produced was destroyed by the action of the gas.

Cultures were treated in the same way with hydrogen and carbon dioxide. The results were identical with those obtained when an oxygen atmosphere was maintained.

Effect of temperature on stability of inhibitory substance. The

[3] According to the Corning Glass Works, Manufacturers of Pyrex, the type of glass of which these flasks are made permits "medium ultraviolet transmission, and is highly transparent to visible radiation. . . . . "

filtrate was found to be active after heating at 80°C. for seventy-five minutes and at 90°C. for fifty-five minutes. Heating at 90°C. for sixty minutes destroyed its activity. Heating at 100°C. for five minutes did not destroy the inhibitory activity but after ten minutes at this temperature the filtrate was no longer active.

Attempts to isolate the inhibitory substance by distillation. Attempts to separate the inhibitory substance from the mold filtrate by means of distillation at low temperatures under reduced pressure resulted in a complete loss of the inhibitory material as it could not be recovered from either the distillate or the residue. In this distillation a vacuum pump was used to obtain a reduced pressure sufficient to cause boiling at 42°C. to 52°C.

Attempt to separate the inhibitory substance by dialysis. In repeated experiments, using samples of the active inhibitory filtrate placed in collodion bags and allowed to dialyze into sterile distilled water for thirty-six hours, a considerable amount of the yellow pigment passed into the dialysate, apparently setting up an equilibrium between the dialysate and the residue. Both the dialysate and residue were tested for inhibitory properties with negative results. The combined dialysate and residue showed no inhibitory power.

Relation of pigment to inhibitory substance. Since the pigment and the inhibitory substance usually appear simultaneously, an attempt was made to determine whether or not the pigment is the inhibitory substance or related to it. By adsorption on charcoal the pigment and the inhibitory substance are both removed. This was the only evidence obtained that would indicate a close relationship between the two. On the other hand, chloroform extraction removed some of the pigment without affecting the intensity of the inhibitory properties of the broth filtrate. Control experiments showed that the inhibition was not due to traces of chloroform left in the broth. Continuous extraction with ether and acetone for seventy-two hours failed to remove either the pigment or the inhibitory substance. Holding of the broth filtrate containing the inhibitory principle at room temperature, in the dark, at a pH of approximately 8.0 for three to four weeks resulted in a loss of the inhibitory activity without any

decrease in pigmentation. It has been shown that exposure to light does not allow the production of the inhibitory substance while color production is unaffected. Likewise, oxygen, hydrogen and carbon dioxide prevent the production of the inhibitory substance and do not affect pigmentation. Heating brings about a loss of inhibitory activity with no loss in the intensity of the pigment. Thus it seems that there is no close relationship between these two.

Enzymes present in an active filtrate. That enzymes are associated with normal mold growth has been shown by numerous investigators. In order to obtain more information with regard to the nature of the active filtrate, it was thought desirable to determine what enzymes were present. Tests indicated the presence of erypsin and catylase in large amounts. Lipase was present in moderate amounts while traces of amylase, trypsin and amidase were found. Tests for gelatinase and emulsin were negative.

TABLE 1

Surface tension readings on non-inhibitive and inhibitive media

SYNTHETIC MEDIA BEFORE MOLD GROWTH	INHIBITORY FILTRATE FROM SYNTHETIC MEDIA AFTER MOLD GROWTH	NUTRIENT BROTH + 10 PER CENT FILTRATE-INHIBITED STAPHYLOCOCCUS AUREUS
52.6 dynes	43.1 dynes	46.25 dynes

Effect of filtrate upon charge of bacteria inhibited. Experiments to determine the degree of charge on *Staphylococcus aureus* as influenced by the inhibitory substance were performed. Suspensions of the organisms were made in nutrient broth, in the inhibitory filtrate active in 1:500 dilution, in veal infusion broth and in synthetic broth. The charge on the organism was measured electrophoretically. The degree of charge was found to be the same in each case indicating that the inhibitory substance does not alter the charge on the organism which it inhibits.

Surface tension. As indicated in the literature (Albus and Holms, 1926; Davis, 1927; Gibbs, Batchelor and Lickels, 1926; Kopeloff and Beerman, 1926, 1927; Pizarro, 1927) a lowered sur-

face tension may have an inhibiting effect upon bacteria, especially upon *Staphylococcus aureus* and *Corynebacterium diphtheriae*, both of which are readily inhibited by the filtrate from Fleming's mold culture. Samples of nutrient broth, nutrient broth plus filtrate, undiluted filtrate and synthetic medium were tested. The results obtained after many readings on the Cenco-DuNoüy tensiometer are given in table 1. It seems unlikely that this reduction of surface tension is sufficient to cause the marked inhibitory effect noted.

SUMMARY

1. The production of an inhibitory material by molds is not common. Only one of many species studied produced it.

2. The inhibitory substance studied is selective in its action, affecting relatively few bacteria and these of types apparently not closely related.

3. The pigment produced by Fleming's mold is not identical with the inhibitory material.

4. Light, oxygen, hydrogen and carbon dioxide prevented the production of the inhibitory material or destroyed it.

5. The inhibitory substance is relatively thermo-stable.

6. The inhibitory substance is not obtained free from the rest of the filtrate by dialysis, adsorption or distillation at low temperature.

7. The charge upon the bacteria inhibited by the mold filtrate is not changed by the filtrate.

8. Several enzymes were found present in the filtrate containing the inhibitory substance.

9. The surface tension of the broth upon which Fleming's mold culture is grown is decreased slightly.

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