CHAETOMIN, A NEW ANTIBIOTIC SUBSTANCE PRODUCED BY CHAETOMIUM COCHLIODES

II. Isolation and Concentration[1, 2]

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INTRODUCTION

It has been shown (Waksman and Bugie, 1944) that a certain strain of *Chaetomium cochliodes* produces an antibiotic substance, designated as chaetomin, that is highly active against gram-positive bacteria but has little effect upon gram-negative organisms. A further study of the concentration and purification of the chaetomin yielded a highly potent preparation that inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus* in dilutions of 1:500,000,000 or even greater. The following report deals with the extraction and purification of the chaetomin, as well as with some of the chemical and biological properties of this highly potent preparation.

PRODUCTION AND ISOLATION OF CHAETOMIN

Cultivation of C. cochliodes. The glucose-nitrate or Czapek-Dox medium, supplemented with calcium carbonate and with certain accessory growth substances in the form of corn steep liquor or yeast extract, was found most suitable for the growth of the organism and for the production of the chaetomin. The medium was placed in shallow layers in flasks, sterilized, inoculated with the organism, and incubated at 28 C. After 10 to 15 days the chaetomin was extracted from the medium.

It was soon found that the mycelium of the organism was very rich in chaetomin. When this mycelium was extracted with acetone, the chaetomin obtained was about twenty times as great as the yield of the substance in the culture filtrate. The addition of calcium carbonate was of no advantage in this connection, but the corn steep liquor or yeast extract gave decidedly favorable results. Corn steep liquor is preferred to yeast extract because the latter often leads to troublesome emulsions during extractions. As brought out in table 1, the active substance is produced in the mycelium most rapidly from the sixth to the tenth day of growth, whereas the antibacterial activity of the filtrate remains nearly constant after 6 days' incubation. This leads to the suggestion that the chaetomin is produced in the mycelium and is secreted in the medium until the latter becomes saturated, the degree of saturation depending upon

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various factors, such as growth, nature of nutrients, and reaction. The excess chaetomin accumulates in the mycelium.

For the production of chaetomin, the above medium supplemented with 0.5 per cent corn steep liquor and neutralized to pH 7.0 with sodium hydroxide was used, and the culture harvested in 10 to 12 days. It has been shown elsewhere (Waksman and Bugie, 1944) that the production of chaetomin is characteristic of one particular strain of *C. cochliodes* and is not formed by other species of *Chaetomium* or even by strains of this species when obtained from other sources. In order to demonstrate that this is also true of the mycelium of the organism, the following experiment was conducted:

A strain of *Chaetomium* was isolated from a natural source (an infected piece of cloth suspended in a moist chamber) and tested for antagonistic activity by streaking it across the center of a fungus-agar plate. After growth had appeared in 2 days, *Staphylococcus aureus*, *Bacillus mycoides*, *Bacillus subtilis*,

TABLE 1

Yields of chaetomin per liter of medium

Czapek-Dox corn steep medium with and without $CaCO_3$ used

S. aureus dilution units

INCUBATION	CHAETOMIN FROM FILTRATE		CHAETOMIN FROM MYCELIUM	
	Without $CaCO_3$	With $CaCO_3$	Without $CaCO_3$	With $CaCO_3$
days				
2	200,000		10,000	
4	100,000		800,000	
6	200,000	500,000	3,000,000	3,000,000
8	300,000	500,000	8,000,000	8,000,000
10	200,000	200,000	10,000,000	10,000,000
12	200,000	200,000	10,000,000	10,000,000
14	200,000	100,000	10,000,000	10,000,000
16	100,000	100,000	10,000,000	10,000,000

and *Sarcina lutea* were streaked perpendicularly to the first streak. The first and last organisms were both inhibited to some extent by the growth of the *Chaetomium*.

Two liters of Czapek-Dox medium, containing 0.5 per cent corn steep liquor, and two liters of a glucose-peptone salt solution medium were inoculated with the above *Chaetomium*. After 10 days' incubation at 28 C, the bacteriostatic activity of the culture filtrate and of the acetone extract of the mycelium was determined. The results indicated no activity whatsoever either in the culture filtrates of the two media or in the acetone extracts of the mycelium obtained from the two media. A similar experiment was performed using a strain of *Chaetomium globosum* with the same results.

Extraction of chaetomin from mycelium. The procedure for the extraction of chaetomin from a culture of *C. cochliodes* is shown diagrammatically in figure 1. The liquid medium is filtered off, and the mycelium is collected and extracted

at room temperature three times for one day each with acetone, using a total of 2 liters for the extraction of mycelium from 10 liters of medium. The acetone is evaporated to about 400 ml under diminished pressure, and the aqueous liquid remaining is then extracted with three 200-ml portions of ethyl acetate. The addition of 25 g of sodium chloride before the first extraction helps to prevent the formation of emulsions. The ethyl acetate extracts are combined and washed twice with a 5 per cent solution of sodium bicarbonate, then twice with a 5 per cent solution of sodium carbonate, and finally with water, until neutral. The washed extract is evaporated to dryness under diminished pressure, and the residue dried in a vacuum desiccator over sulfuric

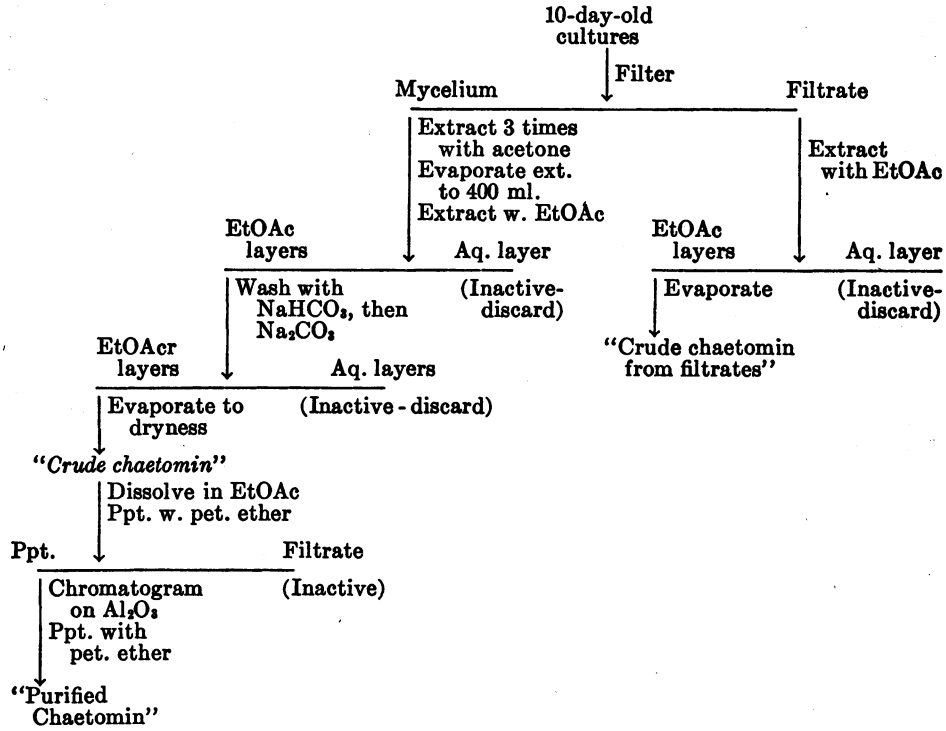

FIG. 1. PURIFICATION OF CHAETOMIN

acid to constant weight. From 1.0 to 1.5 g of residue, termed *crude chaetomin*, is usually obtained.

The crude chaetomin is dissolved in 10 ml of ethyl acetate and slowly added with stirring to 100 ml of ice-cold petroleum ether ("Skelley-solve"). The precipitate is centrifuged off, washed twice with cold petroleum ether, and dried *in vacuo*. The dried solid forms a light tan powder, with a melting point of 200 to 210 C. About 1 g of material is usually obtained from the mycelium of 10 liters of medium. The material is further purified by chromatography on a column of Merck's alumina according to Brockmann. For this purpose the chaetomin is dissolved in 50 ml of benzene and poured upon a column 2 cm in

diameter and 18 cm long. The column is then washed once with 50 ml of benzene and twice with 100 ml of benzene containing 10 per cent absolute methanol. The benzene-methanol eluates are collected and evaporated to dryness *in vacuo*. The residue is dissolved in 5 ml of ethyl acetate and slowly added with stirring to 30 ml of ice-cold petroleum ether. The precipitate is centrifuged off and dried in a vacuum desiccator over sulfuric acid. The yield ordinarily amounts to about 350 mg. The activity of this material is equivalent to 90 per cent or more of the total bacteriostatic activity of the original acetone extract of the mycelium.

Extraction of culture filtrate. The chaetomin present in the culture filtrate has also been extracted by the following procedure: Ten liters of filtrate were treated three times with ethyl acetate, using a total of 2 liters of the solvent. The extract was concentrated to about 500 ml by evaporation under diminished pressure and washed three times with 200-ml portions of 5 per cent sodium bicarbonate, three times with 200-ml portions of sodium carbonate, and finally with water, until neutral. Evaporation of the ethyl acetate under diminished pressure gave a brown, gummy residue of crude chaetomin, weighing about 100 mg. This material was found to possess the same bacteriostatic spectrum and the same general stability to chemical reagents as the chaetomin obtained from the mycelium; the two preparations are, therefore, considered as identical in nature.

BIOLOGICAL AND CHEMICAL PROPERTIES OF CHAETOMIN

Bacteriostatic spectrum. The chemical separations of the various chaetomin fractions described above were always accompanied by determinations of their bacteriostatic activity, using the agar plate dilution method. A series of typical results, expressed in dilution units, are given in table 2.

When the crude chaetomin was tested against various gram-negative bacteria such as *Escherichia coli* and *Proteus vulgaris*, no activity was observed at as low a dilution as 1:10,000. It had some activity, however, against *Mycobacterium phlei*, comparable to that against *B. mycoides*; the activity against both avian and human strains of *Mycobacterium tuberculosis* was equivalent to 0.1 per cent of the activity against *B. subtilis*. As pointed out previously, chaetomin resembles penicillin in many respects, although it shows much greater activity against *B. mycoides*.

Chemical properties of chaetomin. The purified chaetomin sintered at 200 to 205 C and melted at 218 to 220 C to a red liquid that did not solidify on cooling. It was readily soluble in acetone, ethyl acetate, chloroform, benzene, dioxane, and pyridine; less soluble in ether and methyl or ethyl alcohol; and insoluble in water and petroleum ether. Chemical analyses indicated that the material was probably not yet in a completely pure state as different lots were found to vary in nitrogen content from 10.0 to 10.5 per cent and in sulfur content from 12.5 to 14.0 per cent. A typical lot contained 54 per cent carbon and 4.6 per cent hydrogen. The material has not been obtained as yet in a crystalline form.

Solutions of chaetomin in alcohol may be prepared by warming the chaetomin to 40 to 50 C. It is best dissolved in a little acetone and diluted with alcohol to the required volume.

True aqueous solutions cannot be prepared, but stable aqueous emulsions can readily be obtained. If small amounts of alcohol are not objectionable, emulsions stable for several hours may be prepared by diluting an alcoholic solution of the substance with distilled water. A solution of 10 mg of chaetomin in 1 ml of alcohol gives a satisfactory emulsion when added to 100 ml of water.

Alcohol-free emulsions may be prepared with the help of gum acacia and will remain stable in the refrigerator for weeks. Ten mg of chaetomin in 1 ml of alcohol were added to 5 ml of a sterile (Seitz filtered), 3 per cent solution of gum acacia. Two drops of amyl alcohol were used to prevent frothing, and the solution was evaporated to dryness in a vacuum desiccator over calcium chloride. The dried residue formed a stable emulsion on rubbing with 5 ml of water. Such solutions can be diluted further, and solutions of higher concentration can be obtained by using less water.

TABLE 2

Yield of chaetomin in various extracts of culture of C. cochliodes

Dilution units per gram of preparation

	S. AUREUS	B. MYCOIDES	B. SUBTILIS	SARCINA LUTEA
Acetone extract of mycelium.....	80,000	30,000	80,000	100,000
Crude chaetomin.....	100,000,000,	50,000,000	100,000,000	200,000,000
Purified chaetomin.....	500,000,000	100,000,000	500,000,000	1,000,000,000
Culture filtrate.....	300	200	300	300
Crude chaetomin from filtrate....	50,000,000	20,000,000	50,000,000	100,000,000

Chaetomin is not impaired in activity by heating to 100 C in suspension in water for 10 minutes, and no spontaneous loss in activity has been observed when it stands in alcoholic solution at room temperature. The substance was found to be stable to 0.01 M alcoholic hydrochloric acid or 0.1 M aqueous citric acid at room temperature for 24 hours. Its activity was destroyed completely under the same conditions by 0.01 M alcoholic potassium hydroxide or 5 per cent aqueous sodium carbonate.

Chaetomin resembles gramicidin in solubility, in bacteriostatic spectrum, and in having a high melting point, but it differs greatly in composition. Chaetomin gives no biuret test for peptide groups, even by the sensitive procedure of T. B. Osborne; and Millon's reaction for tyrosine or tryptophane and the dimethylaminebenzaldehyde test for tryptophane were negative.

The bacteriostatic properties of chaetomin were compared with those of penicillin as reported by Waksman and Bugie (1944). Among chemical differences the following may be noted: Chaetomin does not seem to be an acid but a neutral compound, as the method of purification and also the nonimpairment of its activity *in vitro* by treatment with diazomethane indicate. Also, it is not

affected by the enzyme penicillinase, although the preparation used destroyed penicillin rapidly. The greater stability of chaetomin to heat and acid is another important difference from penicillin.

Like gliotoxin, chaetomin is a neutral sulfur compound. The sulfur of chaetomin is much more firmly bound than that of gliotoxin, for no lead sulfide is formed on heating with sodium plumbite and no sulfide or sulfate is liberated on heating with aqueous alkali. It also differs from gliotoxin in its bacteriostatic spectrum, since gliotoxin acts against certain gram-negative bacteria against which chaetomin is inactive.

SUMMARY

The results of this study on the isolation and purification of chaetomin may be summarized as follows:

Chaetomin, an antibacterial substance produced by *Chaetomium cochliodes*, is found both in the culture filtrate and in the mycelium of the organism.

Chaetomin has been found to be present in much larger quantities in the mycelium than in the culture filtrate.

Chaetomin is extracted from the filtrate by ethyl acetate and from the mycelium by acetone, and is repurified by washing with sodium bicarbonate and sodium carbonate. It is further purified by treatment with petroleum ether and by chromatographic absorption.

Chaetomin contains nitrogen and sulfur, but differs greatly in biological activity from gliotoxin and penicillin. Although it is similar in some respects to tyrothricin, it differs considerably from this antibiotic substance as well.

Chaetomin is largely active against gram-positive bacteria and has little if any effect upon gram-negative organisms. Preparations having 500,000,000 or more *Staphylococcus aureus* units have been obtained.

REFERENCE

WAKSMAN, S. A., AND BUGIE, E. 1944 Chaetomin, a new antibiotic substance produced by *Chaetomium cochliodes*. I. Formation and properties. J. Bact., 48, 527.