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CYATHIN, A PREVIOUSLY UNKNOWN ANTIBIOTIC COMPLEX

by

© BHAVDISH NARAIN JOHRI

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cyathin, A Previously Unknown Antibiotic Complex" submitted by Bhavdish Narain Johri in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

A new antibiotic complex from *Cyathus helenae*, named cyathin is described herein. Of this complex, six components have been purified to date. Empirical formulae for four of these have been determined. These are:

Cyathin B, $C_7H_6O_4$ (2,4,5-trihydroxybenzaldehyde)

Cyathin A_5, $C_{20}H_{26}O_5$

Cyathin A_4, $C_{20}H_{30}O_4$

Cyathin A_3, $C_{20}H_{30}O_3$

Cyathin has a broad spectrum of antimicrobial activity; it is active against Actinomycetales, gram-positive and gram-negative bacteria (Eubacteriales) and many fungi including 'dermatophytes'. Cyathin has been found to be highly heat-stable and is little affected by pH. Like many other known antibiotics, cyathin is bacteristatic at low concentrations, and is bactericidal at higher concentrations.

The production of cyathin is mainly limited to *Cyathus helenae*, but is also a characteristic of *C. striatus*. However, the chromatographic spectra of the culture broth of the two species differ as do also the titres of cyathin. Cyathin production is not a property of all cultures of *C. helenae*; not all strains tested exhibited the presence of antibacterial substances. Different sectors of one strain of *C. helenae* (1500-102) were found to produce different titres of cyathin. The chromatographic spectrum obtained from the concentrated

culture broth did not differ greatly from that obtained from the mycelium suggesting a close relationship among the sectors.

Cyathin appears to exert its antibacterial effect through the inactivation of -SH groups. However, it is possible that it impairs the synthesis of macromolecules.

For fungal growth and cyathin production, dextrose, maltose, d-fructose and starch proved to be suitable carbon sources; suitable nitrogen sources were sodium nitrate, ammonium nitrate and calcium nitrate when they were used along with asparagine. The maximum cyathin titres were achieved by using dextrose along with calcium nitrate and asparagine. Chromatographic analysis of the culture broth obtained when various carbon and nitrogen sources were used, revealed that the change of a single component in the medium can alter the production of the various components of the cyathin complex; the appropriate combination of carbon and nitrogen sources makes possible a high yield of any particular component of the cyathin.

Cyathin is toxic to roots of some green plants even at low concentrations. A concentration as low as 6 µg/ml of cyathin retarded the growth of tomato roots *in vitro*. Root hairs appear to be markedly affected. However, the shoots of the plants tested withstood cyathin concentrations as high as 1.25 mg per plant.

ACKNOWLEDGEMENTS

I wish to thank Dr. H. J. Brodie for his advice and encouragement during the course of this study. His help with the photography and editing of the manuscript has been most generous.

Sincere thanks are due to Dr. F. D. Cook for his invaluable suggestions and providing the shaker and cultures of the micro-organisms. Dr. L. L. Kennedy, Dr. J. W. Carmichael and Dr. F. L. Jackson were very kind to provide the cultures of micro-organisms and this help is gratefully acknowledged. Thanks are also due to Dr. G. D. Weston for carrying out the test on excised tomato roots.

The chemical aspects of cyathin reported in this thesis were carried out by Mr. Hubert Taube, Dr. A. D. Allbutt and Dr. W. A. Ayer of the Chemistry Department. I would like to thank them for the collaboration and allowing me to use some aspects of the chemical work in writing this thesis.

Financial support in the form of a Graduate Service Assistantship and later through the NRC funds from Dr. Brodie is gratefully acknowledged.

Finally, I wish to thank Miss Jean Andruchow for taking up the enormous task of typing the manuscript in the short time at her disposal.

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Chemistry of the Cyathin Complex

INTRODUCTION

For some years biologists have recognized that many kinds of fungi produce a great variety of antibiotic substances; for a time, however, the number of species of fungi tested (or 'screened') was much smaller than the number of Actinomycetes screened. Lately, emphasis has shifted towards attention to the fungi, and this has been especially so following the report by Beneke (1963) of the production of calvacin by *Calvatia gigantea*.

Changes in the focus of attention have also occurred in other respects. In 1951, when Brian reviewed the status of the antibiotics produced by fungi, research was being directed mainly towards the search for substances which had anti-bacterial activity. However, when Broadbent (1966) published a survey of the antibiotic activity and of the kinds of antibiosis exhibited by fungi, it was realized not only that the list of fungi known to have antibiotic properties had become markedly augmented, but also that among the fungi there are some which produce anti-tumor substances. Ruelius *et al.* (1968) when describing poricin (an anti-tumor 'principle' obtained from *Poria corticola*) were able to report that more than 7000 species of the Basidiomycetes had been screened for anti-tumor principles in their laboratory. Recently a scrutiny of fungi derived from forest soils and cultivated soils regarding anti-mycotic activity has been reported by Blunt and Baker (1968). These two reports

direct attention to the fact that extensive surveys are necessary in order that drugs having various kinds of antimicrobial and anti-tumor activity may continue to be discovered. The continuing need for new antibiotics arises, at least partly, from the survival and development of strains of bacteria (and other kinds of organisms) which are resistant to an antibiotic to which a particular micro-organism population was originally susceptible. This problem has been posed frequently in medical and other publications and a discussion of it will not be belabored here.

Examination of reviews of research concerning antibiotics, such as that published by Brian (1951) and that by Broadbent (1966), reveals that, of the antibiotics--exclusive of anti-tumor substances--synthesized by fungi, the majority are produced by the 'microfungi', i.e. those groups of fungi in which, mostly, there are not developed large fruiting structures (Bilai, 1963); 'macrofungi', especially the Basidiomycetes, appear not to have interested researchers to a great extent as possible sources of antibiotics. The reason for this may be that most of the latter fungi are more difficult to manipulate in culture than are the 'microfungi', such as the Deuteromycetes. For example, the spores of most members of the Agaricales or of the Gasteromycetes germinate slowly and in small proportion and their mycelia are slow-growing. In contrast, spores of the Deuteromycetes commonly germinate rapidly, and the ensuing mycelium usually develops rapidly.

One of the most complete surveys devoted to the antibiotics produced by Basidiomycetes was reported by Wilkins (1952, 1954) whose investigation of the possible antibiotic activity of the Basidiomycetes dealt especially with the families Polyporaceae and Agaricaceae. Wilkins confined his attention to a search for bacteristatic substances and he made no attempt to seek the equally important anti-virus, anti-tumor and anti-fungus potentialities of the fungi screened. To some extent, the gap was bridged by the work of Bekker *et al.* (1961) and of Bekker and Suprun (1962) who examined wood-destroying fungi as possible sources of anti-phytopathogens and of cytotoxic substances.

With minor exceptions, almost all antimicrobial activity among 'higher' Basidiomycetes is, at present, ascribed to members of the families Polyporaceae and Agaricaceae. There is one exception, as far as the writer is aware; some note should be taken of this because it is relevant to the main topic of this thesis. In his preliminary survey of the presence or absence of antibiosis among the Basidiomycetes, Wilkins (1954) reported tests involving three members of the gasteromyceous family Nidulariaceae, namely, *Crucibulum laeve* (his "*C. vulgaris*"), *Cyathus olla* and *C. striatus*: of these, only *C. striatus* exhibited any demonstrable bacteriostasis. No further investigation of the activity of the latter fungus was published by Wilkins and, apparently, the report has

gone un-noticed.

In 1966, Brodie (1966) described a previously unrecognized species of Bird's Nest Fungus (from the Canadian Rockies) which he named *Cyathus helenae* Brodie (see Plate I). Brodie grew the fungus in pure culture from single basidiospores which yielded monokaryon mycelia; also, dikaryon mycelia were obtained by the planting of whole peridioles.

A. Olchowecki, studying *Cyathus helenae* in culture, early in 1967, made the chance observation that the mycelium inhibited the growth of bacteria with which some culture plates had accidentally become contaminated. Because investigation into the nature of the observed antibiosis was not germaine to the main subject of Olchowecki's thesis (by then almost completed) he did little more than some preliminary testing of the action of the antibiotic.

However in that thesis (Olchowecki, 1967) he was able to report the results of testing the action of the crude filtrate, obtained from mycelium of *C. helenae* grown on liquid medium, against a few bacteria; these were *Staphylococcus aureus*, *Micrococcus* spp., *Escherichia coli*, *Serratia* sp.

The antibiotic, which was named 'cyathin', inhibited the growth of most of the tested organisms; however, it was clear that the action of cyathin was more pronounced against some micro-organisms than against others.

It was further reported by Olchowecki (1967)

PLATE I *Cyathus helenae* Brodie

The photograph shows the essential macroscopic features of the species; *viz.* the vaguely fluted nature of the silvery interior of the fruit body; the massive basal 'emplacement'; the peculiar tufted tomentum and the elliptical, rough peridioles. The photograph is provided by courtesy of Dr. H. J. Brodie (from the type specimen). x 4.5

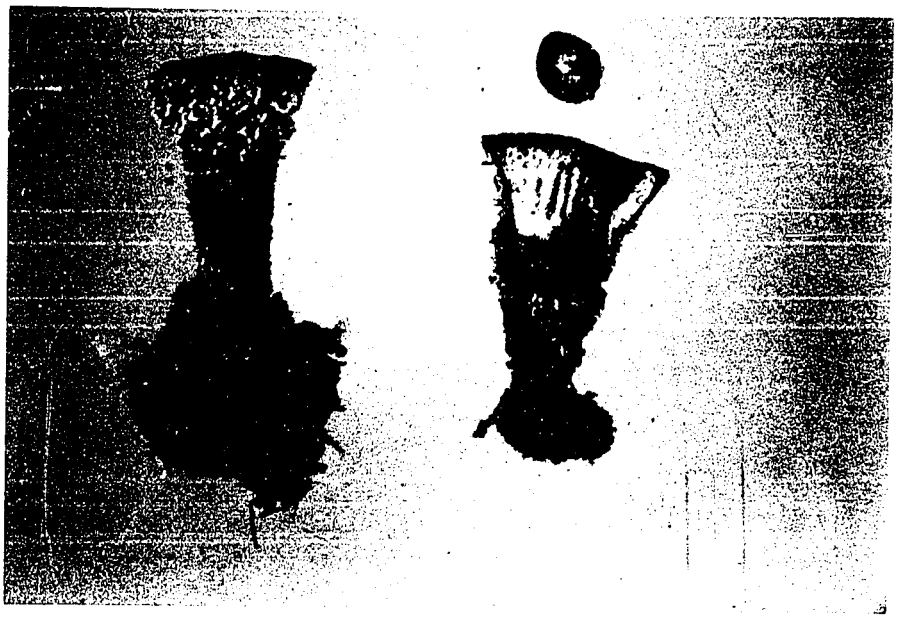

PLATE I

that cyathin is quite heat-stable and that its production is, apparently, enhanced by using a medium five times as concentrated as that employed for growth in routine culture.

Of other species of the genus *Cyathus* screened for antibiosis, some indication of positive reaction was reported by Olchowecki for *C. striatus*, *C. limbatus* and *C. poeppigii*.[1]

Olchowecki and Brodie (1968) recently published a genetic and morphological study of *Cyathus helenae* and of *C. striatus* which indicated the relationship between the two species. In the original description, Brodie (1966) pointed out this relationship, and he stated, as well, that there are marked differences between the two species which allow each one to be readily recognized. A further indication of physiological relationship is afforded by Olchowecki's report that both species are the only species of the genus *Cyathus* known to produce antibiotics in appreciable amount.

The purpose of and plan for the investigations reported hereunder evolved from consideration of the facts and postulates presented in the preceding pages; briefly, the original intentions were:

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[1] It was not so stated in Mr. Olchowecki's thesis, but Dr. Brodie reported (personal communication) that the antibiosis referred to above was barely detectable, except for the antibiosis produced by *C. striatus*; even that was feeble in comparison with the effect produced by *C. helenae*.

(1) To examine the antimicrobial action spectrum of cyathin.

(2) To develop a completely chemically-defined culture medium which might give good growth of the fungus as well as cyathin titres.

(3) To examine the effects of environmental factors (physical and chemical) upon the production and yield of cyathin, making use of the chemically-defined medium.

(4) To isolate, purify and identify chemically the antibiotic substance(s) at present called 'cyathin'.[1]

(5) To re-examine other species of *Cyathus* and various strains of *C. helenae* for possible antibiosis with a view to establishing differences between species or strains regarding antibiotic activity, if such differences exist.

---

[1] The purely chemical aspects of the project were carried out by Dr. W. Ayer, Dr. A. D. Allbutt and Mr. Hubert Taube of the Department of Chemistry, University of Alberta. The chemical work is referred to in various places throughout the thesis but the details are recorded in the Appendix. All purely biological work, however, was done by the writer in the laboratories of the Department of Botany.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Cultures of *Cyathus* spp. were provided from the collection of Prof. H. J. Brodie. *Cyathus helenae*, strain 1500-102 (Haploid), was the strain used chiefly throughout the investigations reported herewith. Other fungus cultures used for testing the action of cyathin were obtained from the collection of Dr. L. Kennedy or from the Department of Plant Pathology, University of Alberta. The cultures of Actinomycetes and some bacterial cultures were provided by the kindness of Prof. F. D. Cook of the Soils Department. Most of the cultures of bacteria were obtained from Prof. F. L. Jackson of the Department of Bacteriology. Cultures of 'dermatophytes' were obtained from Dr. J. W. Carmichael of the Provincial Laboratory in Edmonton.

Cultures of *Cyathus* were maintained on normal 'Brodie Medium' which has the following composition: Dextrose--2.0 g; Maltose--5.0 g; Yeast Extract--2.0 g; Asparagine--0.2 g; Peptone--0.2 g; Glycerol--6 ml; KH_2PO_4 --0.5 g; $Ca(NO_3)_2 \cdot 4H_2O$--0.5 g; $MgSO_4 \cdot 7H_2O$--0.5 g; $Fe_2(SO_4)_3$--trace; Distilled Water--1 liter. Other fungus cultures were maintained on the latter medium and also on Sabouraud's Maltose Agar (Difco). Dermatophytes also were maintained on Sabouraud's medium. Except for pathogenic forms, other bacterial cultures as well as actinomycetes were maintained on Difco Nutrient Agar slants. For pathogenic bacteria a Difco Blood Agar medium was used. All cultures were stored at 5°C

in a cold room and to maintain vigor, were transferred to fresh slants from time to time. Unless otherwise indicated, all media were autoclaved at 15 lb/sq inch pressure for 15-20 min at a temperature of 120°C. The ingredients of the media were either Difco 'certified' products or reagent--grade chemicals from the Fisher Company.

Extraction of and Large-Scale Production of Cyathin

Transfers of mycelium of the fungus *Cyathus helenae* were grown throughout as 'Still-Surface' cultures. Brodie Medium, without agar and containing 2% dextrose (but no maltose), was used during the earlier part of the work. A quantity of 200 ml or 400 ml of this medium was dispensed into a 500 ml or a 1000 ml Erlenmeyer flask and autoclaved. The medium was inoculated with three or four 8.0 mm discs of mycelium of *Cyathus helenae* taken from a 16 to 18-day-old colony growing on Brodie solid medium (see Plate II for cultural characteristics). During the summer of 1968, as much as 45 liters of broth was extracted. The mycelium was removed by passing the broth from cultures (25-day-old) through a thick layer of cheesecloth. The broth was then extracted using an equal volume of ethyl acetate. The yellowish ethyl acetate extract, along with some aqueous dark-colored impurities, was again passed through a thick layer of cheesecloth. The resulting yellow liquid was reduced to dryness under vacuum at 35°C. This operation produced a reddish-brown powder, highly soluble in acetone or ethyl

PLATE II Cultural Characteristics of *C. helenae* (1500-102)

Fig. 1. Growth on Brodie's Agar medium. Note the regular fluffy growth of the fungus. Mycelium appears slightly yellow-brown in color. Reverse gives a dark red-brown color due to the release of cyathin and other pigments.

Fig. 2. Growth on chemically defined medium with added yeast extract. Note irregular growth and formation of 'sectors'. The medium gives a dark red-brown color due to the cyathin complex and some other pigments. Actual size.

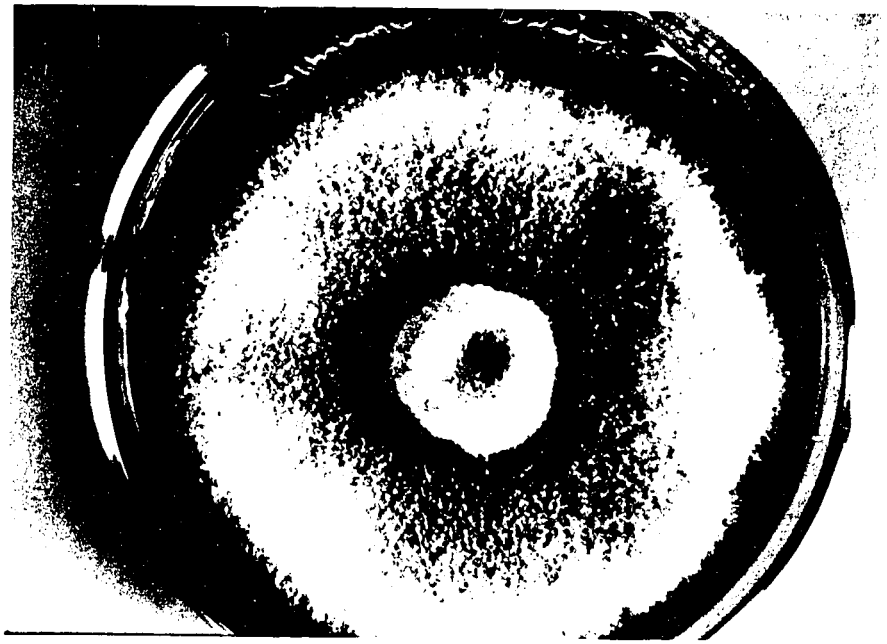


Fig. 1

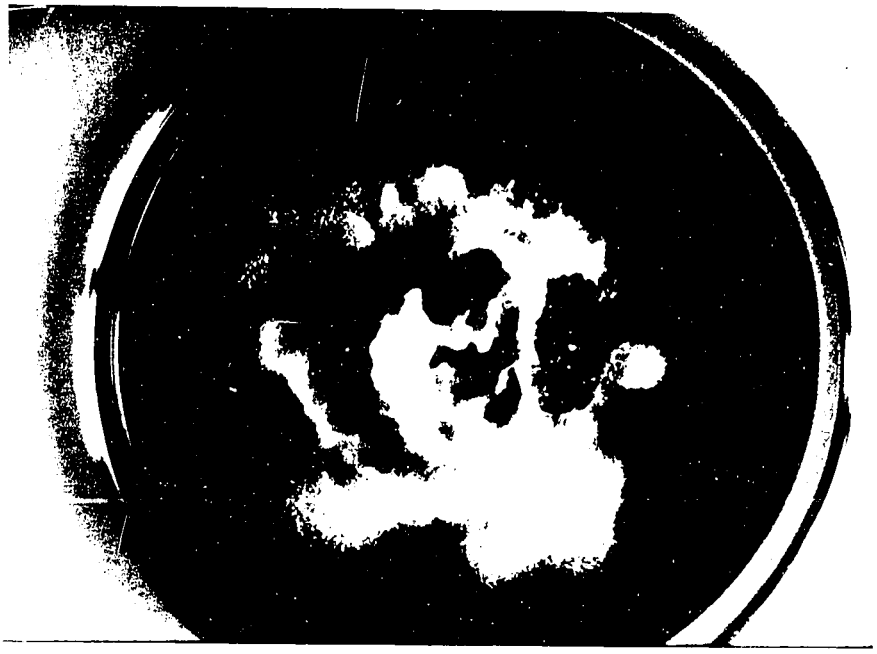


Fig. 2

PLATE II

acetate. Further purification was achieved by passing the extract through activated charcoal to remove colored impurities. For further analysis, this residue was submitted to the members of the Chemistry Department for isolation and characterization of the active substances.

During the latter part of the study, a chemically-defined medium was developed. The filtrate from cultures grown on this medium yielded a higher titre of cyathin than when Brodie liquid medium was used. Further large-scale extraction was then carried out using the broth from the chemically-defined medium. The procedure for extraction was the same in either case. The defined medium has the following composition: Dextrose--30 g; Asparagine--1.5 g; KH_2PO_4--1.0 g; $Ca(NO_3)_2 \cdot 4H_2O$--0.5 g; $MgSO_4 \cdot 7H_2O$--0.5 g; $ZnSO_4 \cdot 2H_2O$--250 µg; Thiamine Hydrochloride--150 µg; Distilled water to make 1 liter.

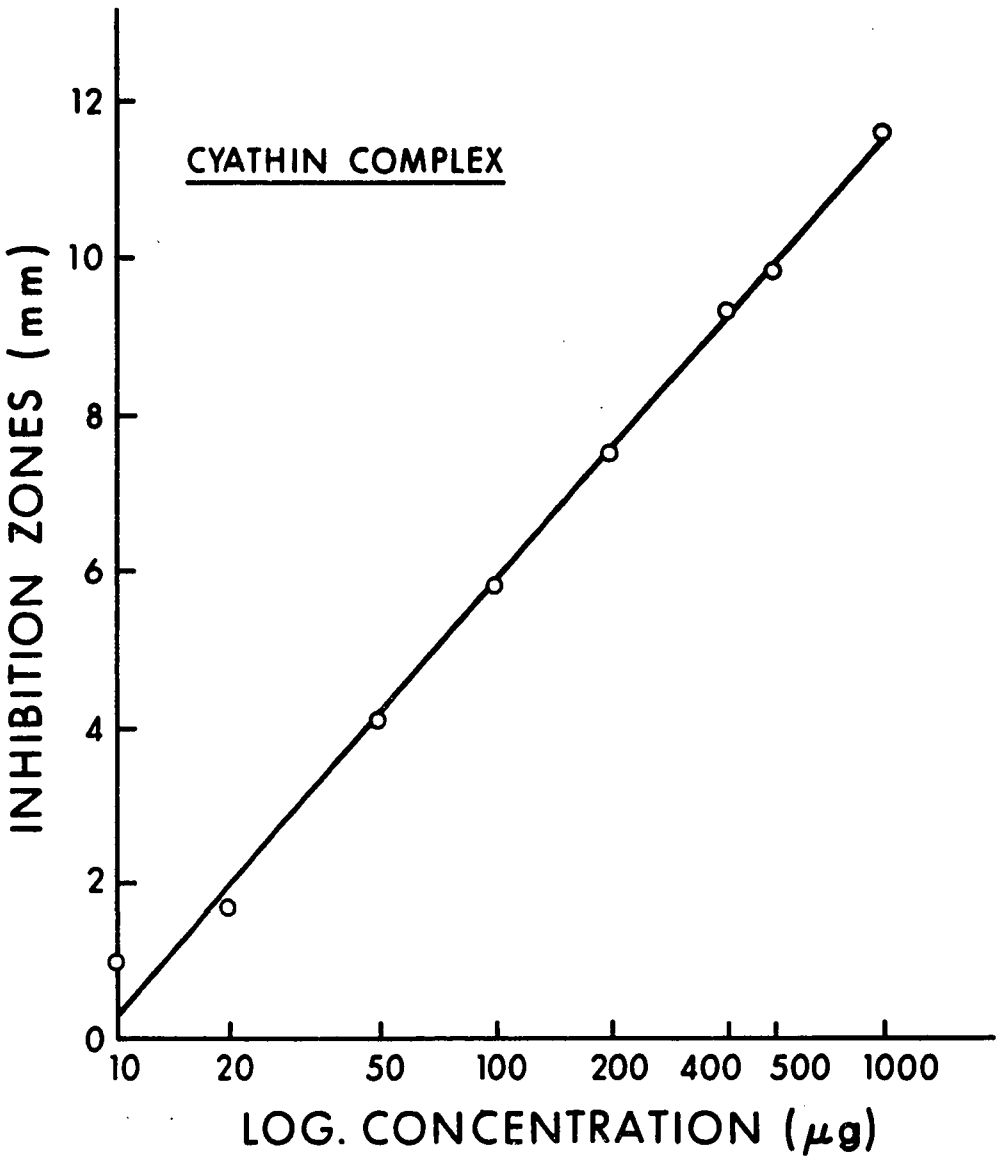
As much as 125 liters of medium was subjected to extraction to provide sufficient amounts of cyathin for microbiological as well as chemical studies.

From one quantity of filtrate derived from a culture grown on the defined medium, a working 'standard' curve for the activity of cyathin was obtained by measuring the inhibition zones produced by different concentrations of cyathin incorporated on to filter paper discs (Plate III). The test organism used was *Staphylococcus aureus*. The procedure of microbiological assay is dealt with in the following section.

PLATE III Concentration Curve of Cyathin

Obtained by the microbiological assay (See Materials and Methods for details) against *Staphylococcus aureus*.

PLATE III


CYATHIN COMPLEX
INHIBITION ZONES (mm)
0
2
4
6
8
10
12
10
20
50
100
200
400
500
1000
LOG. CONCENTRATION (µg)

Microbiological Assay of Cyathin

The procedures for antibiotic testing prescribed by the Food and Drug Administration (US/FDA) and the Pharmacoepia of the United States and as mentioned in the Difco Manual (1966) were followed throughout this investigation.

A paper-disc-agar-plate method for the assay of antibiotic substances (DeBreer & Sherwood, 1945) was used. The test organism was a strain of *Staphylococcus aureus* (2053) which is characterized by its resistance to 10 µg penicillin discs (obtained from Difco). Disposable plastic Petri plates (100 x 20 mm) were used and 20 ml of Difco Antibiotic Medium No. 2 were poured as a base layer. After solidification of this base layer a top layer of Difco Antibiotic Medium No. 1 was poured. The top layer included the inoculum which had been prepared by suspending bacterial cells from a 24 hr-old nutrient-agar slant in 5 ml of sterile saline solution and adding this aseptically to 1 liter of the medium. A quantity of 5 ml of this inoculum was poured on top of the base layer to provide an even surface and a uniformly luxuriant growth of bacteria. Clear, sharp zones of inhibition were obtained using the method described. Plates were stored in a cold room at 5°C and removed only when required for cyathin testing.

Filter paper discs, 12.7 mm diameter, made of highly absorbent paper were obtained from Carl Schleicher and Schnell Co., U.S.A. Discs were dipped in the test solution, drained free of excess liquid and three discs were

placed on each bacteria-seeded plate. Plates were incubated at 37.5°C ±1 and the inhibition zones were measured after 18 hr of growth. For standard curves, discs were not dipped in the cyathin solutions; rather, known amounts of cyathin were pipetted on to the discs which were then placed on the seeded plates. Discs bearing only the solvent were used as control.

The minimum inhibitory concentration (abbreviated MIC) of cyathin (or of one of its fractions) was determined by a serial dilution procedure using Bacto-Penassay Broth (Difco).

Antimicrobial Spectrum

Stock cultures of actinomycetes and bacteria were maintained on Nutrient Agar Slants (Difco); cultures of fungi were maintained on Sabouraud's Maltose Agar or on Brodie medium.

Inoculum from the actinomycetes and bacteria was prepared as follows: cells from a fresh slant were washed either in sterile distilled water (for actinomycetes) or with sterile saline (for bacteria). The light transmittance of a 'Spectronic-20 Colorimeter' was adjusted to 20% at 600 mμ, and 1 ml of the suspension was added to 100 ml of the proper medium. Sterile glass Petri plates were used, and 20-25 ml of seeded medium were poured into each plate. Discs soaked with cyathin were placed on each plate, and observations were recorded after 24-48 hr, depending upon the growth rate of the test organism. For fungi, the suspension was

added to 100 ml of the medium. Normally, discs were not placed on plates immediately; a 24-48 hr period elapsed before the cyathin-bearing discs were placed on plates; observations were made after 24-72 hr. The inhibition zones surrounding each of ten discs were measured for any one particular test. The diameters of the inhibition zones did not deviate from one another by more than 0.1-0.3 mm.

Chromatography and Bio-Autography

Although, during the earlier stages of the research, paper chromatography was employed, thin-layer chromatography (TLC) proved to be of much more value; therefore, in the latter part of the work, only TLC was used. Either chromatograms from Gelman Co., coated with silica gel G of 250 μ thickness or glass plates were used. Preparation of glass plates and the procedure of TLC was followed as outlined by Stahl (1963). All solvents used were Fischer reagent-grade chemicals. Glass chambers lined with filter papers were used, and plates were deactivated at room temperature before being used. Gelman sheets were more useful in bio-autography than plates, because the silica gel with a binder did not adhere to the agar surface. Bio-autography of glass plates was unsuccessful using the techniques available from the literature (Narsimhachari and Ramchandran, 1967; Hamilton and Cook, 1968). For bio-autography of Gelman sheets, standard assay plates were used. Chromatograms were placed on the assay plates for 30 min to 1 hour at 5°C to allow the diffusion of antibiotics into the medium.

Plates were then incubated at 37.5°C ±1 for 18 hr and the bioactive spots were located. Thin-layer plates were sprayed with a 30% solution of sulfuric acid and heated at 100°C from 5-10 min. The spray proved very useful, and the spots were differently colored at first, although the colors faded if the plates were left too long. At times, iodine solution was also used for improving the distinctness of the spots on chromatograms. The spots were mostly brownish or bluish.

Environmental Factors and Cyathin Production

A chemically-defined medium (abbreviated DMIII) with the following composition was used throughout this work; any deviations are mentioned in the Results section: Dextrose--30 g; Asparagine--1.5 g; KH_2PO_4--1.0 g; $MgSO_4 \cdot 7H_2O$--0.5 g; $Ca(NO_3)_2 \cdot 4H_2O$--0.5 g; Thiamine HCl--150 µg; Distilled Water--1 liter.

A quantity of 30 ml of the defined medium was poured into each 125 ml Erlenmeyer flask. Either cotton plugs or sterile disposable foam plugs were used for closure. Each flask was inoculated with an 8.0 mm disc of mycelium of *Cyathus helenae* taken from a 16-18-day-old culture growing on Brodie agar medium. The size of the mass of inoculum and the procedure for growing the fungus were constant throughout the study.

Fungal growth was measured by the dry-weight method. For each record, 3-4 flasks were used. The results are expressed on the graphs in the form of bars to show the range of variation of results. After the required

number of days of growth, mycelium was removed and placed on a pre-weighed filter paper. The filtrate was used to test the concentration of cyathin. Mycelium from each flask was washed with 150 ml of distilled water and air-dried. After drying in air, it was placed in an oven at 100°C for 24 hr and dried to constant weight. A second weight of the filter paper plus mycelium was measured, and the dry weight of the fungus was calculated. The pH of the broth was also determined from time to time.

Method of Studying the Effect of Cyathin on Seed Germination and Growth of Seedlings

(i) Seed Germination

Seeds of Timothy (Canada No. 1), Tomato (Bounty), Pea (variety unknown) and Radish (Scarlet globe) were obtained from Robertson Seeds Ltd., Edmonton.

Seeds were pre-soaked for a half hour in distilled water and were then placed on a filter paper moistened with cyathin solution (2 ml); finally, they were placed in a glass Petri plate. The lid of the Petri plate was lined with filter paper moistened with water to maintain high humidity. Plates were placed in a controlled growth chamber providing a light intensity of 660 ft c and a day length of 12 hr. Temperature was maintained at 65°F during the daytime and 55°F during the night.

Germination of seeds was recorded at 24 hr intervals up to a period of 10 days. Two different concentrations

of cyathin, 180 µg/ml and 360 µg/ml were used in this experiment.

Another experiment was run at room temperature to observe the effect of presoaking seeds of the various green plants in cyathin and the subsequent effect on their germination. A cyathin solution of concentration 90 µg/ml was used, and all the seeds were left for a 24 hr period in this solution. They were then washed thoroughly with distilled water and allowed to germinate, as in the first experiment. Radish seeds grew fast and gave 100% germination if soaked in distilled water; consequently the effect of 24-hr presoaking of Radish seeds in various concentrations of cyathin and their subsequent germination pattern were also studied.

(ii) Seedling Growth

In one set of experiments, 3-day-old seedlings of radish were transferred to a Petri plate lined with absorbent cotton moistened with 10 ml cyathin solution at a concentration of 360 µg/ml. Subsequent effects on the growth of the seedlings were recorded.

To avoid direct contact of the whole plant with cyathin, in another set of experiments, 6-day-old seedlings of radish, tomato and pea plants were inserted through cotton plugs into 25 x 52 mm glass tubes containing 10 ml sterile Hoagland's solution each plus an amount of cyathin in the following five concentrations: 10 µg/ml, 18 µg/ml, 38 µg/ml, 45 µg/ml, and 340 µg/ml.

For each concentration, five replicate seedlings

were used. Tubes were placed in a controlled growth chamber and the morphological effects on root and shoot system were observed at intervals. Control seedlings in each case were kept in Hoagland's solution without added cyathin. During the later period in the experiment, when the total amount of the solution declined by evaporation or absorption, any addition of Hoagland's solution was without cyathin; thus the above five concentrations added to the original 10 ml of solution represent also the total amount provided to each seedling during its growth.

In all the seed germination or seedling studies, a partially purified sample of cyathin was used. (organic solvent extracted)

Effect of Cyathin on Excised Tomato Roots

A tomato root tip 10 mm in length and obtained from a single clone, was inoculated into 50 ml of sterile modified White's Medium (Sheat *et al.*, 1959) dispensed in a 125 ml Erlenmeyer flask. Cyathin solutions of four different concentrations were added aseptically after autoclaving the medium at 15 lb/sq inch for 8-10 min. Five replicate flasks were used for each cyathin concentration. The flasks were incubated at 27.5°C ±1 for 7 days and growth of the main axis was recorded.

RESULTS

Antimicrobial Spectrum

The results of the activity of cyathin against Actinomycetes, Bacteria and Fungi are presented in Table I. Six Actinomycetes were tested and all of them were sensitive to cyathin. Among these *Mycobacterium rhodoerinchii* showed the largest zones of inhibition. The most striking of all was the reaction of *Streptomyces griseus* (Plate IV) as this organism itself is known to produce several antibiotics (Herr and Reusser, 1967; Szeszak and Szabo, 1967).

Gram-positive as well as Gram-negative bacteria responded to cyathin, but the former were more sensitive. All species of *Micrococcus* (Plate V) tested proved to be sensitive. *Staphylococcus aureus* 'penicillin-sensitive' as well as 'penicillin resistant' strains were found to be sensitive (Plate V). However, *Escherichia coli* showed only small zones of inhibition at the concentration at which all the organisms were tested. This was also true for *Serratia* sp. and *Proteus mirabilis*. Among pathogenic bacteria, only *Pneumococcus multocida, Corynebacterium diphtheriae, Clostridium welchii, Diplococcus pneumoniae, Streptococcus pyogenes, Neisseria meningitidis* and *Haemophilus parainfluenzae* were sensitive and they are listed here in the decreasing order of sensitivity to cyathin (Plate VI). It may be noted that none of these pathogenic forms, however, exhibited large zones of inhibition. On the other hand, *Agrobacterium tumefaciens*--the cause of

TABLE I

Antimicrobial Action Spectrum of Cyathin[1]

Organisms Tested	Inhibition Zone (mm)[2]
Actinomycetales	
4463 (unidentified)	14.5
4471 (unidentified)	13.5
Unidentified	12.5
J_2 (unidentified)	22.0
Mycobacterium rhodoerinchii	24.5
Streptomyces griseus	19.0
Eubacteriales	
Aerobacter aerogenes	2.0
Agrobacterium tumefaciens	30.5
Bacillus pumilis	5.0
B. subtilis	14.0
Escherichia coli	1.5
Lactobacillus casei	9.5
Micrococcus aurantiacus	14.5
M. candidus	14.5
M. conglomeratus	13.0
M. cyaneus	12.0
M. denitrificans	12.5
M. flavus	12.0
M. freudenerichii	8.5
M. luteus	10.5
M. roseus	14.0
M. varians	12.0
Pediococcus cerevisae	7.0
Proteus mirabilis	2.5
Pseudomonas denitrificans	13.5
Serratia sp.	5.0
Streptococcus faecalis	7.5
Human Pathogens[3]	
Clostridium welchii	++
Corynebacterium diphtheriae	++
Diplococcus pneumoniae	+
Escherichia coli	-
Haemophilus parainfluenzae	+
Neisseria meningitidis	+
Pneumococcus multocida	+++
Proteus sp.	-
Pseudomonas sp.	-
Salmonella enteritidis	-
S. thompsoni	-
Shigella sonnei	-
Staphylococcus aureus (penicillin resistant)	+++

Table I. Continued

Streptococcus sp.	-
S. pyogenes	+
Fungi	
Aspergillus amstelodami	3.5
A. leporis	++
A. nidulans	+
A. niger	3.0
Candida krusei	+
Chaetomium sp.	16.0
Dipodascus sp.	1.5
Fusarium solani	8.0
Gliocladium aureus	6.5
Mucor genevensis	+
Penicillium claviforme	++
P. commune	++
P. digitatum	7.0
P. ducklauxii	+++
P. expansum	+
P. lilacianum	4.0
P. notatum	6.0
P. raistrickii series	+
P. restrictum	5.0
P. roqueforti	-
Phoma sp.	7.5
Rhizopus sp.	+
Schizosaccharomyces octosporus	2.5
Rhodotorula sp.	+
Torulopsis sp.	+
Trichoderma album	7.0
T. lignorum	6.0
Ustilago hordei	+
Zygosaccharomyces daperi	+
Dermatophytes	
Arthroderma uncinatum	+
Microsporum canis	9.0
M. cookii	5.5
Trichophyton mentagrophytes	3.0
T. rubrum	7.0
T. terrestre	8.5

[1]Average of ten observations
[2]Cyathin concentration 480 µg per disc
-No inhibition
+Zones not well defined; (++, +++: Comparatively well marked inhibition)
[3]Tests were carried out at the Department of Bacteriology (Courtesy Dr. F. L. Jackson)

PLATE IV Action of Cyathin against Actinomycetes

Fig. 3. *Streptomyces griseus*.

Fig. 4. An unidentified actinomycete (4483).

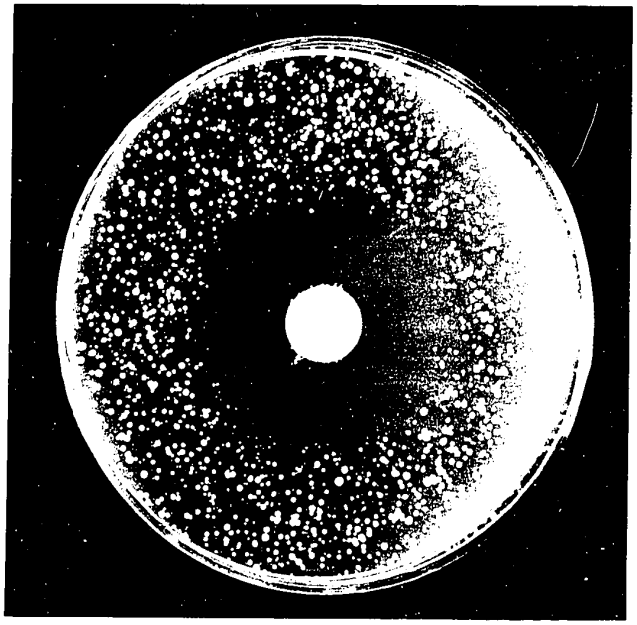


Fig. 3

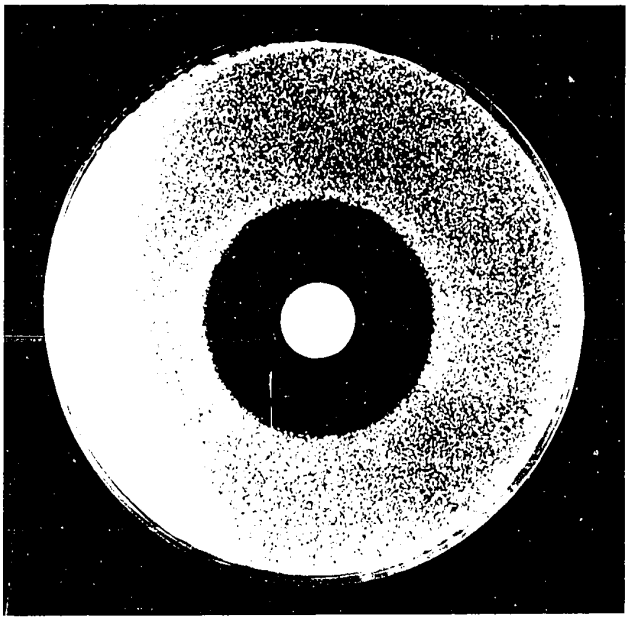


Fig. 4

PLATE IV

PLATE V Action of Cyathin against Bacteria

Fig. 5. Action of chemically synthesized cyathin B (see details in the text) against a penicillin-resistant strain of *Staphylococcus aureus*.

Fig. 6. *Micrococcus luteus*.

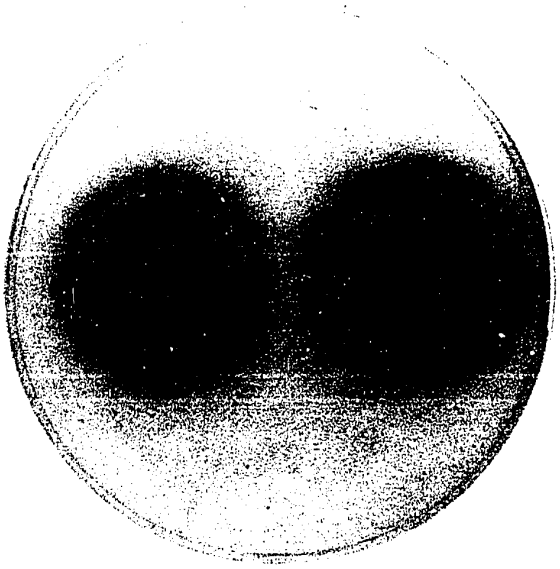


Fig. 5

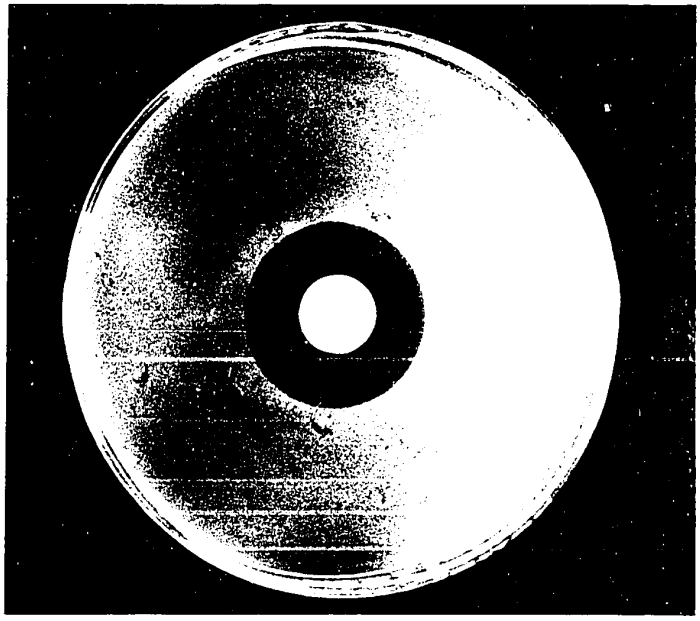


Fig. 6

PLATE V

PLATE VI Action of Cyathin against Bacteria

Fig. 7. *Corynebacterium diphtheriae.*

Fig. 8. *Pneumococcus multocidia.*

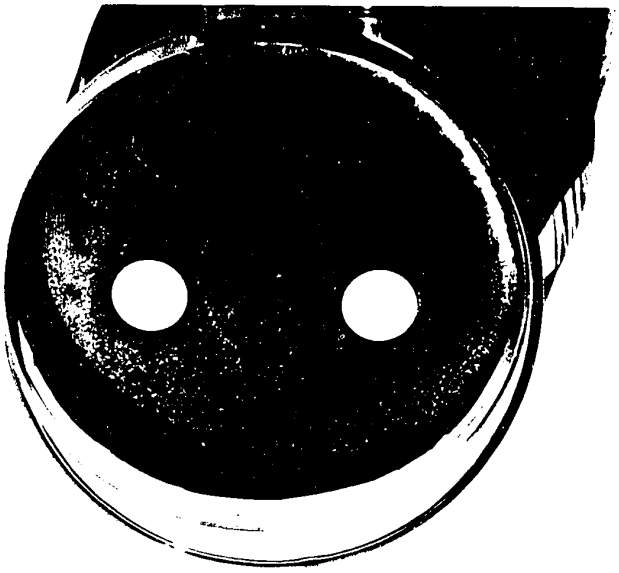


Fig. 7

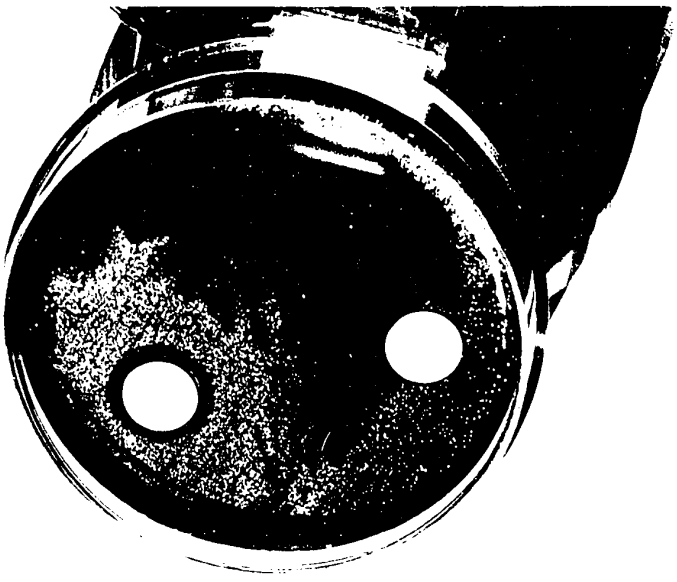


Fig. 8

PLATE VI

Crown Gall disease of many horticultural plants was found to be the most sensitive of all the organisms screened. Although no further efforts regarding *Agrobacterium* have been made in this study, it would be interesting to do so, as Crown Gall is a serious plant disease frequently of economic importance.

All the fungi tested against cyathin were sensitive to some degree. Most of the sensitive ones belong to the class Deuteromycetes (Alexopoulos, 1961); the maximum number of antibiotic-producing fungi also belong to this group. The most striking examples of fungi sensitive to cyathin include species of *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, *Chaetomium* and *Gliocladium* (Plate VII). A species of *Chaetomium* was found to be the most sensitive fungus. However, none of the members of the Phycomycetes proved to be sensitive to cyathin. This was true for the yeasts also, as none of them showed marked zones of inhibition.

Seven pathogenic and skin-disease-causing fungi ('Dermatophytes') were tested and all were found to be sensitive to cyathin. The most striking examples were *Microsporum canis*, *Trichophyton rubrum* and *T. terrestre*; all these three showed zones of inhibition varying from 7 to 9 mm.

Screening of *Cyathus* Species and Strains of *C. helenae* for Cyathin Production

Although the early studies of cyathin were conducted using one strain of *C. helenae* (1500-102), it was assumed that different strains might well have different

PLATE VII Action of Cyathin against Fungi

Fig. 9. *Trichoderma lignorum.*

Fig. 10. *Penicillium digitatum.*

Fig. 9

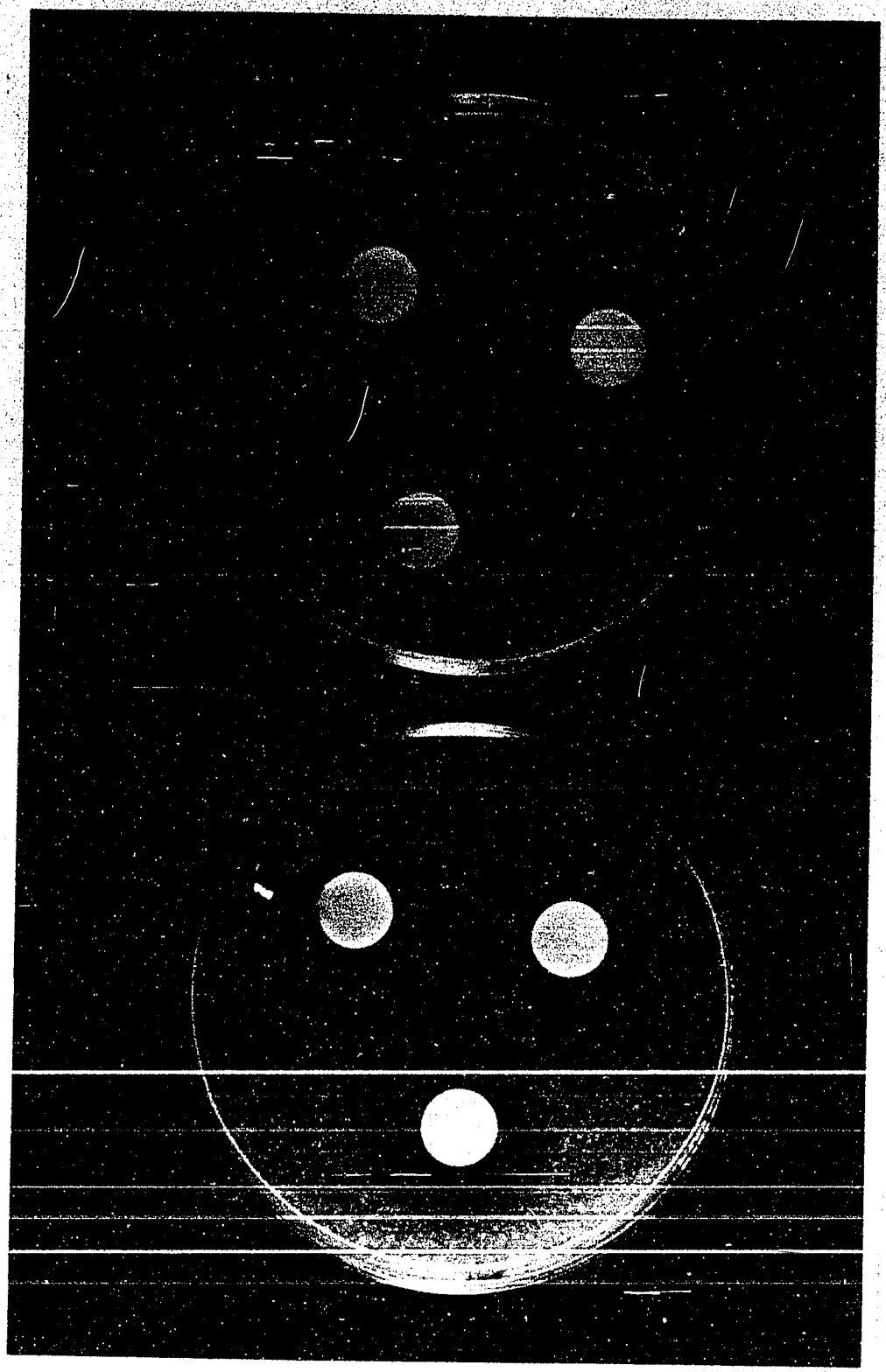


Fig. 10

PLATE VII

capacities to produce cyathin, and it is very likely that a higher yielding strain of *C. helenae* could be found. The numbers 1500 and 67076 refer to two different collections of *C. helenae*. Comparison of different strains for cyathin production was made on a liquid Brodie medium containing dextrose as the only sugar at a concentration of 10 g per liter. The results are presented in Table II. In all, 14 strains were tested, 8 representing collection 1500, and 6 representing 67016. Three strains from 1500 namely, 5, 110 and 102f and two strains from 67016 namely, 4 and 5, were found to produce cyathin. The amount of cyathin varied according to the strain; also the time of highest cyathin titres showed differences. In this respect the strain 67016-4 is most striking: for this strain cyathin production continued for 35 days, whereas in all other strains it dropped between 22-26 days. The maximum titres of cyathin, however, were noted in 67016-5 which gave a value of 245 µg/ml of cyathin at 22 days, although no cyathin could be detected on the 26th day.

Brodie (1966) emphasized the relationship of *C. helenae* to *C. striatus*. The production of antibacterial substance(s) from the latter fungus has also been reported (Wilkins, 1954). It was tempting to try to find out whether or not the pattern of antibiotic production was comparable in the two species. In Table II, it may be seen that the strain of *C. striatus* tested did produce the antibacterial substance and that the pattern resembled that of the other

TABLE II

Screening of Species of *Cyathus* and Strains of *C. helenae* for Cyathin Production

Organism	Cyathin (µg/ml)[1]					
	Days					
	15	20	22	26	30	35
C. helenae						
1500-5	-	33.5	41.5	-	-	-
-6	-	-	-	-	-	-
-22D	-	-	-	-	-	-
-102F	105.0	28.5	90.0	-	-	-
-110	130.0	+	42.5	-	-	-
-120	-	-	-	-	-	-
-127	-	-	-	-	-	-
-150	-	-	-	-	-	-
67016-	-	-	-	-	-	-
-2N	-	-	-	-	-	-
-3	-	-	-	-	-	-
-4	-	+	150.0	?	45.0	27.0
-5	22.5	70.0	245.0	-	-	-
-6	-	-	-	-	-	-
C. striatus						
66145-6	-	47.5	95.0	-	-	-

- No cyathin production
+ Inhibition zones small and undefined
[1] Average of triplicate observation
? No observation recorded

strain of *C. helenae* in the 'time *vs* cyathin' relationship. However, the level of cyathin production was not very high for *C. striatus* and the duration of production was also slightly shorter. A comparison of *C. helenae* 1500-102f, 67016-5 and *C. striatus* (66145-6) with that of the most used strain of *C. helenae* (1500-102) was also made chromatographically (TLC) in Benzene-Acetone-Glacial Acetic Acid (75:25:1). The chromatogram is shown in Plate VIII. The pattern of the various spots does differ, but this is also true for all the four strains. Therefore, to draw any conclusions on this basis alone regarding the relationship or the differences between *C. helenae* and *C. striatus* is rather difficult, although the latter fungus does have a different chromatographic pattern and the striking spot with pink-brown color (Rf-0.4) is absent from chromatograms of any strain of *C. helenae*.

Sectors and Cyathin Production

Olchowecki and Brodie (1968) demonstrated the formation of sectors (saltants) in *Cyathus helenae*. During the present work also, morphological variations in cultures growing on Brodie medium were noticed. The possibility that physiological strain differences might be associated with morphological variation led to the examination of some sectors which appeared to be different from the parental stock culture. Seven different types of sectors were selected for growing on Brodie liquid medium (dextrose 10 g

PLATE VIII Chromatogram of the culture broth from *Cyathus helenae* (1500-102, 1500-102f, and 67016-5) and *C. striatus* (66145-6) at the 22nd day. Solvent system--Benzene:Acetone:Acetic Acid (100:25:1). Letters for different colors of the spots have been abbreviated as follows: B--Black; Br--Brown; Y--Yellow; G--Green; Gr--Grey; Li--Light; Bl--Blue; P--Pink. Numbers 1, 2, A_5, B, A_3 and A_4 on the left side of the plate correspond to different components of cyathin (refer to Table VII).

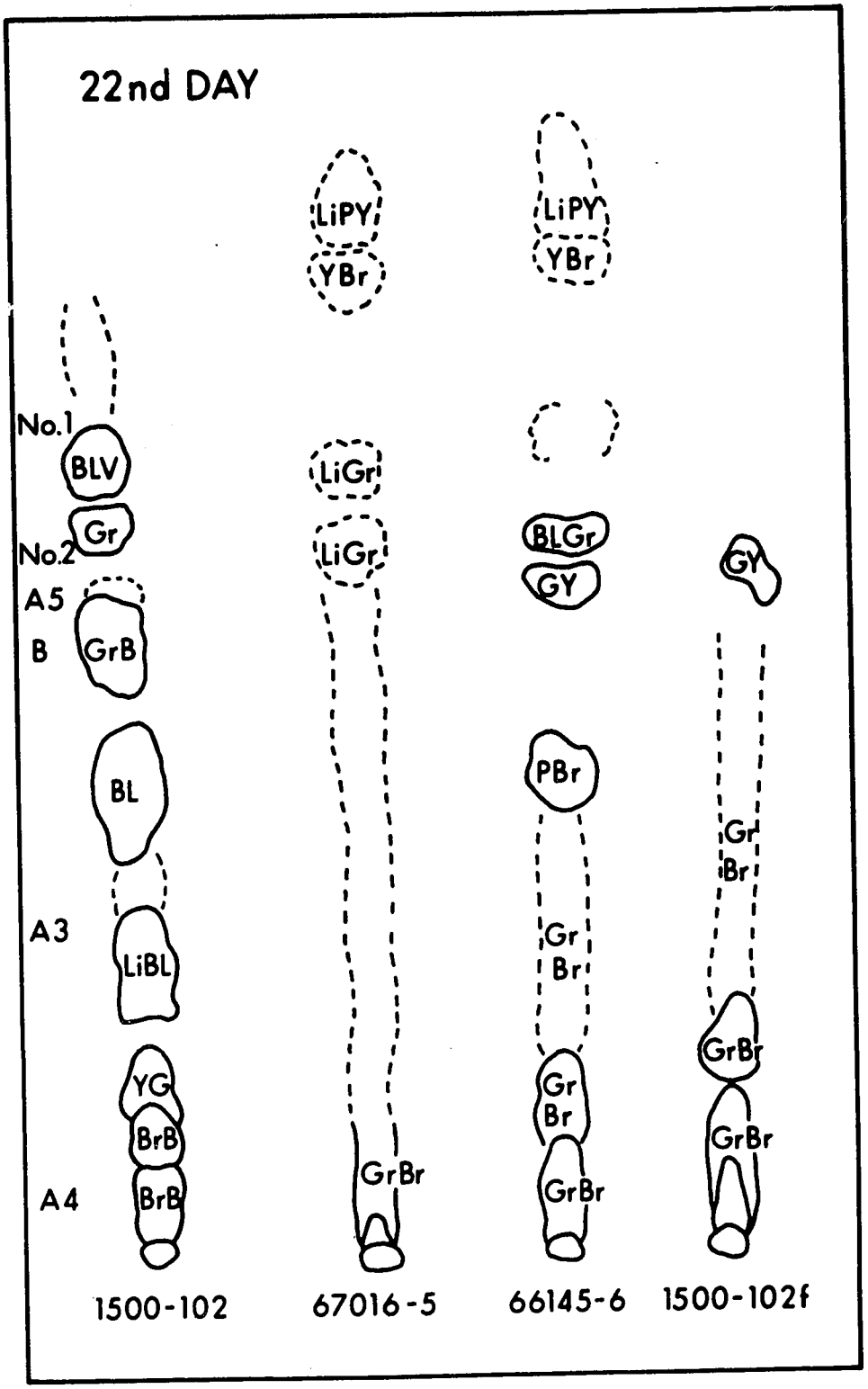

22nd DAY
No.1
BLV
Gr
No.2
A5
B
GrB
BL
A3
LiBL
YG
BrB
BrB
A4
LiPY
YBr
LiGr
LiGr
GrBr
LiPY
YBr
BLGr
GY
PBr
Gr
Br
Gr
Br
GrBr
GY
Gr
Br
GrBr
GrBr
1500-102
67016-5
66145-6
1500-102f

PLATE VIII

per liter; maltose deleted). The levels of cyathin production for each were measured at different day intervals. The results are presented in Table III. The numbers given to various sectors are purely arbitrary figures. It was observed that all seven sectors differed in the titres of cyathin; indeed one of them did not produce any noticeable amount of cyathin (No. 1). Sectors No. 3 and No. 6 were also unusual as to the amount of cyathin produced and the time required to reach this level. Maximum titres were obtained from Sector No. 0.

The difference in cyathin titres derived from the various sectors, led to a comparison of the nature of the cyathin complex from culture broth (exogenous) and from mycelium (endogenous), using TLC. The solvent system was Benzene:Acetone:Acetic Acid (75:25:1).

Two 'harvests' were taken, one at 20 days and the other one at 25 days. The culture broth was extracted using ethyl acetate in each case, and then reduced under vacuum. Residue was taken up in the minimum amount of acetone. Mycelium in each case was washed thoroughly with distilled water, air dried and ground using a mortar and pestle. Fine quartz sand was used to homogenize the mycelium. The homogenized mycelium was extracted with 50% methanol in acetone and filtered through a double layer of thick filter paper. The clear supernatant liquid was reduced in volume under vacuum and the residue dissolved in minimum amount of acetone. It should be noted that sector No. 4 is not

TABLE III

Screening of Sectors from *C. helenae* (1500-102) for Cyathin Production

Sectors	Cyathin (µg/ml)					
	Days					
	12	15	20	25	30	35
0	16.0	27.0	70.0	+	-	-
1	-	-	-	-	-	-
2	-	-	31.0	25.5	-	-
3	-	-	-	21.5	-	-
4	33.5	33.5	47.5	+	-	-
5	16.0	22.5	53.0	17.5	-	-
6	-	-	17.5	-	-	-

- No cyathin production

+ Zones of inhibition small and not well defined

listed among those for which chromatograms are given. The reason for this is that the flasks became contaminated during the experiment. Concentrated culture fluid extract as well as mycelial extract was spotted on to 500 μ thick silica gel G plates. The plates were developed in the above mentioned solvent system, dried and sprayed with 30% H_2SO_4. They were then heated at 100°C from 5-10 min. The chromatograms are reproduced in Plates IX and X. At the 20-day interval, cyathin B, A_3 and A_4 (for terminology, refer to Table VII) were all released into the culture medium while cyathin No. 1, No. 2 and A_5 were present mainly in the mycelium. One noteworthy feature of both chromatograms is the release of only polar materials to the medium. The non-polar materials mainly remained within the mycelium; whether this is the result of the solubility of different fractions in water or the diffusibility of separate fractions, has not been determined. At the 25 day interval the chromatographic spectra remained more or less the same, except that some of the non-polar fractions (cyathin No. 1, 2, A_5) leached out into the medium. Two new non-polar spots D and E also developed during this five day period. From the chromatographic analysis, it is quite certain that the relationship between different sectors seems to be fairly close, as far as the cyathin complex is concerned. The apparent absence of some spots from chromatograms of some sectors very likely reflects the low concentration of that fraction rather than its absence. A difference

PLATE IX Chromatogram of exogenous (broth) and endogenous (mycelium) cyathin from 'sectors' of *C. helenae* (1500-102), at the 20th day interval. Numbers 1-5, refer to various components of Cyathin (see Table VII). Spot C refers to an unidentified component. The top line of the illustration represents the solvent front.

PLATE IX

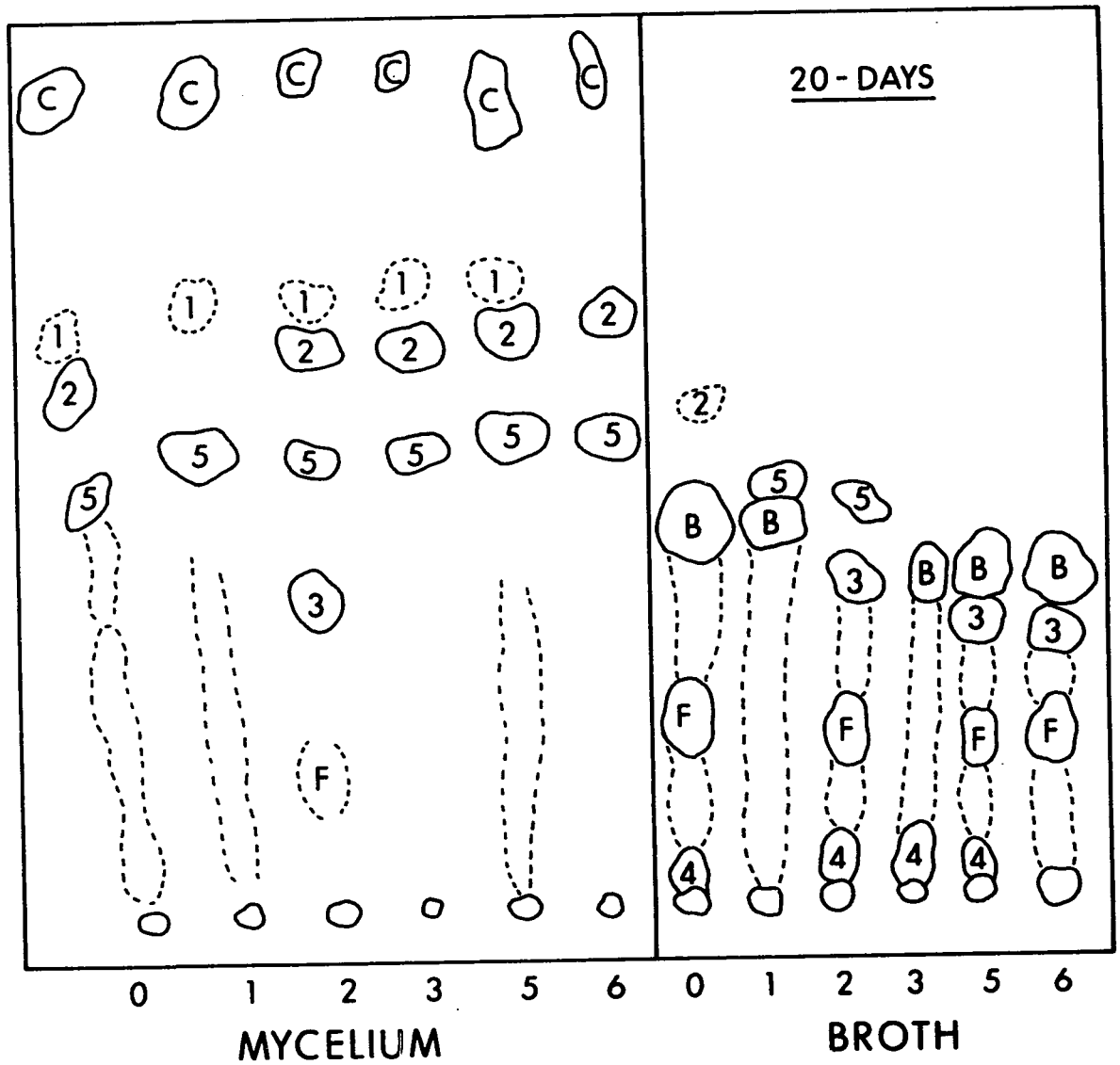
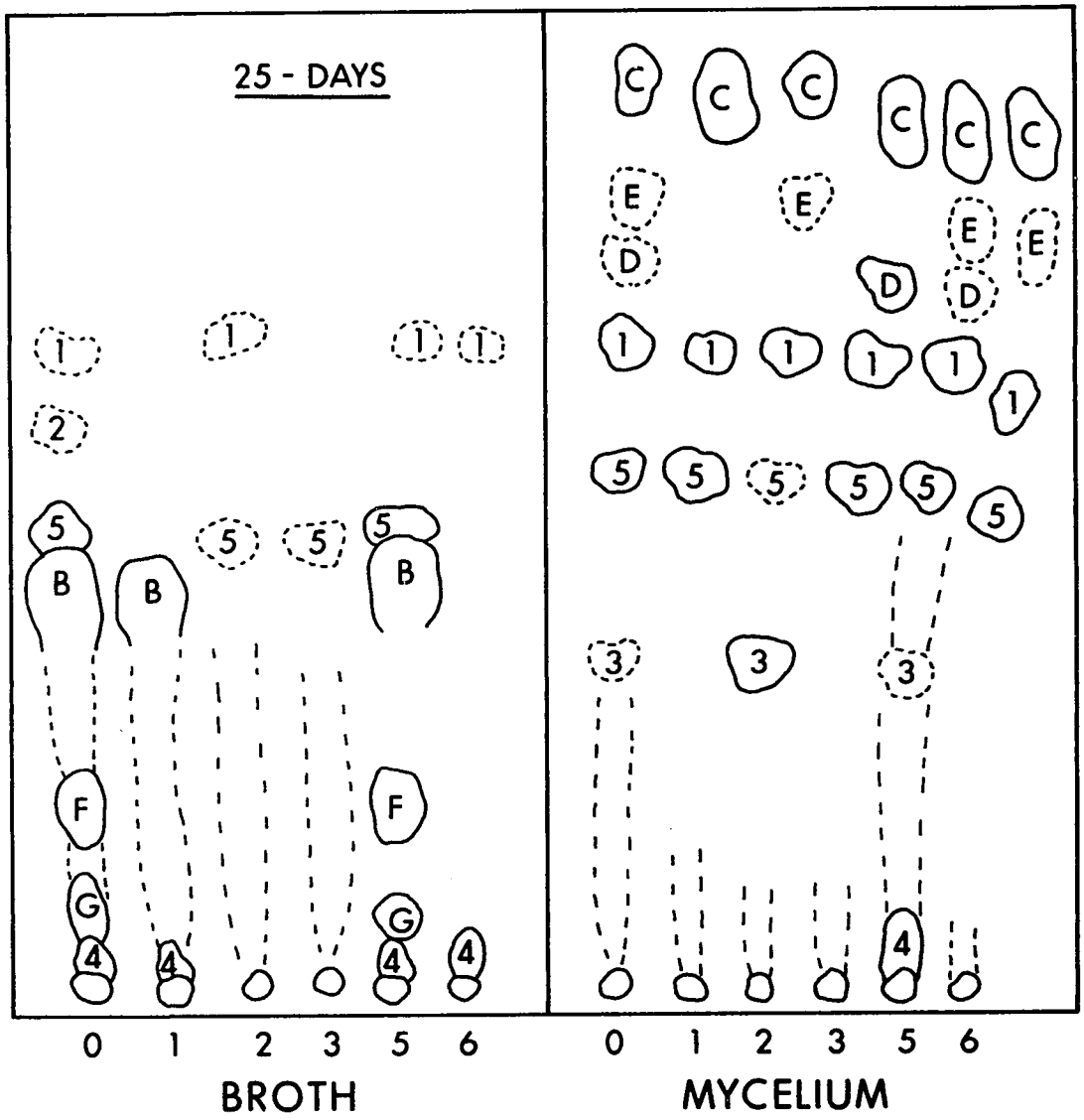


PLATE X Chromatogram of exogenous (broth) and endogenous (mycelium) cyathin from 'sectors' of *C. helenae* (1500-102) at the 25th day interval. Details same as in Plate IX. Spots C, D, E, F and G refer to unidentified components. The top line of the illustration represents the solvent front.

PLATE X



in the titres of cyathin from various sectors does not necessarily mean a difference in the components of the complex; probably, some sectors have a more active system for synthesizing cyathin than do others which could partly depend on the nutritional conditions of the medium.

The Cyathin Complex

An Approach to Systematic Analysis

It is common experience in research on antibiotics to find that some supposedly new antibiotic turns out to be a known one. In order to avoid this difficulty, an effort was made at an early stage to identify the active principles of cyathin and also to assess the probability that the antibiotic in question is not an already known one.

An instant thin-layer chromatographic (ITLC) method, devised by Aszalos *et al.* (1968) proved to be useful. The method makes use of movement of an antibiotic in a specific solvent system. The first three solvent systems α, β, and γ (refer to Table IV) yield four primary groups which are then subdivided on the basis of the use of 11 additional solvent systems. Aszalos *et al.* examined 84 known antibiotics by this procedure. Their method was employed *in toto* for the systematic analysis of cyathin. As may be seen from the Table IV, cyathin exhibited movement in all the first three solvent systems (α, β, and γ). Of the additional 11 solvent systems, cyathin did not

TABLE IV

ITLC of Cyathin

Groups	Solvent System	Rf
α	Methanol	0.4-0.8
β	10% Methanol in Chloroform	0.0-0.28 0.29-0.75
γ	Chloroform	0.0-0.08 0.14 0.48
Ia	Pyridine:Water (1:1)	0.4 0.61
Ib	Pyridine:Water:Ethanol (1:1:1)	0.44 0.48-0.72
Ic	Pyridine:Water:Ethanol (1:1:3)	0.4-0.64
IIa	n-Butanol:Methanol	0.72
IIb	Chloroform:Methanol	0.39 0.49-0.8
IIc	Absolute Ethanol	0.49-0.74
IIIa	Methanol:Benzene (12:88)	0.38 0.64
IIIb	Methanol:Benzene (6:94)	0.0
IIIc	Methanol:Benzene (4:96)	0.0
IVa	Methanol:Benzene (1:99)	0.0
IVb	Methanol:Benzene:Chloroform (1:49:50)	0.0

exhibit any movement in solvents IIIb, IIIc, IVa and IVb (Plate XI). This would place cyathin in subgroup IV-I of the scheme of Aszalos *et al*. This subgroup contains only four antibiotics, *viz*, actinobolin, DON, psicofuranine and spiramycin. Table V shows the Rf of these four antibiotics and of cyathin in solvents α, β, γ, IVa and IVb. It may be noted that cyathin did not match any of the four known antibiotics in all the solvents. Thus one big advantage this method offers is to cut down the choice of identities (four in the case of cyathin). Having established this much, cyathin was chromatographed on buffered silica gel sheets impregnated with 0.5 M KH_2PO_4 buffer solution of pH 2 or pH 11. The chromatograms were developed using the least polar solvents in which cyathin showed movement (Table IV). The results are expressed in Table VI. Chloroform, which separated cyathin very well on ordinary sheets, showed two bands at pH 2 and none at pH 11, when chromatographed on buffered sheets. This points to the fact that whole of the cyathin complex behaves as though basic in nature. In methanol, however, two bioactive spots were produced at pH 2 and one long band at pH 11. The latter solvent system which indeed is the least polar one in which cyathin shows movement, would indicate that both basic as well as acidic substances are present in the complex, or else that it has amphoteric substances, which no doubt, would appear acidic, basic or neutral in the two pH system. Despite all its advantages, the ITLC

PLATE XI Chromatogram of systematic analysis of cyathin complex. Eastman chromagram sheets were used and active principles identified by bio-autography against *Staphylococcus aureus*. For details refer to Table IV.

PLATE XI

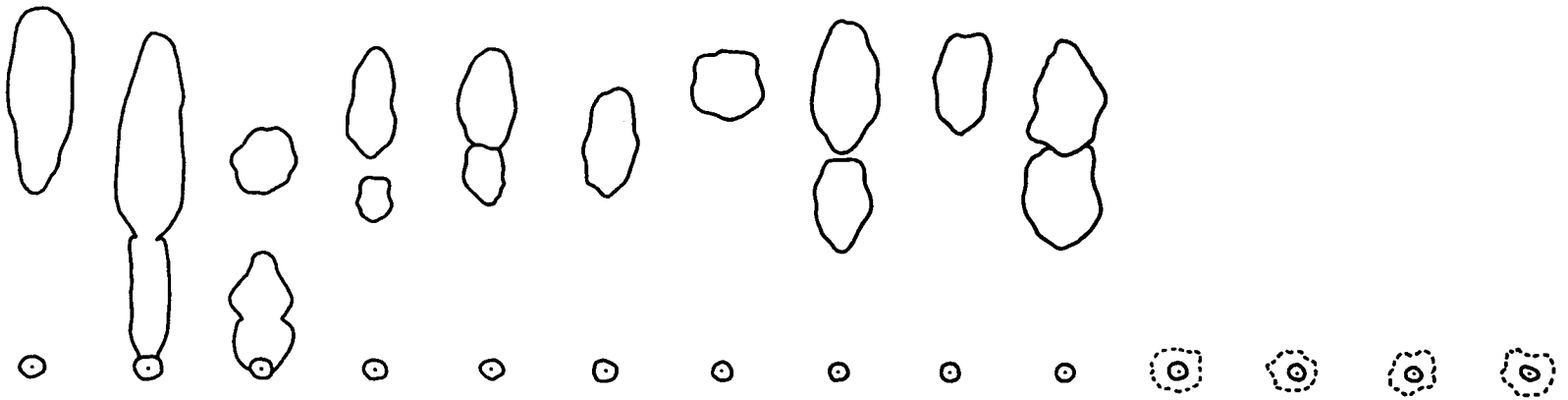


TABLE V

Comparison of Cyathin with Known

Antibiotics of Subgroup IV-I

Antibiotic	Solvent System				
	α	β	γ	IVa	IVb
Actinibolin	0.42	0.2	0.08	0	0
DON	0.78	0.76	0.65	0	0
Psicofuranine	0.7	0.15	0-0.1	0	0
Spiramycin	0.6	0.4	0-0.3	0	0
Cyathin	0.4-0.8	0-0.28 0.29-0.75	0.08 0.14 0.48	0	0

TABLE VI

Buffered ITLC of Cyathin

Solvent System	Rf	
	pH 2.0	pH 11.0
Chloroform	0-0.29 0.31-0.64	0
Methanol	0.39 0.78	0.34-0.99

system cannot differentiate chemically one antibiotic from another and also would not identify an individual antibiotic, if used alone. Further microbiological and chemical tests would permit the few antibiotics of a subgroup to be distinguished from each other. Of the two tests, the former has been dealt with in the section on Antimicrobial Spectrum, while the latter will be dealt with in brief below.*

Purification and Yield of Cyathin Components

For routine chemical analysis, a preparative thin layer chromatographic (TLC) procedure was used, the details of which are dealt with in the Appendix. To date, four active principles have been obtained in pure form and their empirical formulae determined; another two active principles have also been purified but their empirical formulae have yet to be determined. Until all the ingredients of the cyathin complex are known and chemically characterized, it is necessary to assign some arbitrary name to each fraction for the sake of simplicity while dealing with them in detail. This has been done in Table VII, which also shows the Rf values of the six purified fractions in the two solvent systems used. Table VIII gives a picture of the yield of various fractions in purified form obtained from the crude ethyl acetate extract. The amount of material crystallized from each purified fraction is also shown.

---

* A detailed account of chemical studies is presented in the Appendix.

TABLE VII

TLC of Cyathin and Terminology Adopted

Terminology	Solvent System	
	Benzene:Acetone:Acetic Acid (75:25:1)	Benzene:Dioxane:Acetic Acid (100:25:1)
Cyathin No. 1 ($A_1 + A_{51}$)*	0.67[1]	0.67
Cyathin No. 2	0.63	0.6
Cyathin A_5 ($C_{20}H_{26}O_5$)	0.54	0.5
Cyathin B ($C_7H_6O_4$)	0.51	0.44
Cyathin A_3 ($C_{20}H_{30}O_3$)	0.27	-
Cyathin A_4 ($C_{20}H_{30}O_4$)	0.06	-

* Possibilities exist that Cyathin No. 1 is a mixture of two components

[1] The data represents Rf values

TABLE VIII

Yield of Purified Fractions of Cyathin

Fractions	Crude Cyathin (gm)	Purified (mg)	% Yield	Crystalline (mg)	% Yield
Cyathin A_1	0.83	32.0	4.0	-	-
Cyathin A_{51}	1.0	15.0	1.5	-	-
Cyathin B	0.7	200.0	28.5	36.0	18.0
Cyathin A_3	3.0	236.0	7.8	-	-
Cyathin A_4	3.0	312.0	10.0	-	-
Cyathin A_5	0.83	217.0	26.2	35.0	16.0

It will be seen in Table VII that cyathin No. 1 contains two active principles which for the sake of simplicity, have been named A_1 and A_{51}. Cyathin A_3, A_4 and A_5, all have a C_{20} chain: the terminology was based on the number of oxygen atoms in each substance. Cyathin B is the most completely analysed active principle: for this the empirical formula is $C_7H_6O_4$ and the structure is also now known *viz* 2,4,5-trihydroxybenzaldehyde. Pure material obtained from crude broth or by chemical synthesis, yields deep-yellow brown crystals. A separate symbol 'B' has been used for the C_7 compound, rather than including it in Cyathin 'A' series. Another possibility, *viz* to name it "chromocyathin" (owing to its color) is under consideration. The name "cyathin" or cyathin complex unless otherwise stated, refers to the <u>whole complex</u> of unpurified and other unidentified principles.

The yield of crude cyathin from surface cultures varies not only in relation to the nutritional conditions, but also with the different batches used for extraction. Yields of crude material obtained from four different harvests and two different media are presented in Table IX. The chromatograms of some batches are reproduced in Plates XII and XIII. This variation is limited not only to cyathin, but has been reported recently for patulin (produced by *Penicillium urticae* Bainier) by Norstadt and McCalla (1969). Because up to the present a complete analysis of the active principles is not available, it is

TABLE IX

Yield of Cyathin in Batch Culture

Harvest	Medium	Volume of Culture Fluid[4] (liters)	Yield (in gm)
1	Undefined[1]	17	5.767
2	Defined[2]	11	2.54
3	Undefined	15.5	3.54
4	Defined[3]	6	1.0

[1] Dextrose 20 g/liter in Brodie medium (no maltose)

[2] Chemically defined medium (dextrose 30 g/liter)

[3] Chemically defined medium (maltose 30 g/liter; no dextrose)

[4] Broth was extracted after 25 days of growth.

PLATE XII Chromatogram of four different batch cultures of of cyathin. Large scale extraction was achieved by using ethyl acetate and evaporation under vacuum. Note the change in pattern of the complex. Solvent system--Benzene:Dioxane:Acetic Acid (100:25:1). L-R: Undefined, defined, undefined and undefined medium. Numbers and letters on the right side of the chromatogram correspond to various components of cyathin (refer to Table VII). Only the labelled spots are known to have antibiotic activity.

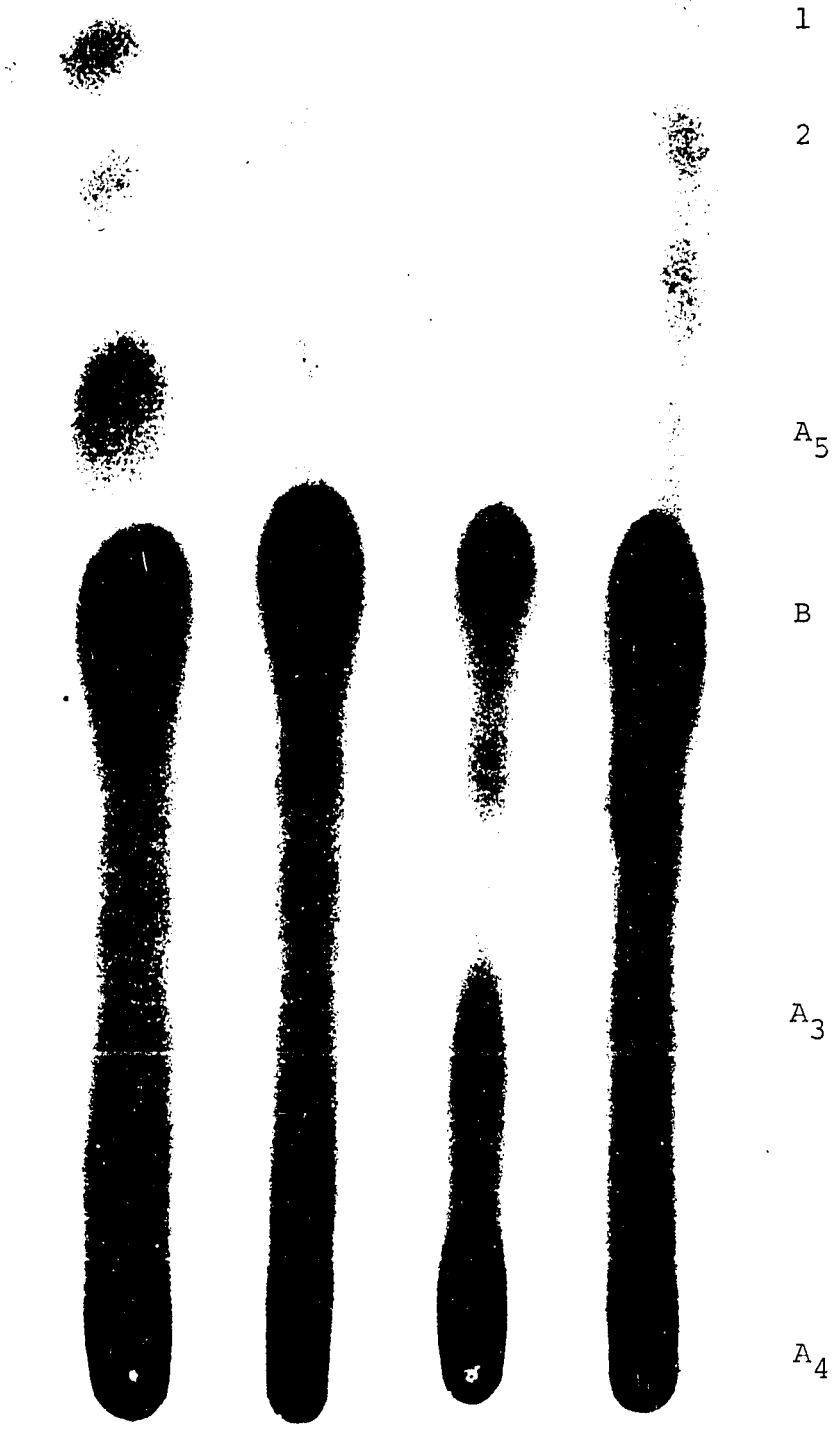


PLATE XII

PLATE XIII Chromatogram of four different batch cultures of cyathin. Solvent system--Benzene:Acetone:Acetic Acid (100:25:1). Note the slightly better separation of polar components of cyathin. Details same as in Plate XII.

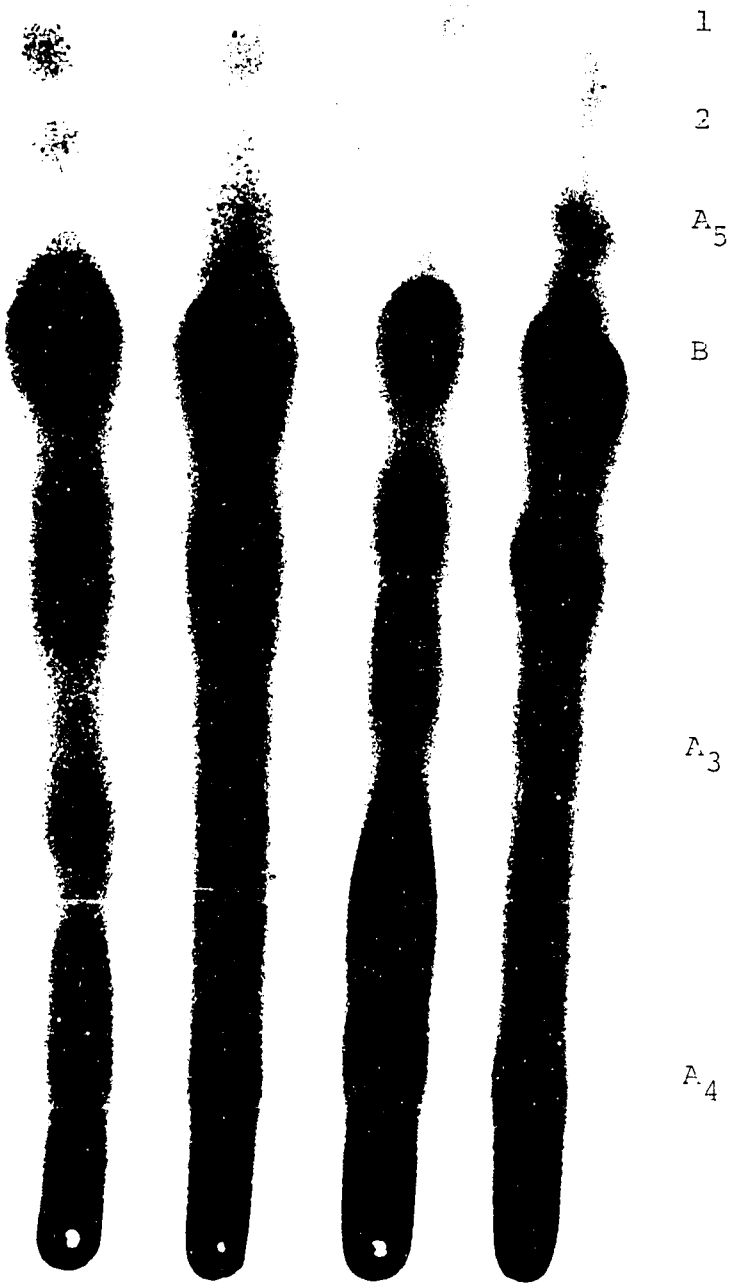


PLATE XIII

difficult to estimate the proportion in which each of the six purified fractions is present in cyathin. However, examination of Table VIII does give some idea as to the proportion of the purified fractions. The per cent yield has been calculated by taking the crude material as 100%. The maximum yield is that of cyathin B (28%), which has now been obtained in chemically synthesized form. Cyathin A_4, which is extremely polar gives a yield of 26.2%. These two fractions (cyathin B and cyathin A_4), therefore, make up more than half of the cyathin complex, while the remaining four fractions make up only 23% of the total. If these figures are added, the total is nearly 78%, and 22% of the material is unaccounted for. As would be suspected, a proportion of the original crude extract is in the form of impurities, although the Plates XII and XIII of the chromatograms would seem to show that there still remain a few more spots between cyathin A_3 and A_4 which are yet to be characterized.

All six purified fractions were tested at 500 µg concentration against *Staphylococcus aureus* for their comparative effectiveness. Cyathin A_{51}, of which the yield is lowest, shows the most effective inhibition; cyathin A_1 is second in effectiveness. As compared with the cyathin complex only cyathin A_{51} produces equal inhibition zones.

In Table X are shown the results of a comparison of cyathin fractions with a few well-known antibiotics. Discs of known concentrations were obtained from Difco

TABLE X

Comparison of Cyathin Activity with that of Known Antibiotics

Antibiotic	Amount (µg)	Inhibition Zone[1] (mm)
Aureomycin	10	16.5
Chloromycetin	10	4.0
Cyathin A_1	500	8.5
Cyathin A_3	500	1.5
Cyathin A_4	500	4.5
Cyathin A_{51}	500	10.0
Cyathin B	500	8.0
Erythromycin	10	6.5
Novobiocin	10	10.5
Penicillin	10	-
Polymyxin	10	-
Sulfadiazine	10	-
Tetracycline	10	8.5

- No inhibition

[1] Average of four replicates

(U.S.A.) It can be realized that even the best fraction of cyathin, cyathin A_{51}, is nearly fifty times less effective against *S. aureus* than aureomycin, novobiocin, and tetracycline.

Four fractions from the above mentioned six were also tested against *S. aureus* by a serial dilution method to establish their minimum inhibitory concentrations (MIC). The results are presented in Tables XI, XII, XIII and XIV. It is common practice to observe the growth of microorganisms only after 18-24 hr. The concentration at which turbidity in the medium does not appear is referred to as the 'MIC' of the substance in question. However, in the present study bacterial growth was observed at various intervals, the final observation being taken after 5 days. There are two advantages of doing this: one, a substance which may show a bactericidal effect at the 24 hr interval may not be active if observed again after 48 hr; secondly if two active principles have the same MIC, it is possible to decide which one of the two would be the more effective. Both of these advantages are quite obvious from the tables of the MIC for various cyathin fractions. Cyathin B (Table XII), cyathin A_1 (Table XIII) and cyathin A_{51} (Table XIV), all have a MIC equal to an antibiotic concentration of 25 µg/ml of the medium. However, observations made at 10 and 12 hour intervals show that cyathin A_{51} is the most active of the three as even a concentration as low as 1.56 µg/ml inhibits growth of bacteria for 12 hr,

TABLE XI

MIC Determination for the Cyathin Complex

Concentration (µg per ml)	Time (hr)					
	5	10	24	30	48	120
100.0	0	0	0	0	0	0
50.0	0	0	0	0	0	0
25.0	0	0	+	+	+	+
12.5	0	+	+	+	+	+
6.25	+	+	+	+	+	+
3.12	+	+	+	+	+	+
1.56	+	+	+	+	+	+
0.78	+	+	+	+	+	+
0.39	+	+	+	+	+	+
0.0 (Inoc.)	+	+	+	+	+	+
0.0 (Non-inoc.)	0	0	0	0	0	0

+ Growth, medium turbid

0 No growth, medium clear

TABLE XII

MIC Determination for Cyathin B

Concentration (µg per ml)	Time (hr)					
	5	10	24	30	48	120
100.0	0	0	0	0	0	0
50.0	0	0	0	0	0	0
25.0	0	0	0	0	0	0
12.5	0	+	+	+	+	+
6.25	+	+	+	+	+	+
3.12	+	+	+	+	+	+
1.56	+	+	+	+	+	+
0.78	+	+	+	+	+	+
0.39	+	+	+	+	+	+
0.0 (Inoc.)	+	+	+	+	+	+
0.0 (Non-inoc.)	0	0	0	0	0	0

+ Growth, medium turbid

0 No growth, medium clear

TABLE XIII

MIC Determination for Cyathin A_1

Concentration (µg per ml)	Time (hr)				
	12	20	24	48	120
100.0	0	0	0	0	0
50.0	0	0	0	0	0
25.0	0	0	0	0	0
12.5	0	0	0	0	+
6.25	0	0	0	+	+
3.12	0	+	+	+	+
1.56	+	+	+	+	+
0.78	+	+	+	+	+
0.0 (Inoc.)	+	+	+	+	+
0.0 (Non-inoc.)	0	0	0	0	0

+ Growth, medium turbid

0 No growth, medium clear

TABLE XIV

MIC Determination for Cyathin A_{51}

Concentration (µg per ml)	Time (hr)				
	12	20	24	48	120
100.0	0	0	0	0	0
50.0	0	0	0	0	0
25.0	0	0	0	0	0
12.5	0	0	0	0	+
6.25	0	0	0	0	+
3.12	0	0	0	+	+
1.56	0	+	+	+	+
0.78	+	+	+	+	+
0.0 (Inoc.)	+	+	+	+	+
0.0 (Non-inoc.)	0	0	0	0	0

+ Growth, medium turbid

0 No growth, medium clear

while for cyathin B the same effect is observed only at a concentration of 25 µg/ml. Cyathin A_1 has activity similar to that of A_{51} except that the lowest concentration which inhibits bacterial growth to any extent is 3.12 µg/ml. The mutual antagonism of the various fractions when present together, as in the cyathin complex, is quite evident here for the MIC for the whole complex is 50 µg/ml.

Properties of Cyathin Complex

Cyathin is highly soluble in organic solvents, such as acetone, methanol, n-butanol, and ethylacetate. The solubility in diethyl ether is half that in the above mentioned solvents. The production of antibiotic in the culture broth as well as in the mycelium, points to the low solubility of cyathin in water. The most suitable solvent for extraction proved to be ethylacetate, although n-butanol was equally effective as a solvent. Cyathin is stable over a wide pH range and highly heat-stable. Autoclaving at 15 lb/sq inch for 20 min destroyed only 25% of the activity. Storage at low temperatures or even at room temperatures for several months did not result in loss of activity.

Cyathin B

When crystalline cyathin B (but not chemically synthesized) became available some preliminary studies using this compound were undertaken. It was desirable to see

whether or not it moved as a single bioactive spot on the chromatogram, as a test of purity. The solvent systems employed and Rf obtained on Eastman Chromagram sheets are given in Table XV. Except where the solvent system was Benzene-Dioxane-Acetic Acid, cyathin B not only moved as a single spot, but also did not show any trailing which is a common occurrence in the chromatography of so many other antibiotics. The bioactivity of the spot left no doubt that cyathin B was really a single active substance. The MIC for cyathin B has already been given in Table XII and a concentration curve has been presented in Plate XIV.

The next step was to study the effect of various concentrations of cyathin B on the growth of *Staphylococcus aureus*. This was done by following the growth of bacteria in a Bacto-Penassay Broth (Difco) in the presence and absence of cyathin B. The growth was measured by the optical density method at wave-length 600 mμ. The results are presented in Plates XV and XVI. As was expected from its MIC, concentrations of cyathin B lower than 25 μg/ml were bacteristatic only; growth of bacteria resumed as soon as the action of antibiotic was finished. Compared with the growth-curve without the antibiotic, the slope of the curve is very regular over a concentration range of 5 μg/ml to 25 μg/ml. The latter concentration did not allow the growth of bacteria at all.

In the above experiment, cyathin B was added to the medium at the zero hour and, therefore, during their

TABLE XV

ITLC of Cyathin B

Solvent Systems	Rf
Benzene-Dioxan-Acetic Acid (100:25:1)	0.0-0.49
Acetone	0.86
Isopropanol-Water (10:0.5)	0.8
n-Butanol-Methanol (1:1)	0.71
Chloroform-Methanol (1:1)	0.88
n-Butanol-Acetic Acid-Water (2:1:1)	0.75
Ethyl Acetate-Methanol (1:1)	0.68

PLATE XIV Concentration curve for cyathin B (crystalline sample). Known amounts of antibiotic were incorporated into the filter paper discs and inhibition zones measured against *Staphylococcus aureus*.

PLATE XIV

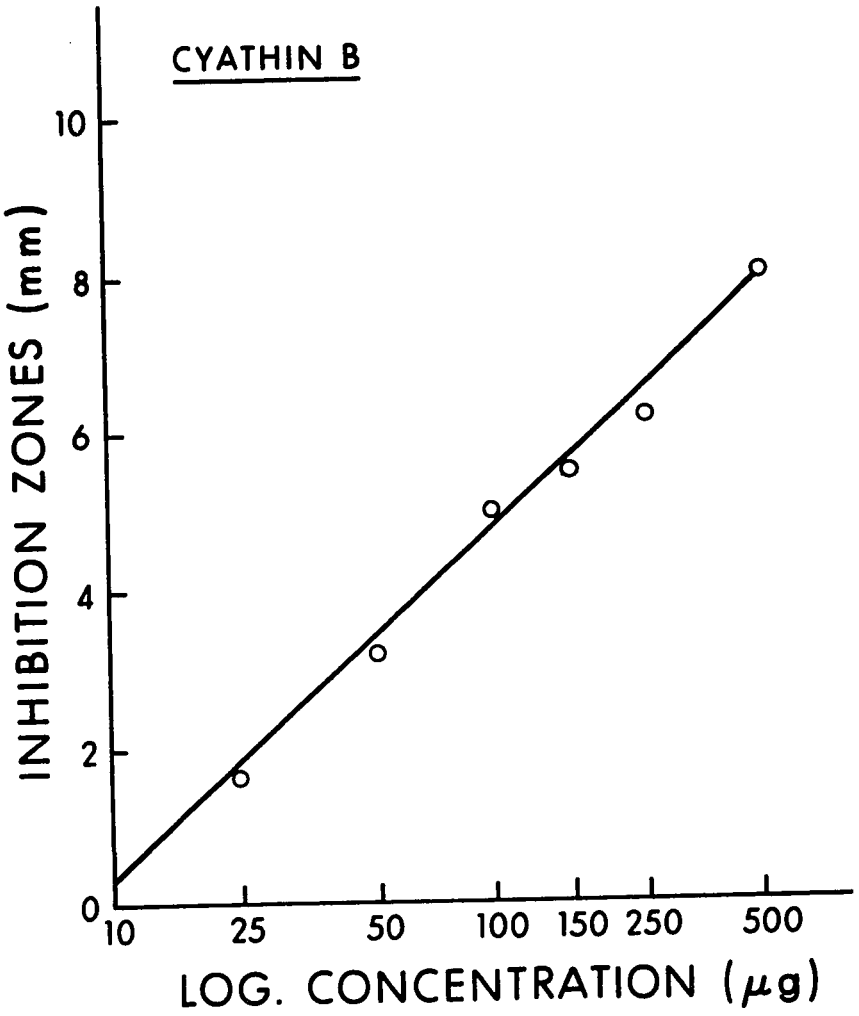

CYATHIN B
INHIBITION ZONES (mm)
0
2
4
6
8
10
10
25
50
100
150
250
500
LOG. CONCENTRATION (μg)

PLATE XV Effect of various concentrations of cyathin B on the growth of *Staphylococcus aureus*. Additions of antibiotic were made at zero hour in the Bacto-Penassay Broth. Growth was followed in a Spectronic 20 Colorimeter at 600 mμ. The data point represents an average of three replicates.

PLATE XV

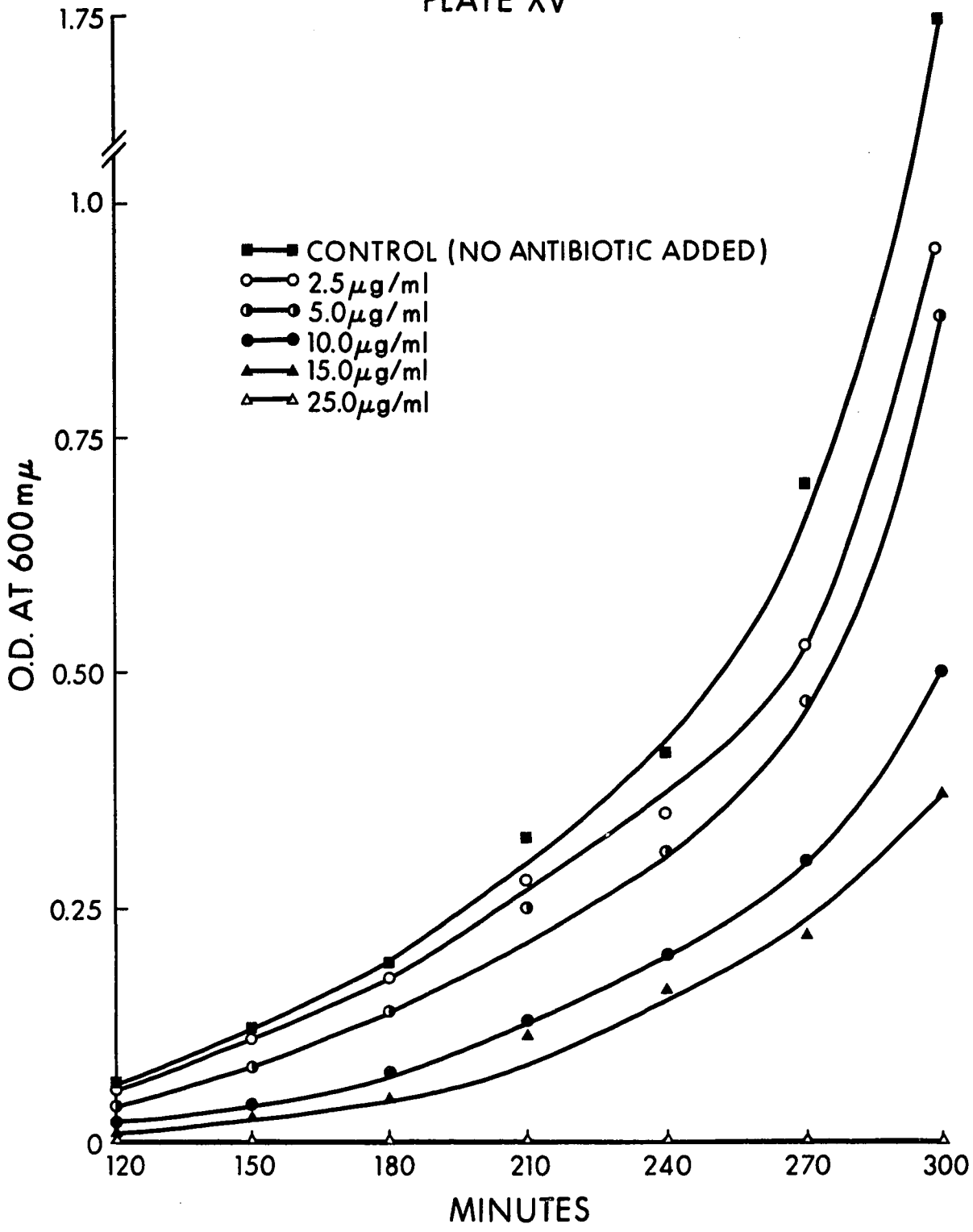
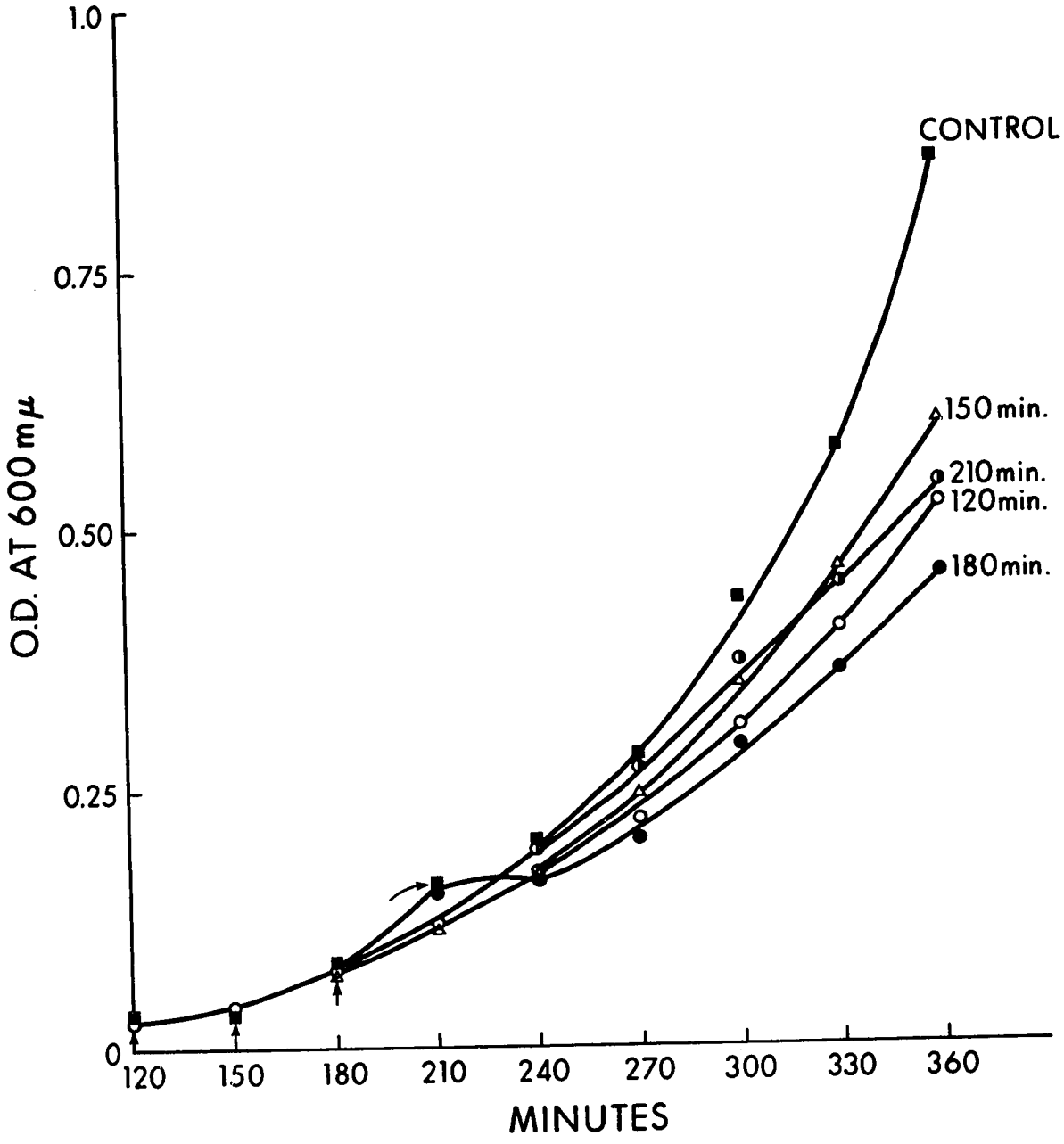

1.75
1.0
0.75
0.50
0.25
0
O.D. AT 600mμ
CONTROL (NO ANTIBIOTIC ADDED)
2.5μg/ml
5.0μg/ml
10.0μg/ml
15.0μg/ml
25.0μg/ml
120
150
180
210
240
270
300
MINUTES

PLATE XVI Effect of time of addition of cyathin B (10µg/ml) on the growth of *S. aureus*. Additions were made at 120, 150, 180 and 210 minutes and the growth of bacteria followed in a Spectronic 20 Colorimeter at 600 mµ. The data point represents an average of four replicates.

PLATE XVI


1.0
0.75
0.50
0.25
0
O.D. AT 600 mμ
CONTROL
150 min.
210 min.
120 min.
180 min.
120
150
180
210
240
270
300
330
360
MINUTES

entire growth period bacteria were in contact with antibiotic. Under the usual conditions for the use of antibiotics bacteria are already dividing and a useful antibiotic should be effective enough to check the growth of a microorganism. In order to evaluate more closely the effect of cyathin B, a concentration giving nearly 70% inhibition of bacterial growth up to 6 hr was chosen (10 µg/ml) and incorporated into the medium at various time intervals. The subsequent growth of bacteria was followed by measuring the optical density. The results are presented in Plate XVI. The slope of the growth curve in the control in this experiment was purposely brought down, so that the action of cyathin B could be followed for a longer period. The growth pattern, however, is exactly comparable in the Plates XV and XVI. Cyathin B added at either 120 minute intervals when growth of bacteria had hardly begun, or during the rapid growth-phase, *viz* at 180 minutes and 210 minutes, showed the optimum effect against bacterial growth.

Effect of pH on Cyathin B

A cyathin B solution at a concentration of 10 mg/ml was made and reduced to 5 mg/ml by the addition of this solution to an equal volume of 0.1 M Na_2HPO_4 - KH_2PO_4 buffer adjusted to various pH levels. The original solution of cyathin B was made up in acetone due to the low solubility of the antibiotic in water. Standard assay plates were used for assessing the activity of cyathin B at various pH levels. The activity was compared with that of

the original acetone solution, which was taken as 100%. Triplicate measurements were made at every pH level and the results are given in Table XVI. Even under very acidic or highly basic conditions, 82% of the original activity of cyathin B can be observed. This leaves no doubt that cyathin B is highly pH stable. Stability was also tested by adjusting the pH of the Difco Antibiotic Medium No. 2 (original pH 6.6) before autoclaving, to various ranges and testing the activity of cyathin B using *S. aureus*. In all the pH tests paper discs impregnated with 400 µg of cyathin B were used. Any change in pH during autoclaving was not taken into account. The results are presented in Table XVII. Activity of 100% was assigned to the pH 6.6 level which was the original pH of the medium used throughout the standard microbiological assay. The results are somewhat unexpected: the activity of cyathin B increases by 34% and 40% at a pH of 5 and 8 respectively. An increase of 21% at pH 7.0 is also quite remarkable, although a slight decrease (3%) is noted at pH 6.0. It is known that bacteria grow better at a higher pH as compared with fungi and the normal pH used in bacterial work varies between 6.5 - 7.0. Therefore, an increase of 21% at pH 7.0 as compared with 6.6 could be accounted for by the difference in pH and also to higher sensitivity of bacteria at pH 7.0. It is rather difficult to explain the situation at pH 5.0 and pH 8.0 as one level is quite acidic and the other quite basic. Although no quantitative

TABLE XVI

Cyathin B Activity at Various pH Levels

	pH of Antibiotic Solution						
	2	4	6	7	8	10	12
% activity*	82	86.5	90	82	79	79	82
% loss of activity	18	13.5	10	18	21	21	18

* 100% activity attributed to an acetone solution of Cyathin B

TABLE XVII

Effect of pH of the Assay Medium on Cyathin B Activity

	pH of the Medium				
	5	6	6.6	7	8
% activity*	134.3	97	100	120.9	140.3
% increase	34.3	0	0	20.9	40.3
% decrease	0	3	0	0	0

* 100% activity attributed to the medium with pH 6.6

data were obtained, observation of the test plates showed that the bacteria were growing and that they produced uniform growth on the plates comparable to control plates (pH 6.6) at both these pH levels. As the observation does not give any evidence about the rate of growth, it might be suspected that the bacteria grew comparatively slowly, and that this gave a better opportunity for cyathin B to diffuse more rapidly. Another possibility might be the inactivation of inhibitors in the agar at these pH levels, which resulted in a faster diffusion of antibiotic. It would be interesting to seek for an explanation for the pH effect, because it might increase the effect of cyathin B on an agar medium.

Evaluation of the Effect of Various Substances on the Action Cyathin by Paper Strip Technique

Although qualitative in nature, a survey of the relation of certain substances including cations, anions, sugars, amino acids, oxidising agents and reducing agents on the action of cyathin was made using *Staphylococcus aureus* as the test organism. The procedure used was very simple; it involved only two strips (3.5 x 0.5 cm) of Whatman No. 1 filter paper. One strip was impregnated with a cyathin solution of a concentration of 10 mg/ml, while the other strip was impregnated with a 1% solution of the substance being tested. The antibiotic strip was placed on an assay plate; across it, at the lower end, was placed

the strip of substance being tested. The effect of test substance was evaluated as explained in Plate XVII. The results of such a test are presented in Table XVIII. Twenty five substances, in all, were tested by this procedure. Of these, six had no effect; ten depressed the antibacterial action of cyathin, while the remaining stimulated the action of cyathin.

The purpose underlying such tests was to reveal a substance(s) which would either stimulate the action of cyathin (*e.g.* diphenylamine) or would depress the action of cyathin (*e.g.* cysteine hydrochloride); such a discovery might provide some indication as to the site of action or the mechanism of action of cyathin against *S. aureus*. Of the substances tested, the most striking action was that of cysteine hydrochloride; this compound gave a marked depression of the zone of inhibition produced by cyathin. However, because a hydrochloride salt of cysteine was used, it was difficult to interpret the result. There were two possibilities:

(i) the hydrochloride salt influenced the pH of the medium, thereby affecting cyathin action

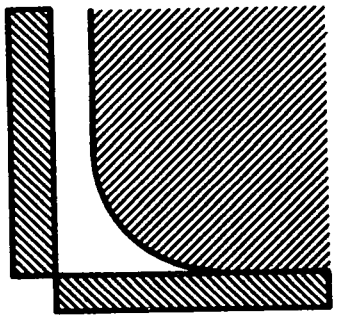
(ii) the -SH groups of the cysteine played some role in the action of cyathin

To examine each of the above possibilities, another test was made using l-cysteine to exclude the first possibility. The inhibition of cyathin action was still marked. By this time a chemically synthesized sample of

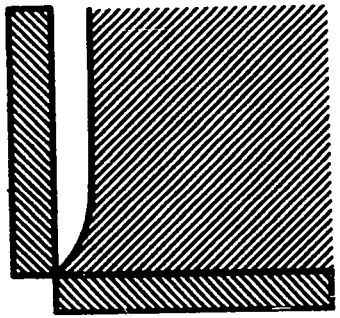
PLATE XVII Evaluation of the effect of various substances on the action of cyathin.

PLATE XVII

NO EFFECT



STIMULATION



DEPRESSION

TABLE XVIII

Influence of Various Substances on the Action of Cyathin

Compounds Tested	Response
dl-Alanine	0
p-Aminobenzoic acid	-
Ascorbic acid	-
Asparagine	0
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0
Calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$]	+
*l-Cysteine	+
*Cysteine hydrochloride	+
Diphenylamine	-
Galactose	+
Lactose	+
Laevulose	+
Magnesium chloride ($MgCl_2$)	0
Maltose	+
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	-
Mannitol	+
dl-Methionine	0
Nicotinic acid	-
Oxalic acid	-
Potassium phosphate (K_2HPO_4)	+
Sodium nitrate ($NaNO_3$)	0
Sodium tricitrate	+
Starch	+
Tartaric acid	-

0 No effect

+ Inhibition

- Stimulation

* Most marked inhibition of cyathin action

cyathin B had become available. Therefore, some of the compounds listed in Table XVIII including l-cysteine, were tested for their effect on the action of cyathin B. The depression by l-cysteine or the stimulation by diphenylamine was suggestive but not as clearly pronounced as in the case of cyathin. It has been mentioned elsewhere that cyathin is a complex of active principles and the action of l-cysteine on cyathin would suggest that some other fraction of cyathin complex is depressed more strongly (than cyathin B) to produce such marked depression of cyathin action. It may be pointed out that only a partially purified sample of cyathin was employed in these tests. Reduced glutathione (GSH) was another compound to be tested for its action since l-cysteine is one of the components in the biosynthesis of this -SH compound. As expected, glutathione also depressed cyathin action. To test the proposition that only -SH compounds are active in protecting the bacteria from the action of cyathin, glutamine and glycine (both used in glutathione formation, beside l-cysteine) were also tested and found to have no effect whatsoever. The latter tests were indicative of the action of cyathin on -SH groups. However, how effective l-cysteine or glutathione were in 'protecting' -SH groups, remained to be determined. To make the results more quantitative, tests then were performed using filter paper discs impregnated with cyathin or cyathin B at a

concentration of 400 µg. Different amounts of l-cysteine (0.1 M) or glutathione (0.1%) were incorporated into the antibiotic discs, and sensitivity tests carried out using the standard microbiological assay (see Materials and Methods). The per cent reversal of cyathin activity was calculated on the basis of the inhibition zones produced by the antibiotics without added -SH compounds (this value taken as 100%). The results are expressed in Table XIX. The addition of even 0.05 ml of l-cysteine resulted in a 55.6% reversal of cyathin action, while cyathin B showed a reversal only of 20.6%. Using 400 µg of antibiotics, 90% of cyathin activity could be reversed by the addition of 0.1 ml of a 0.1 M l-cysteine solution.

The results appear to indicate that cyathin acts by the inactivation of thiol (-SH) groups and that l-cysteine and glutathione reversed the cyathin action, thus protecting the -SH groups. l-Cysteine, however, is also known to be metabolized. Therefore, doubts still persisted as to whether or not l-cysteine is protecting -SH groups or reversing cyathin action by itself being involved in the metabolism of the bacterial cells. To choose between the propositions above mentioned, dithiotheritol (Cleland's reagent, DTT) instead of l-cysteine was used. DTT is a well known protector of -SH groups and is also known not to be self metabolized. If the reversing

TABLE XIX

Reversal of Cyathin Activity

Addition to the Discs (ml)	% Reversal of Activity*	
	Cyathin B (400 µg)	Cyathin (400 µg)
l-cysteine (0.1 M)		
0.05	20.6	55.6
0.08	20.0	83.3
0.1	28.8	90.0
Glutathione (0.1%)		
0.05	13.7	22.2
0.08	16.5	24.5
0.1	4.0	25.6

* 100% activity attributed to the zones produced by 400 µg of antibiotic

action of cyathin were observed even with DTT, it would leave no doubts that l-cysteine too protects the -SH group and is not metabolized. A 0.1 M solution of DTT was made in water, and amounts of 0.05, 0.08, and 0.1 ml were added to the filter paper discs impregnated with 400 µg of cyathin or cyathin B. The discs were tested on the standard assay plates. Surprisingly enough, DTT itself proved inhibitory at all levels (of the 0.1 M solution) to the growth of *S. aureus* and, therefore, resulted in the stimulation of cyathin action. As the latter work was performed very late in the experimental period, no further attempts were made to find another '-SH' protective reagent which is not inhibitory to bacterial growth.

In the absence of conclusive results, it can be stated that l-cysteine and glutathione are the only known compounds capable of reversing the action of cyathin; whether this effect is exerted by the protection of thiol groups or by its metabolic fate, remains to be elucidated.

The Production of Cyathin by *Cyathus helenae* under Laboratory Conditions

Comparison of Static and Shake Culture

In the production of secondary metabolites by micro-organisms usually 'shake culture' process has been used to increase the yield of the substance sought. For the production of cyathin, shake culture did not seem to be a useful technique. A comparison of the yield of

cyathin under 'static surface cultures' and under 'shake culture' is shown in Plate XVIII. For shake culture, an automatic shaker was used to provide 20 strokes per minute. As will be seen from Plate XVIII, on static medium the production of cyathin was five times as great as in the shake culture. One reason for this difference in cyathin titres may be that the mycelium in shake culture tends to clump; compact, tough globular bodies are formed and probably the clumping does not allow the fungus to grow properly and to be able to release desirable amounts of the secondary metabolites. Another important factor is the submerged state of mycelium, which may become slightly anaerobic once the dissolved oxygen is consumed; no outside supply of air was supplied to the flasks. Even in the static cultures it was noted that submerged mycelium gave low yields of cyathin as compared with surface cultures.

Undefined Medium Compared with Chemically-Defined Medium

During the early part of this work, cyathin was produced by the fungus grown on Brodie's semi-synthetic liquid medium. At that time effort was made to increase cyathin production by varying the concentration of sugars in the medium. The results are presented in Plate XIX. Fig 11 shows cyathin production on 'normal' Brodie medium (NB). Fungal growth, as measured by dry weight has also been indicated in the results. An increase in sugar concentration from 2 g dextrose and 5 g maltose (as in NB) to 20 g dextrose (2 DNB) resulted in an increase in cyathin

PLATE XVIII A comparison of the production of cyathin in 'static' culture and 'shake' culture. Note the difference in the quantity of cyathin produced under the two conditions. The data point represents an average of four replicates.

PLATE XVIII

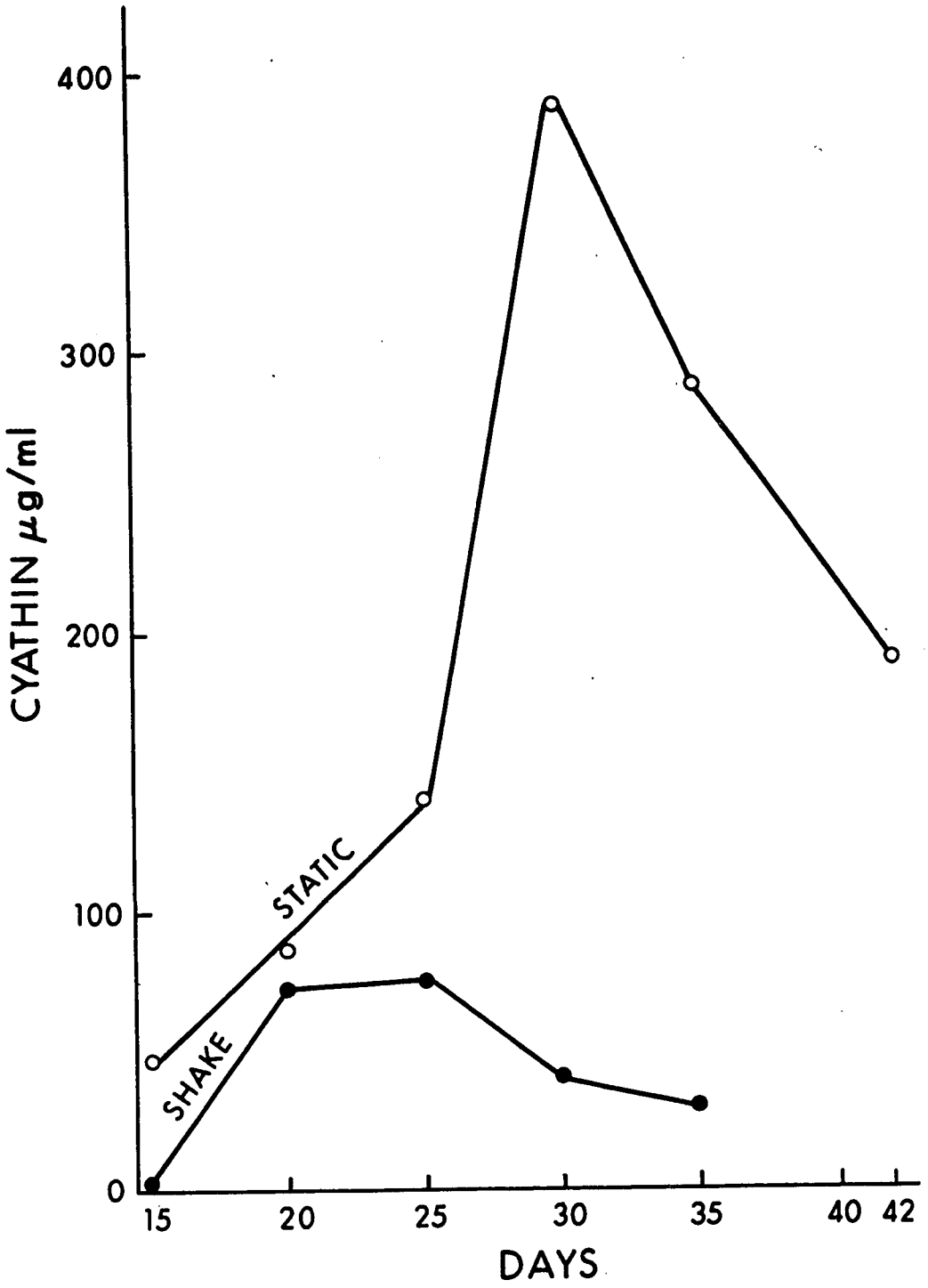

400
300
200
100
0
CYATHIN µg/ml
STATIC
SHAKE
15
20
25
30
35
40
42
DAYS

PLATE XIX Production of Cyathin on Undefined Media

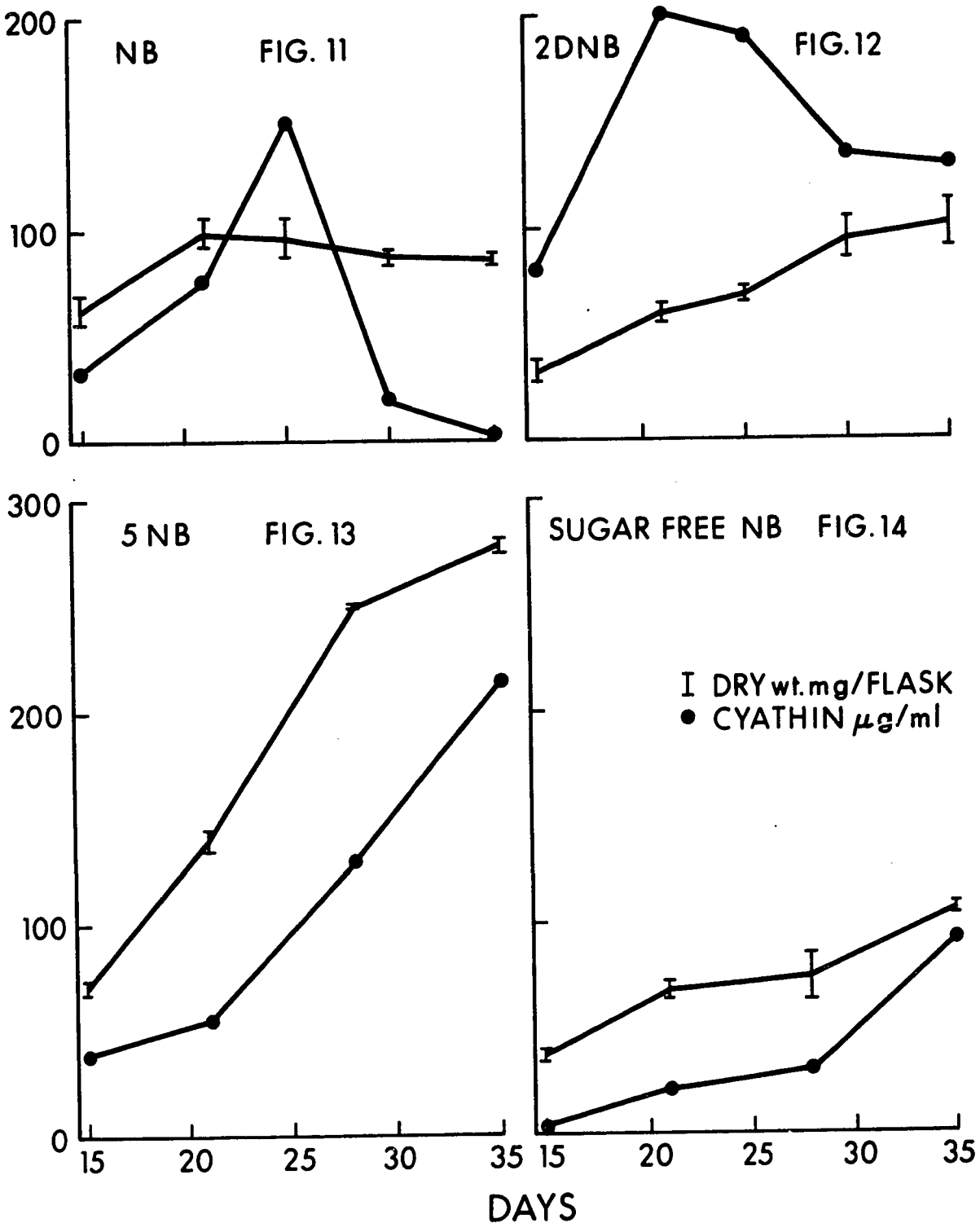
Fig. 11. Normal Brodie medium (NB)

Fig. 12. Normal Brodie medium having dextrose 20 g/litre, no maltose (2DNB).

Fig. 13. Brodie medium at five times its normal concentration (5NB).

Fig. 14. Brodie medium without any sugars, dextrose and maltose both deleted. Note fungus still produces sufficient quantity of cyathin after getting adapted to the environment.

PLATE XIX


200
100
0
NB
FIG. 11
2DNB
FIG. 12
300
200
100
0
5 NB
FIG. 13
SUGAR FREE NB
FIG. 14
I DRY wt. mg/FLASK
● CYATHIN μg/ml
15
20
25
30
35
DAYS

production (Fig. 12). It may however be noted that there is no correlation between the growth of the fungus and cyathin production on these two media. However, Fig. 13 shows the correlation between fungal growth and cyathin production on a 5 normal Brodie medium (5 NB). As expected, an increase in cyathin content was noted, although again it does not correspond in any proportion to the increase in dry weight. The effect and role of carbon-source in the Brodie medium, was then examined. Maltose and dextrose were both deleted and the growth of fungus and cyathin production were determined at periodic intervals. Fig. 14 indicates strong correlation between fungal growth and cyathin production. One noteworthy point concerns the dry weight; in Fig. 14, at 35 days and at 21 days in Fig. 11, dry weights are nearly the same, suggesting that this fungus can grow even on a sugar-free Brodie medium although two weeks longer were required to produce an equal amount of growth as compared with the normal medium. However, cyathin production in sugar-free medium never reached close to the value obtained on normal medium. This would suggest that sugars are important for increasing cyathin production, although not essential, as a sugar-free medium was also able to produce a sufficient amount of cyathin.

Later, during the nutritional work, several chemically-defined media (DM) were tested regarding cyathin production. The results obtained with three of them are presented in Plate XX. All three media are essentially of

PLATE XX Production of Cyathin on Chemically Defined Media

Fig. 15. Defined medium (see Materials and Methods) having 20 g dextrose per liter.

Fig. 16. Defined medium having 25 g dextrose per litre

Fig. 17. Defined medium having 30 g dextrose per liter. This medium has been used in all the later work (abbreviated DMIII).

Fig. 18. Effect of Cu^{++}, Fe^{++} and Mn^{++} on production of cyathin. Basal medium refers to DMIII from which thiamine had been deleted.

PLATE XX

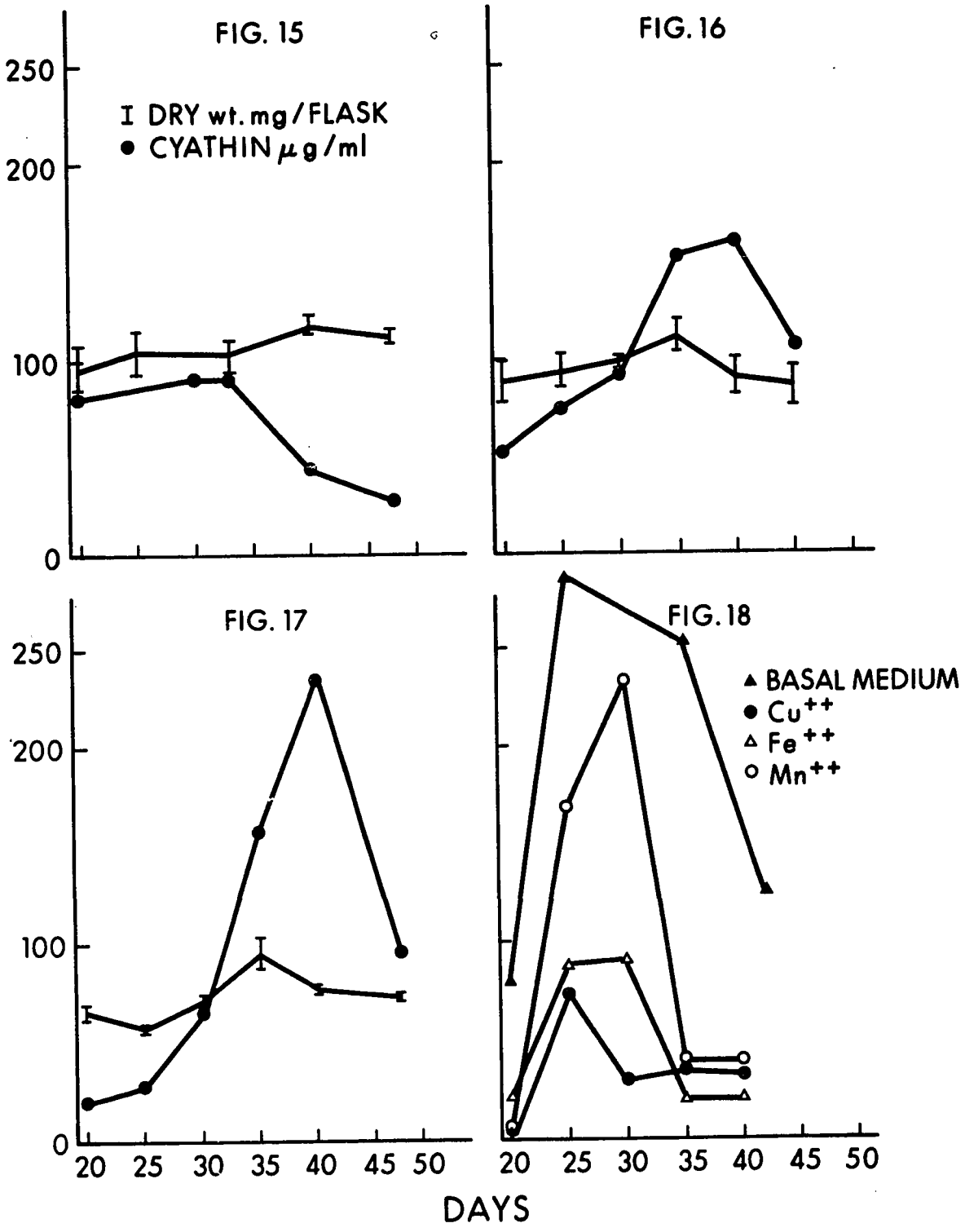


FIG. 15
FIG. 16
I DRY wt. mg/FLASK
● CYATHIN μg/ml
250
200
100
0
FIG. 17
FIG. 18
▲ BASAL MEDIUM
● Cu++
△ Fe++
○ Mn++
250
200
100
0
20 25 30 35 40 45 50
20 25 30 35 40 45 50
DAYS

the same composition, the difference being solely in the amount of dextrose present. DMI, DMII, and DMIII, respectively, represent 20 g, 25 g, and 30 g of dextrose per liter of the medium. With an increase in the amounts of dextrose there is a corresponding increase in cyathin production. An increase from 20 g dextrose in DMI (Fig. 15) to 30 g dextrose per liter in DMIII (Fig. 17) resulted in 2.5-fold increase in cyathin titres. These results suggest that cyathin synthesis by the fungus *Cyathus helenae* is dependent to a great extent upon the carbon source and its concentration in the medium. The amount of nitrogen supplied was never changed, although an increase in sugar always resulted in increased cyathin production, suggesting that the C/N ratio for antibiotic production is fairly high. However, growth of the fungus did not correspondingly increase and maximum cyathin production seems to coincide with a decline in dry weight (although it has not been a constant feature); this result would be expected if cyathin is a secondary metabolite which is released after autolysis of the fungus cell contents. DMIII, (whose composition has been given in Materials and Methods) was later used in all nutritional experiments and any departure from its composition will be indicated.

Although chemically defined medium (DMIII) contains asparagine as well as calcium nitrate as sources of nitrogen, it was of interest to examine the effect of a single nitrogen source when compared with combined organic

and inorganic nitrogen sources. The results are presented in Plate XXI. From DMIII in one experiment asparagine was deleted completely and in another, calcium nitrate was deleted. The growth of fungus was little affected by the deletion of calcium nitrate (Fig. 19). A comparison with DMIII (Fig. 17) shows that the maximum dry weight production of the fungus occurs at the 28th day when asparagine is present. In the test involving combined nitrogen sources the maximum dry weight is reached after 33 days. However, cyathin production in the former case is only 60% of the latter. Deletion of asparagine (Fig. 20) resulted in very little growth of the fungus, and it was not until 45 days had elapsed that growth reached 50% of the growth produced in nearly half the time on the asparagine-containing medium. Even in the absence of asparagine, the fungus becomes adapted to its environment although a longer time is taken for the adaptation and a good titre of cyathin is produced which is nearly 50% as compared with DMIII. The two deletions from the defined medium reveal that *C. helenae* requires organic nitrogen for its growth, although substitution of organic with nitrate nitrogen increases the yield of cyathin, without affecting the growth of fungus.

Yeast extract mainly as a source of vitamins and peptone, as a nitrogen source, are commonly employed in the nutrient media. It is, however, very difficult to ascertain precisely their effects because the chemical composition of either of these natural products is not fully

PLATE XXI Effect of Composition of the Defined Medium (DMIII) on the Production of Cyathin

Fig. 19. Calcium nitrate deleted. Note that the cyathin production is only little affected.

Fig. 20. Asparagine deleted. Note the drastic reduction in the quantity of cyathin as well as growth of the fungus.

Fig. 21. Yeast extract added to complete DMIII at a concentration of 3 g per liter. Note that the growth of the fungus is comparable to DMIII (Fig. 17) but the cyathin production has been reduced by half.

Fig. 22. Peptone added to complete DMIII at a concentration of 1 g per litre. Fungus growth is reduced, but cyathin titres compare with DMIII.

PLATE XXI

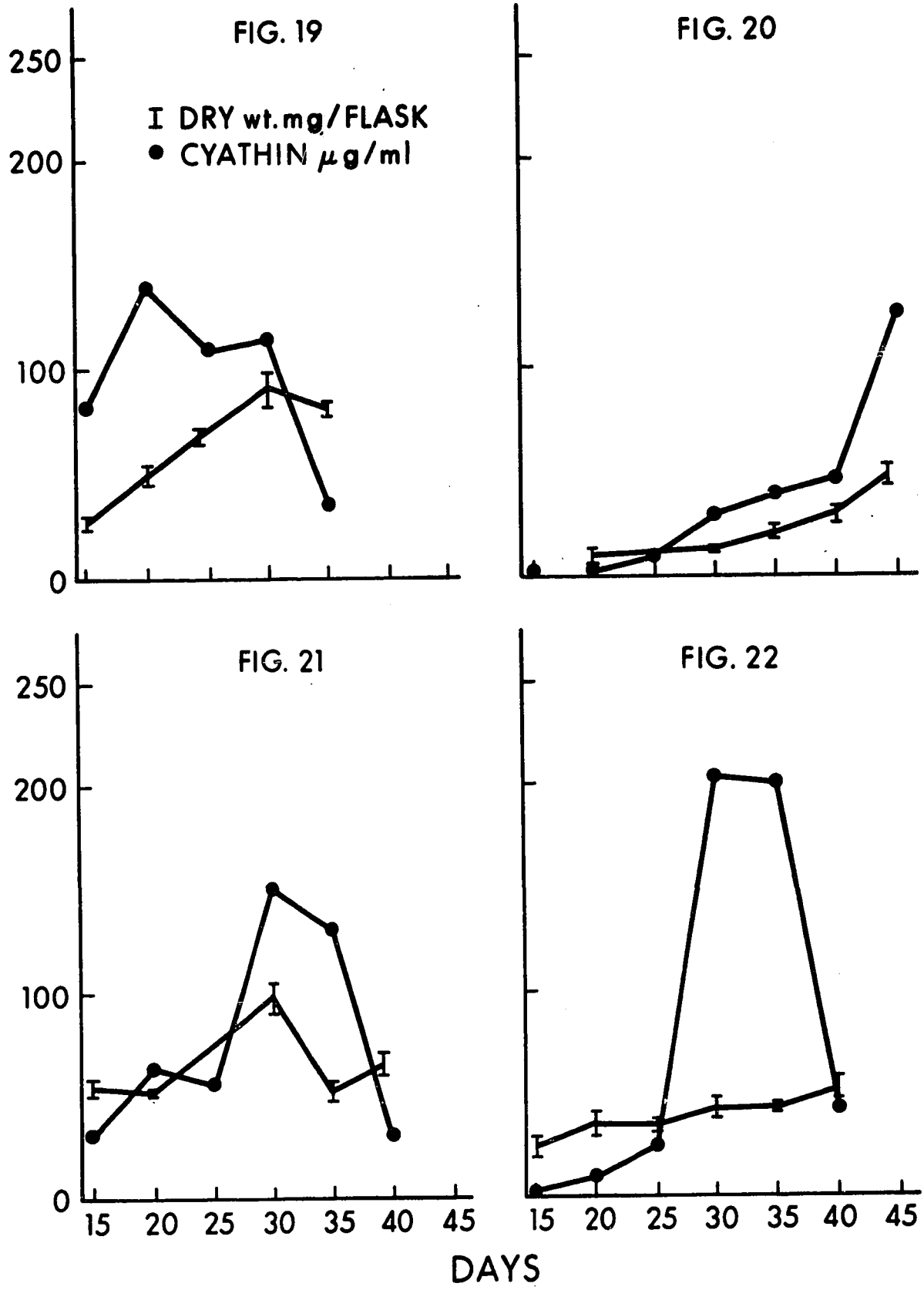


FIG. 19
I DRY wt.mg/FLASK
● CYATHIN μg/ml
250
200
100
0
FIG. 20
FIG. 21
250
200
100
0
15 20 25 30 35 40 45
FIG. 22
15 20 25 30 35 40 45
DAYS

understood. The effect of addition of 3 g yeast extract or 1 g peptone per liter of medium on the production of cyathin were tested separately. The results are presented in Fig. 21 and 22 (Plate XXI). The additions were made to complete DMIII. The titres of cyathin on yeast extract were only 60% as compared to DMIII (Fig. 17). Noteworthy is the correlation between dry-weight and cyathin production. The maximum cyathin titres coincide with the maximum dry weight. The growth of fungus on peptone was rather poor and, even after 40 days of growth, the dry weight was but 50% of that on the DMIII. On the other hand, cyathin titres were higher and reached a value obtained on DMIII. Normally slow growth and less dry weight accumulation results in better yields of cyathin, suggesting that under these conditions utilization of resources is restricted and not all of the nutrients are wasted in the structural build up of the fungus. This results in better yields of secondary metabolites.

Another deletion made from the defined medium was of thiamine. The results of this experiment are presented in Fig. 22 (Basal Medium). When thiamine is deleted from the medium, the increase in cyathin production is quite significant, as it amounts to 22% more than that on DMIII.

Micronutrients and Soil Extract

The effect of the so-called 'minor' mineral elements such as Cu++, Fe++, Mn++ and Zn++ on cyathin

production was studied. Each micronutrient was added one at a time to the basal medium (DMIII minus thiamine except in the case of Zn++ where thiamine was not deleted). The concentrations of the various micronutrients added to the defined medium per liter were as follows:

Cu++ as $CuSO_4 \cdot 5H_2O$----------0.26 ppm

Fe++ as $FeSO_4 \cdot 7H_2O$----------0.13 ppm

Mn++ as $MnSO_4$ ----------0.26 ppm

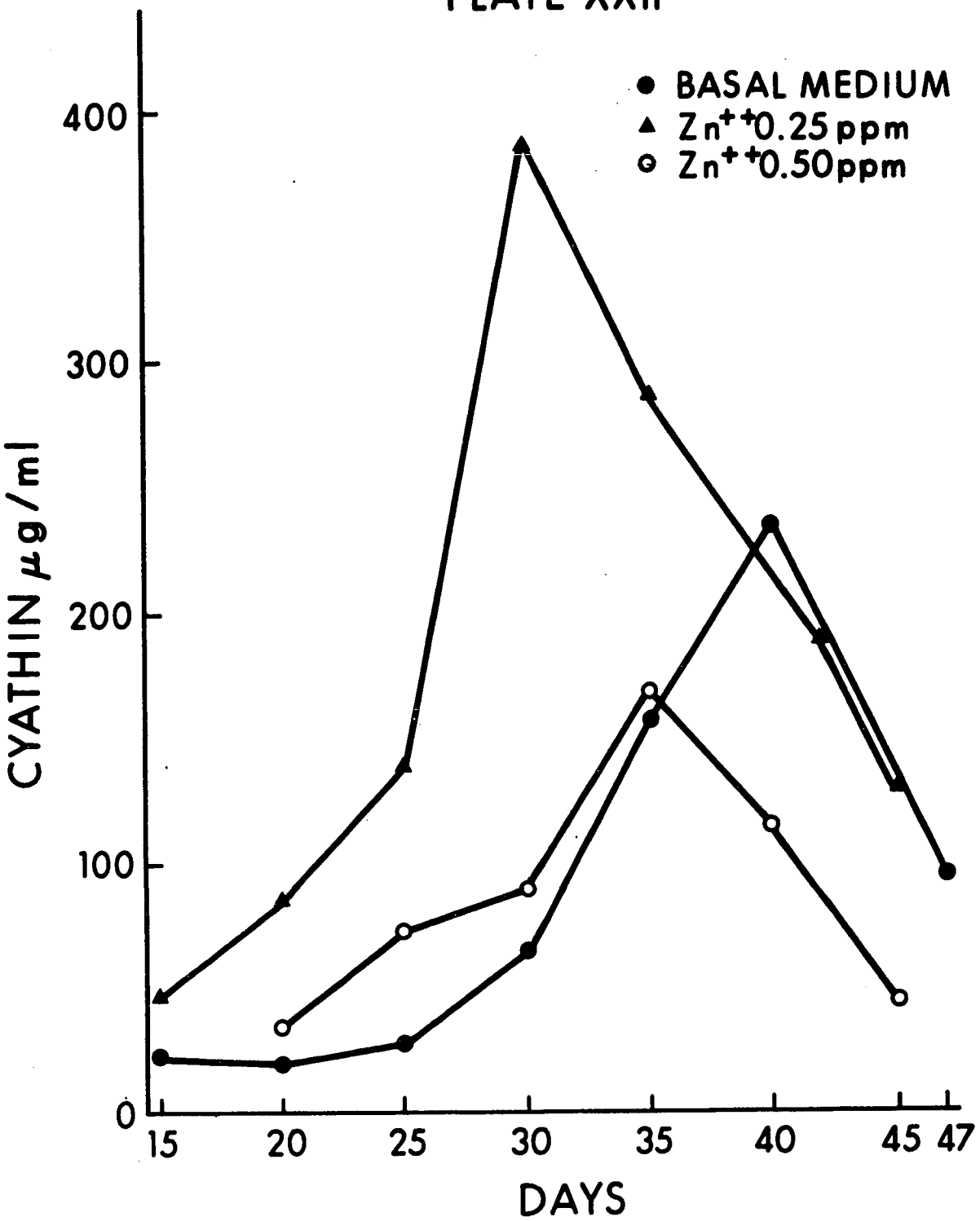
Zn++ as $ZnSO_4 \cdot 7H_2O$----------0.25 ppm

0.50 ppm

The results of this study are presented in Plate XX (Fig. 18) and Plate XXII. Copper and iron were inhibitory. *Cyathus helenae* produced less cyathin and the yields dropped by 74% and 69% respectively. The growth of the fungus was observed only visually and it seemed to be quite normal. Both copper and iron are required in enzyme actions or they are constituents of enzymes. Iron is known to play a leading role in the 'Cytochrome System' as well, and Lilly (1965) also points to the fact that iron is required by virtually all fungi. However, the amounts of iron required by fungi sometimes may be very low and as in these experiments no care has been taken to purify the reagent grade chemicals (traces of iron are always present there) it is quite likely that addition of iron from outside became toxic to the fungus resulting in a loss of cyathin activity. Another possibility is that the fungus, when transferred, carried over sufficient amounts of trace elements from the

PLATE XXII Effect of Zn^{++} on the production of cyathin. Zn^{++} was added to DMIII at a concentration of 0.25 ppm and 0.5 ppm. Note the increase in the titres of cyathin in the former concentration. Also the time of maximum cyathin production is reduced.

PLATE XXII


BASAL MEDIUM
Zn++0.25ppm
Zn++0.50ppm
400
300
200
100
0
CYATHIN µg/ml
15
20
25
30
35
40
45
47
DAYS

medium on which it was growing.

At the concentration employed for testing, manganese did not impede cyathin production and the yields were also quite high. However, addition of Mn++ to the basal medium is not necessary; for in its absence high titres of cyathin are obtained.

The most striking effect of all the micronutrients turned out to be that of zinc (Plate XXII), which caused increase in cyathin production of nearly 60%. Increase of concentration of zinc to 0.5 ppm resulted in a decrease of cyathin titres of more than 50%.

Zinc is a constituent, or is an activator of the action of a number of enzymes (Lilly, 1965). One example is that of alcohol dehydrogenase, which contains 4 atoms of zinc per molecule. Zinc is essential for the functioning of this enzyme. A concentration of zinc of 1 ppm has also been found to influence the synthesis of cytochrome C. Zinc also influences the action of tryptophan synthetase in *Neurospora crassa*. It would appear that, of these several possibilities, activation of cytochrome C could very well influence the entire metabolism resulting in increased cyathin production. Whatevery may be the role of zinc in the production of secondary metabolites, it is clearly essential for the normal growth of the mycelium of *Cyathus helenae* and for the synthesis of cyathin.

An extract of soil is a very good source of micronutrients; however, the chemical composition of soil is

variable or unknown. Amounts of soil extract added to DMIII were: 2 ml, 5 ml, 10 ml and 20 ml per liter of the medium. None of the concentrations of soil extract used in this experiment influenced the cyathin production.

Effect of Hydrogen Ion Concentration

The defined medium (DMIII) used in the nutritional study was at first used on a trial basis and, therefore, it was essential to find a hydrogen-ion concentration which would favor maximum cyathin production. The results of tests relative to pH are presented in Fig. 23 and 24 (Plate XXIII). The maximum cyathin production was obtained when the pH of the medium (after autoclaving) was 4.7. The pH 4.0 level was too acidic for normal growth of the fungus and pH 6.0 was very favorable to the growth of the fungus. Under the latter condition the nutrients were soon exhausted in the construction of the essential structures (*viz*, cell wall) of the fungus rather than in the production of metabolites. Such a postulate could explain the slightly higher cyathin titres at either pH 5 or pH 7. For optimal cyathin production, conditions should be such that the nutrients are not exhausted suddenly so that nothing remains to be converted into 'secondary metabolites'. A slow growth of fungus--which results in slightly less dry weight--apparently yields higher cyathin titres.

Temperature

Because, up to now, no basic physiological studies of the fungus *Cyathus helenae* have been made, it was

PLATE XXIII Effect of Environmental Factors on the Production of Cyathin

Fig. 23. pH of the medium. Note that the maximum cyathin titres were produced at a pH close to 5.

Fig. 24. Change in pH of the medium during the growth of the fungus and the production of cyathin.

Fig. 25. Effect of temperature on the production of cyathin. The growth of the fungus at 15°C was very low.

Fig. 26. Effect of light and dark on the production of cyathin. Apparently cyathin titres remain more or less the same when fungus is grown under continuous darkness and continuous light. However, cyathin titres are reduced under a 12 hr dark and 12 hr light period. The data point represents an average of four replicates.

PLATE XXIII

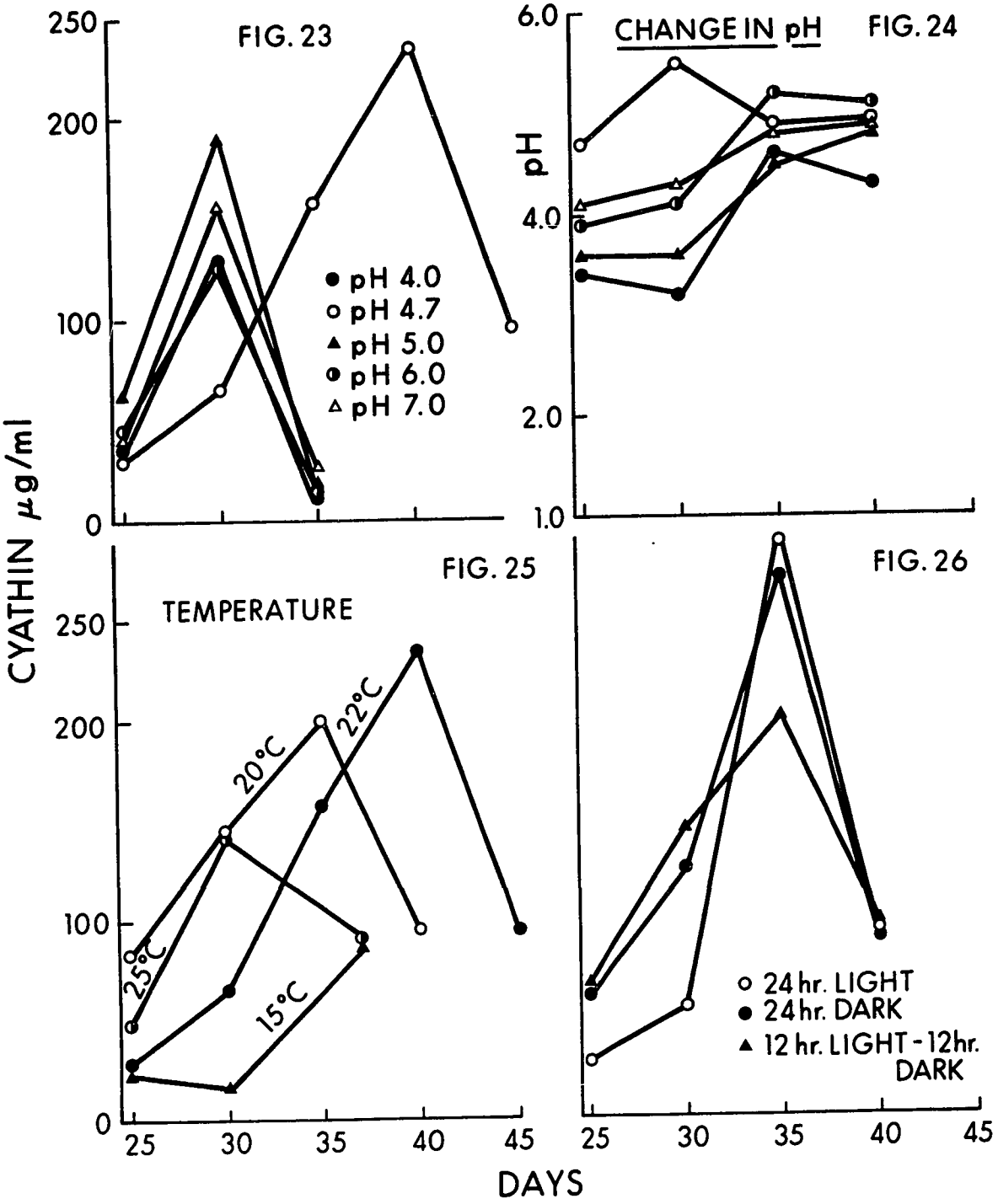


FIG. 23
250
200
100
0
CYATHIN µg/ml
● pH 4.0
○ pH 4.7
▲ pH 5.0
● pH 6.0
△ pH 7.0
CHANGE IN pH
FIG. 24
6.0
4.0
2.0
1.0
pH
FIG. 25
TEMPERATURE
20°C
22°C
25°C
15°C
FIG. 26
○ 24 hr. LIGHT
● 24 hr. DARK
▲ 12 hr. LIGHT - 12 hr. DARK
25
30
35
40
45
DAYS

essential to attempt to describe the ideal temperature conditions under which higher titres of cyathin might be realized. The results of tests are presented in Plate XXIII, Fig. 25. A noteworthy feature of the graphs is the shift in the time required for maximum cyathin production at various temperatures. The maximum yield of cyathin was obtained at 22°C. For good yields, temperature should range from 20-22°. At 25°C, yield of cyathin drops by almost 50% and at 15°C growth of the fungus was very poor. This is reflected in the cyathin titres as well which were three times as low as at 22°C. This difference results from the poor growth of the fungus and a generally low level of metabolism.

Light and Darkness

Cyathus helenae growing on DMIII was grown under three different conditions: (i) 24 hr continuous light where intensity was 60 ft-c during day and 40 ft-c during the night period. (ii) 24 hr darkness. Flasks were wrapped in aluminum foil and kept under dark. (iii) 12 hr light (60 ft-c) and 12 hr dark. The results are presented in Plate XXIII, Fig. 26; none of the first two treatments differed much in their cyathin titres, and the yield of cyathin was quite high. Noteworthy is the total amount of cyathin which was produced over the period of 40 days; it would be slightly higher in the dark grown fungus even though the maximum titres at one time (35 day) are higher in the fungus grown under 24 hr light. The difference,

however, is not very significant. In the third treatment, cyathin titres dropped. The results indicate that for cyathin production the fungus could be grown either in continuous light or continuous darkness without loss of cyathin production.

Vitamins

Seven common vitamins were also tested in examining their effect on cyathin production. These were: p-aminobenzoic acid, biotin, calcium pantothenate, folic acid, pyridoxine, hydrochloride, riboflavin, and thiamine hydrochloride. Four different concentrations for each vitamin per liter of the medium (DMIII minus thiamine) were tested, *viz*, 0.1 ppm; 0.2 ppm; 0.5 ppm and 1 ppm. The results are presented in Plates XXIV and XXV. None of the concentrations of the seven vitamins tested in this study, proved to influence cyathin production except riboflavin (Fig. 32). However, even for riboflavin, only the lowest concentration (0.1/µg/ml) was effective in increasing cyathin titres. This does not, however, imply that an external supply of riboflavin is required by the fungus for its growth, as to date no need for outside supply for this vitamin has been reported (Fries, 1965). In the present study on the other hand emphasis was not on the growth of the fungus, and for this reason not enough care was taken which would normally be required to be sure of the role of vitamins as they are required in very small

PLATE XXIV Effect of Vitamins on the Production of Cyathin

Fig. 27. Calcium pantothenate.

Fig. 28. Pyridoxine hydrochloride.

Fig. 29. Thiamine hydrochloride.

Fig. 30. Folic acid. Note that cyathin production is not influenced by any of the vitamin.

Fig. 31. Biotin.

Fig. 32. Riboflavin. Note the increase in the titres of cyathin at a concentration of 0.1 ppm.

Fig. 33. p-aminobenzoic acid.

PLATE XXIV

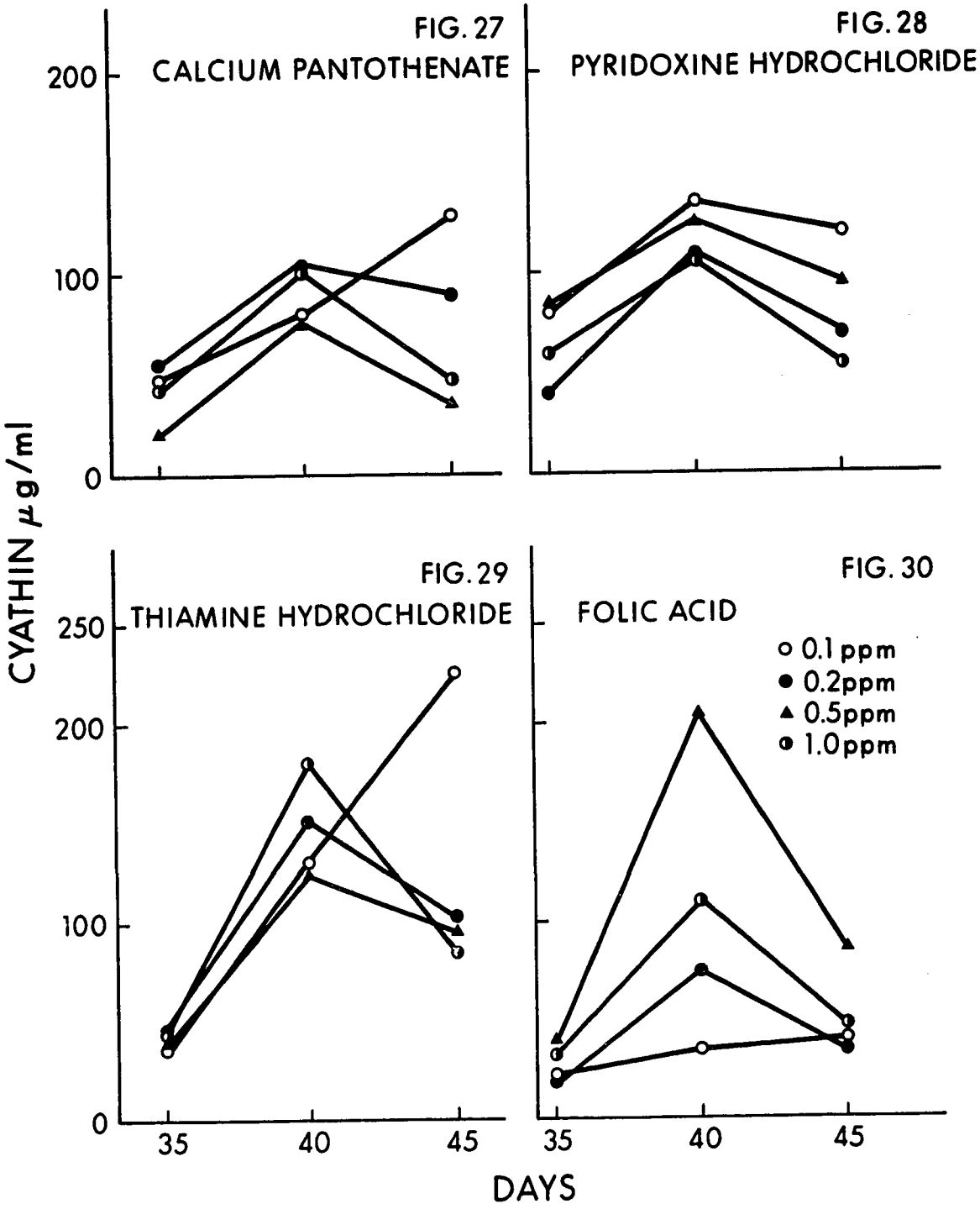


FIG. 27
CALCIUM PANTOTHENATE
FIG. 28
PYRIDOXINE HYDROCHLORIDE
FIG. 29
THIAMINE HYDROCHLORIDE
FIG. 30
FOLIC ACID
CYATHIN µg/ml
200
100
0
250
200
100
0
35
40
45
DAYS
○ 0.1 ppm
● 0.2ppm
▲ 0.5ppm
◐ 1.0ppm

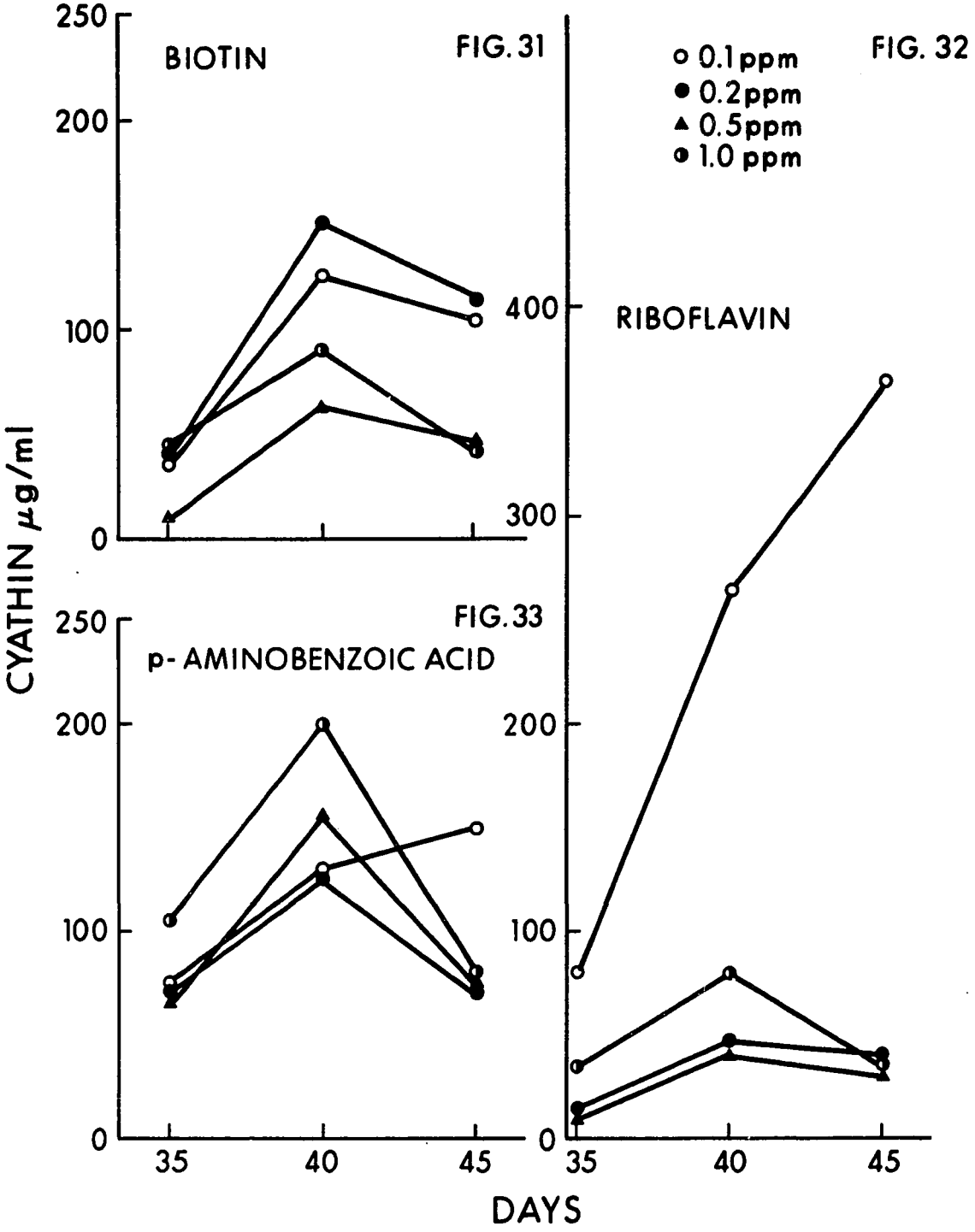
PLATE XXV Effect of vitamins on the production of cyathin

Fig. 31 Biotin

Fig. 32 Riboflavin. Note the increase in the titres of cyathin at a concentration of 0.1 ppm

Fig. 33 p-aminobenzoic acid

PLATE XXV


BIOTIN
FIG. 31
FIG. 32
0.1ppm
0.2ppm
0.5ppm
1.0 ppm
RIBOFLAVIN
FIG. 33
p- AMINOBENZOIC ACID
CYATHIN µg/ml
DAYS
250
200
100
0
400
300
35
40
45

amounts. In none of the cases of vitamins, was maximum production started earlier than 45 days, which was the last observation recorded. There is a pattern of cyathin production regarding all the seven vitamins. Up to 35 days, the cyathin content was always very low; it reached its maximum at 40 days and dropped again at 45 days, except for those cases where vitamins had any effect on cyathin titre. In the latter circumstances, even at 45 days, there was a trend towards increased cyathin production.

It was reported earlier that the deletion of thiamine (0.15 µg/ml) from DMIII resulted in an increased production of cyathin. It seems that an addition of vitamin from outside is not required by this fungus which can synthesize the vitamins necessary for growth from the nutrients available in the medium. An outside supply, therefore, results in a supra-optimal concentration which is inhibitory to fungal growth. This proposition may have value in the light of evidence already existing for a good number of fungi, which are capable of synthesizing vitamins from the nutrients available in the medium.

Carbon Sources

Although dextrose proved to be a good source for cyathin production, the need for testing various carbon sources arose out of certain difficulties which were encountered with the pattern of the cyathin complex on chromatogram, *i.e.* the various components of the complex varied in concentration in different batches. It was felt

that the use of different carbon and nitrogen sources might be utilized to yield a particular fraction of cyathin in large amounts. Six carbon sources including dextrose, maltose, fructose, sucrose, mannitol and starch were incorporated into the basal medium (DMIII) at 30 g per liter. This was followed by recording the dry weight of the fungus, change in pH and yield of cyathin at 5-day intervals, the first observation being made at 15 days. The results are given in Plates XXVI and XXVIII.

As was mentioned above, dextrose is a good source of carbon for fungal growth and cyathin production. From Fig. 17 it is quite clear that there is a long lag-phase in fungal growth which apparently is due to slow utilization of the carbon source at first. The latter condition, often termed "semistarvation" has been found to improve the yield of secondary metabolites because the fungus is not able to utilize all energy and material for its structural growth. On the other hand, maltose (Fig. 36) is utilized very rapidly and the high consumption of sugar during the growth phase of the fungus results in a comparatively smaller yield of cyathin. Fructose (Fig. 34) was a good source of carbon for fungal growth. Consumption was rapid and most of it, therefore, like maltose is probably utilized in structural growth of the fungus, resulting in lower yields of cyathin. The pH for all the three carbon sources remained between 5 and 6 and did not appear to influence the cyathin titres. However, the inability of *C. helenae* to

PLATE XXVI Effect of various sugars on the production of cyathin. Additions were made at a concentration of 30 g sugar per liter of the defined medium (dextrose deleted).

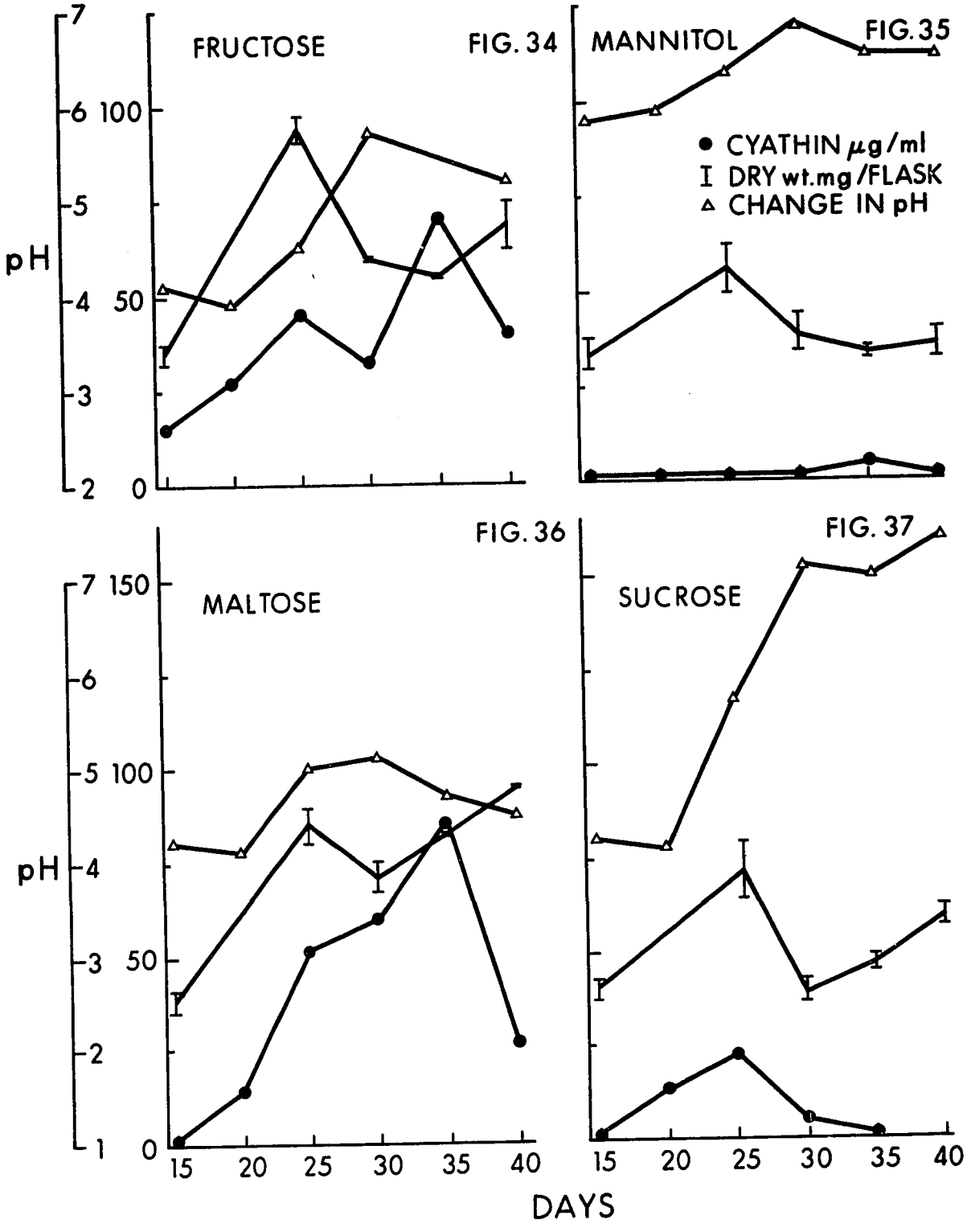
Fig. 34. Fructose (Bacto-Laevulose).

Fig. 35. Mannitol. Note the steady rise in pH of the medium which results in almost no production of cyathin.

Fig. 36. Maltose. Note the short lag-phase in the growth of fungus which brings down the cyathin titres (compared with DMIII, Fig. 17).

Fig. 37. Sucrose. Note the sudden rise in pH of the medium which results in low titres of cyathin.

PLATE XXVI


FRUCTOSE
FIG. 34
MANNITOL
FIG. 35
● CYATHIN μg/ml
I DRY wt.mg/FLASK
△ CHANGE IN pH
pH
7
6
5
4
3
2
100
50
0
FIG. 36
MALTOSE
FIG. 37
SUCROSE
7
6
5
4
3
2
1
150
100
50
0
15
20
25
30
35
40
DAYS

utilize mannitol (Fig. 35) and sucrose (Fig. 37) as sources of carbon could be accounted for by the sudden rise of pH. The original pH for the mannitol medium as well as the sucrose medium was 4.7. The pH rose steadily in the former case, while in the latter case there was a sudden rise at the 20th day when the pH rose to 5.7. This pH is tolerated by the fungus, but another rise within 5 days to 7.1 resulted in a rapid decline in dry weight as well as in cyathin production. The dry weight of the fungus on both carbon sources (mannitol and sucrose) is comparatively less than that produced on dextrose. Cyathin production on mannitol was almost nil although there was slight production of cyathin on sucrose. Soluble starch (Fig. 42) proved to be a very good source of carbon for fungal growth as well as cyathin production. The lag-period of growth was fairly short on starch medium and the fungus reached its maximum dry weight by the 25th day. Cyathin titres corresponded to the increase in dry weight but did not reach the maximum till the 35th day. However, a noteworthy feature is that cyathin titres between the 25th day and 35th day remained nearly constant. If calculated on the basis of yield throughout the course of growth (15-40 days), starch apparently gave the maximum yield of cyathin of all the carbon sources used. Starch is a cheap source of carbon which would be an advantage in the commercial production of cyathin.

Nitrogen Sources

During the early studies on the deletion of asparagine and calcium nitrate from the defined medium, it was observed that *C. helenae* grew best on organic nitrogen and that addition of nitrate resulted in two-fold increase in cyathin production. It was, therefore, desirable to substitute calcium nitrate by some other inorganic and organic sources in order to find a still better combination of organic and inorganic nitrogen source which would yield higher titres of cyathin. In studies on the effect of various nitrogen sources, therefore, additions were made to the basal medium which contained asparagine but lacked calcium nitrate.

Six nitrogen sources were used including ammonium nitrate (NH_4NO_3), ammonium sulphate [$(NH_4)_2SO_4$], calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$], glycine, potassium nitrate (KNO_3) and sodium nitrate ($NaNO_3$). The concentration of nitrogen from the various sources was the equivalent of 0.5 g $Ca(NO_3)_2$ $4H_2O$ per liter of medium. The results are presented in Plates XXVII and XXVIII.

Ammonium nitrate (Fig. 38) did not prove to be a good source of inorganic nitrogen; the growth of the fungus was poor and cyathin titres were low. The maximum cyathin yield was 40 µg/ml as compared to 140 µg/ml produced on asparagine along (Fig. 19). Ammonium sulphate (Fig. 39) more or less followed the same course as ammonium nitrate, although cyathin production reached a titre of 80 µg/ml.

PLATE XXVII Effect of Sources of Nitrogen on the Production of Cyathin

Fig. 38. NH_4NO_3. Addition was made to the defined medium having asparagine only (calcium nitrate deleted).

Fig. 39. $(NH_4)_2SO_4$. Note that the maximum cyathin titres are achieved during declining phase of fungal growth.

PLATE XXVII

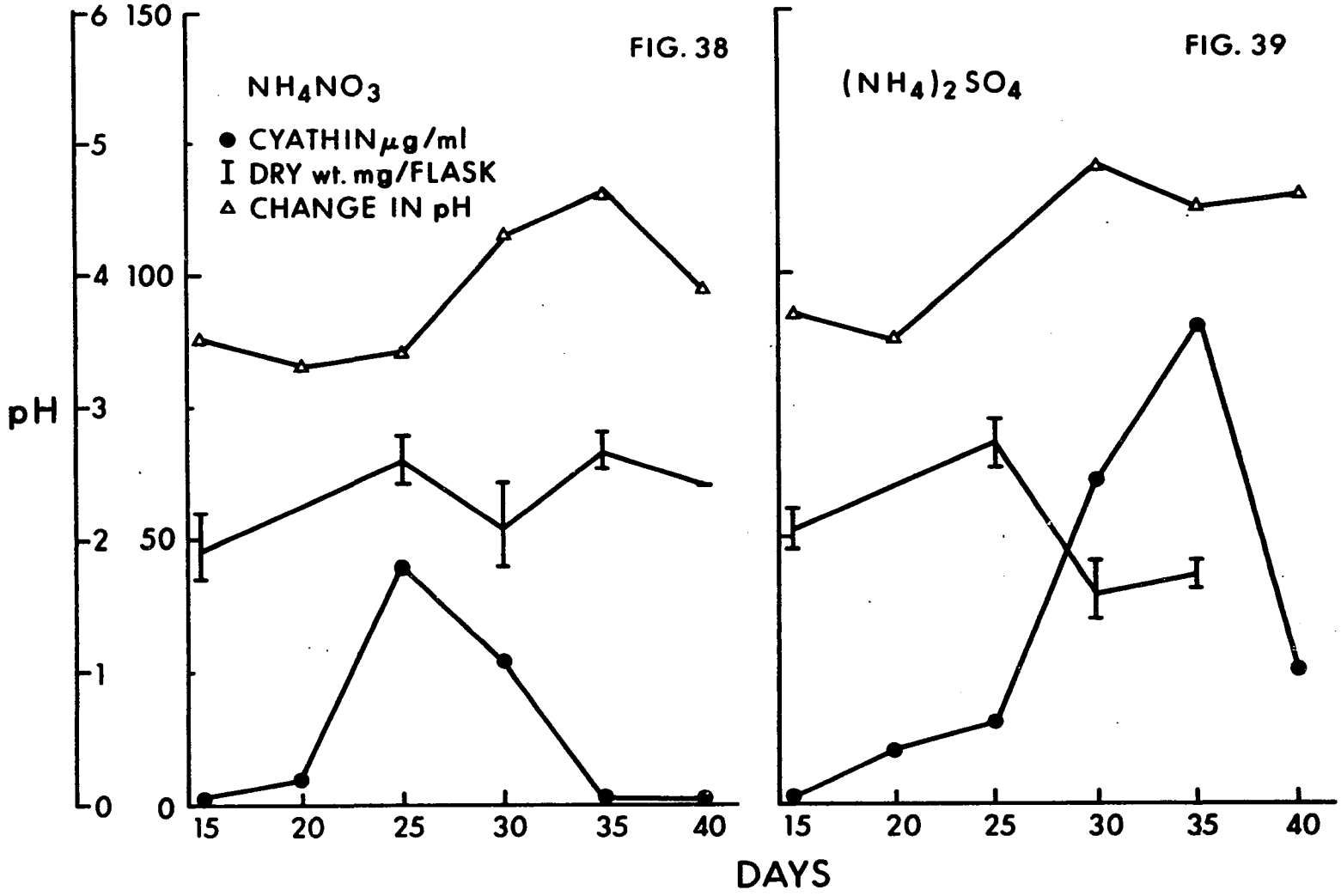


FIG. 38
FIG. 39
NH_4NO_3
$(NH_4)_2SO_4$
● CYATHIN μg/ml
I DRY wt. mg/FLASK
△ CHANGE IN pH
pH
6
5
4
3
2
1
0
150
100
50
0
15
20
25
30
35
40
DAYS

PLATE XXVIII Effect of Sources of Nitrogen and Carbon on the Production of Cyathin

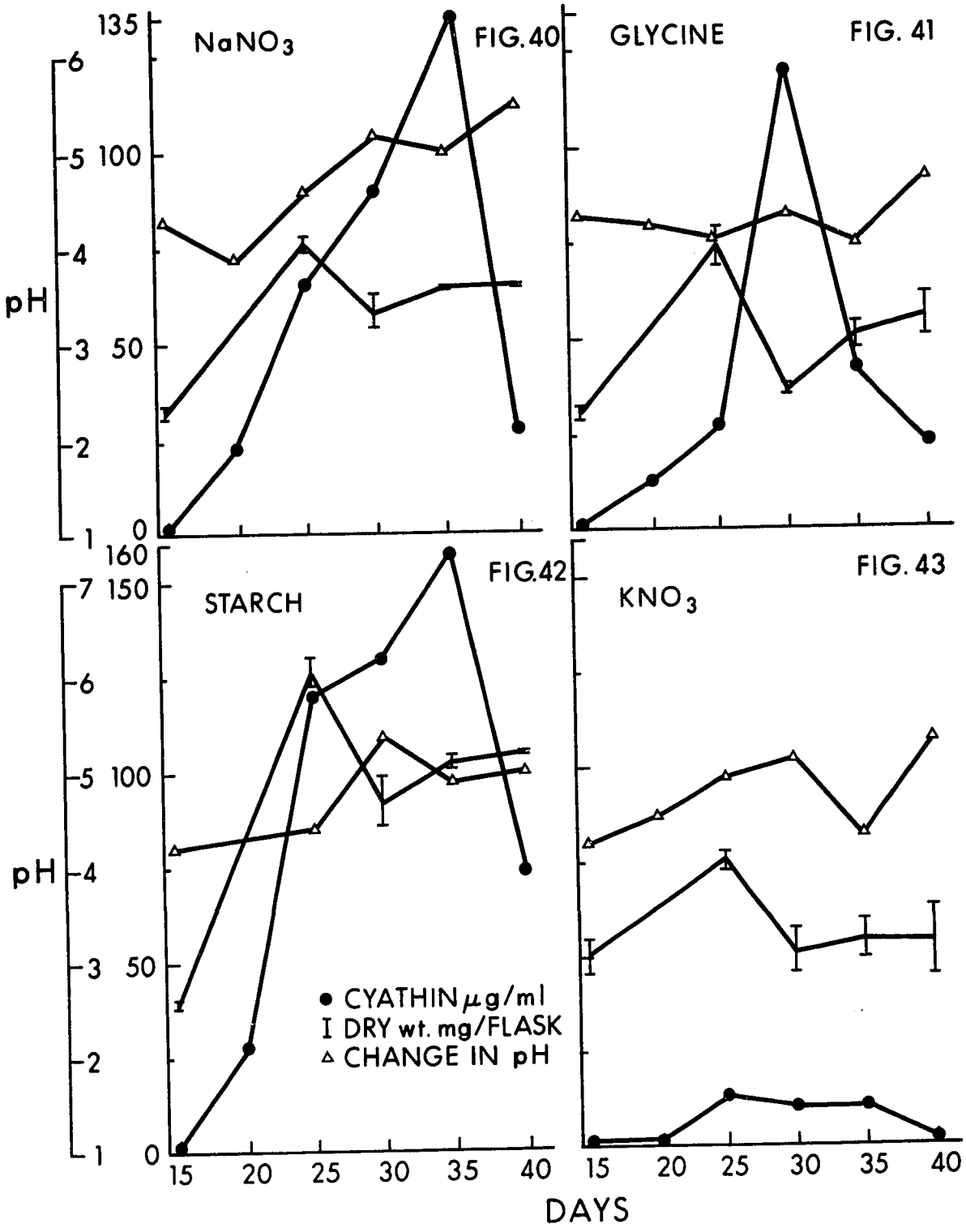
Fig. 40. $NaNO_3$. The maximum cyathin titres are achieved during the declining phase of fungal growth, presumably after autolysis of the cells.

Fig. 41. Glycine. Cyathin production is not influenced very much as compared to that on the asparagine alone (Fig. 19)

Fig. 42. Starch added to the DMIII (dextrose deleted) at a concentration of 30 g per liter. Note that the fungal growth as well as cyathin titres are high. Here also maximum cyathin titres are achieved after presumed autolysis of the fungal cells. Starch would prove a good and cheap source of carbon for commercial production of cyathin.

Fig. 43. KNO_3. Unlike other nitrate nitrogen, potassium nitrate yielded poor titres of cyathin. Moreover almost all of cyathin corresponded to the cyathin B (see Plates XXIX-XXXII).

PLATE XXVIII



For the latter nitrogen source, however, the maximum production did not occur till the 35th day and this coincided with the declining phase of the fungal growth. The pH in both nitrogen sources remained below 5 throughout the growth period.

Calcium nitrate (Fig. 17), on the other hand, proved to be a good substituent for asparagine for growth and for the yield of cyathin. There was not much change, as far as mycelial weight was concerned except that the addition of calcium nitrate resulted in a slightly slower growth of the fungus in the early stages. The maximum growth rate was manifest at 35 days. It is well known that utilization of nitrate is always slower because NO_3^- ions are normally converted to nitrite by the action of nitrate reductase (Nicholas, 1965) and before this reduction takes place growth of the fungus is slow resulting in a long lag-phase as is evident in the case of calcium nitrate. Sodium nitrate (Fig. 40) likewise proved to be a good source of inorganic nitrogen, although cyathin titres were lower as compared with calcium nitrate. However, this is explainable in terms of a comparatively shorter lag-phase which results in slightly faster utilization of sodium nitrate and results in lower cyathin titres. Potassium nitrate (Fig. 43) followed more or less the same course as for sodium nitrate as far as fungal growth and changes in pH were concerned. However, the yield of cyathin was very low in the former case. Nitrate ion, therefore, seems to be

a good substituent of part of the nitrogen source (asparagine) for *Cyathus helenae* in so far as cyathin production is concerned.

Glycine (Fig. 41) as part of nitrogen source (along with asparagine) did not result in any better yields of cyathin than when asparagine alone was used.

Chromatographic Evaluation of Culture Broth from Various Carbon and Nitrogen Sources

In addition to determining the dry weight of the fungus, the pH of the medium and titre of cyathin, the chromatographic spectra of the cyathin complex from the culture broth of various carbon and nitrogen sources were studied to throw some light on the best combination of carbon and nitrogen sources for the production of the cyathin complex or of one particular fraction. Chromatography was carried out by extracting the culture broth with ethyl acetate. The extract was reduced under vacuum and the residue dissolved in the minimum amount of acetone. The extract was then applied to 500 μ-thick silica gel G plates, which were developed in Benzene:Acetone:Acetic Acid (75:25:1). Plates were sprayed with 30% H_2SO_4 and then heated at 100°C for 5-10 min. The spots corresponding to six fractions mentioned in Table VII were recognized by their color and Rf values. Chromatograms made at the 25th, 30th, 35th, and 40th day interval are reproduced in the Plates XXIX-XXXII. An evaluation of these six fractions was also done

PLATE XXIX Chromatogram of the culture broth from various sources of carbon and nitrogen and a comparison of the components of the cyathin (25th day). From L-R: starch, sucrose, ammonium sulphate, sodium nitrate, ammonium nitrate, mannitol, maltose, fructose, potassium nitrate, glycine, and dextrose. Numbers and letters on the right side of the chromatogram correspond to various components of cyathin.

PLATE XXIX

PLATE XXX Legend same as for Plate XXIX (30th day). Note the amount of cyathin B in potassium nitrate.

PLATE XXX

PLATE XXXI Legend same as for Plate XXIX (35th day). Fructose is producing good amounts of cyathin A_3 and A_4 beside cyathin B and other nonpolar fractions.

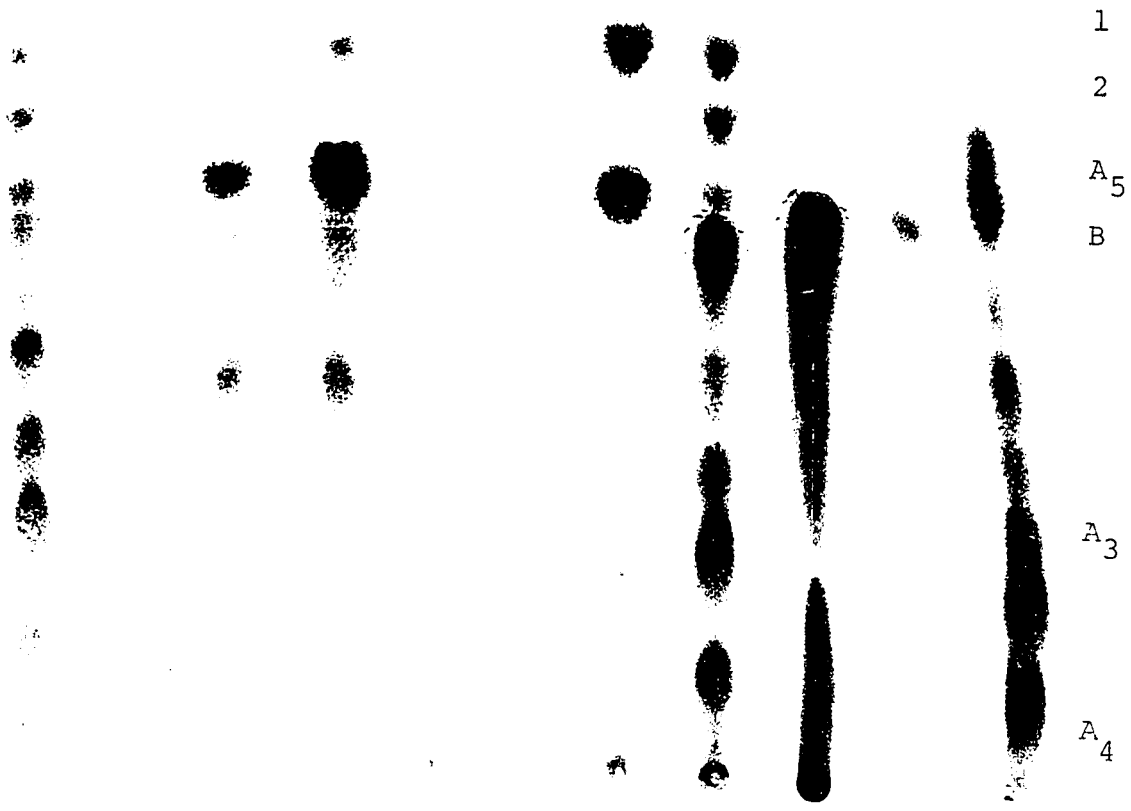


PLATE XXXI

PLATE XXXII Legend same as for Plate XXIX (40th day). Note the bright spot for cyathin A_5 in starch at this late stage. Most of the other fractions in all the sources have started disappearing.

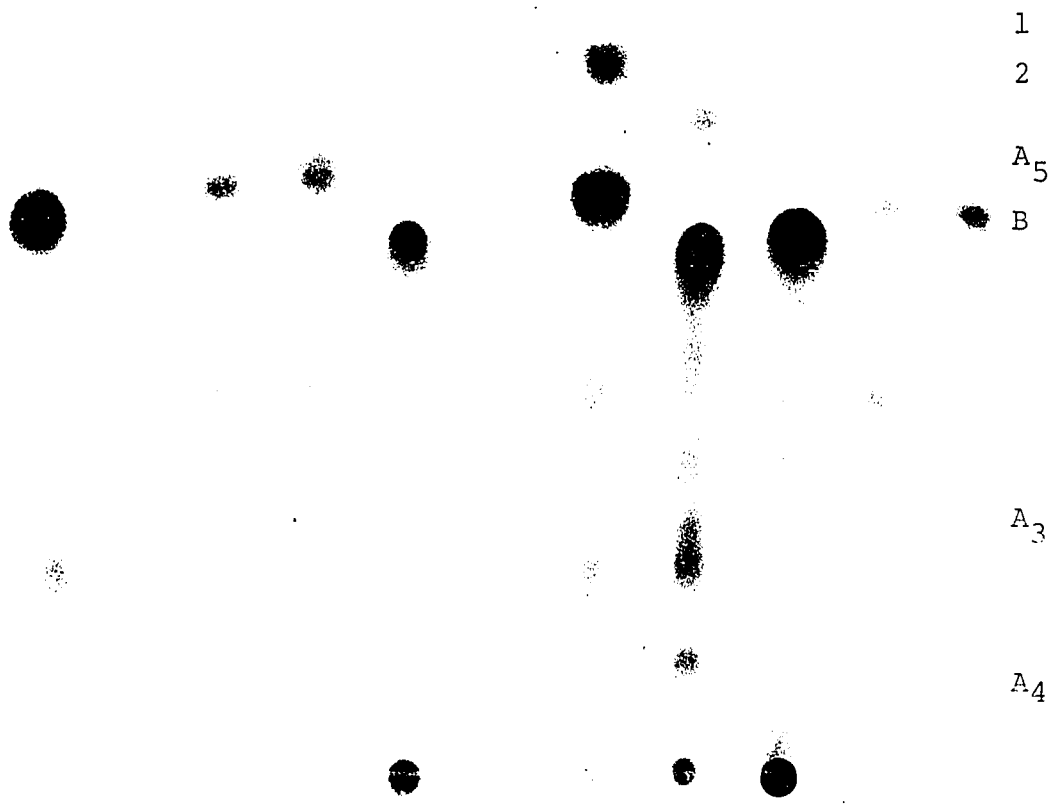


PLATE XXXII

visually on the basis of the intensity and brightness of the spots. The results obtained are given in the Tables XX-XXIII and will be discussed below in some detail.

Cyathin No. 1

This fraction of cyathin (as mentioned elsewhere) gave the maximum inhibition zones when tested against *S. aureus* at 500 µg concentration. Therefore, interest was centered on this fraction to attempt to increase its yield to obtain sufficient quantities for further microbiological work as well as chemical work. It is possible that cyathin No. 1 contains two active principles (A_1 and A_{51}). Although cyathin No. 1 is produced from a good number of carbon and nitrogen sources, sufficient amounts of it were produced only from ammonium nitrate and maltose. Of these two compounds, maltose appears to be the more important; the concentration of cyathin No. 1 remained fairly constant until the 35th day. Mannitol and sucrose produced the least amounts of cyathin No. 1. As it has been mentioned elsewhere, the titres of cyathin were also very low in these two carbon sources.

Cyathin No. 2

This fraction has not yet been purified. It has always been found to occur as a dull yellow-brown spot between cyathin No. 1 and cyathin A_5. An evaluation of this fraction was taken into consideration simply because of the consistency of the spot. This fraction was produced in higher amounts when maltose was the carbon source,

TABLE XX

Evaluation of Chromatographic Spectrum of the Culture Broth from Various Carbon and Nitrogen Sources at the 25th-day

Cyathin Fractions	Starch	Sucrose	$(NH_4)_2SO_4$	$NaNO_3$	NH_4NO_3	Mannitol	Maltose	Laevulose	KNO_3	Glycine	Dextrose
No. 1	-	+	+	+	++	-	+++	+	+	+	+
No. 2	-	+	+	+	+	-	++	-	-	-	+
A_5	+	++	+	++	+++	-	+++	++	-	+	++
B	-	-	-	+	++	-	+	+	+++	-	+
A_3	-	+	+	+	++	-	+	+	-	+	+
A_4	-	-	-	-	-	-	-	-	++	-	+

- Absence of a particular cyathin fraction
+ Amount of a cyathin fraction present based on the intensity and brightness of the spot

TABLE XXI

Evaluation of Chromatographic Spectrum of the Culture Broth from Various Carbon and Nitrogen Sources at the 30th-day

Cyathin Fractions	Starch	Sucrose	$(NH_4)_2SO_4$	$NaNO_3$	NH_4NO_3	Mannitol	Maltose	Laevulose	KNO_3	Glycine	Dextrose
No. 1	+	+	+	+	-	+	++	+	+	+	+
No. 2	-	-	-	-	-	-	-	+	-	-	+
A_5	+	-	++	++	+	-	++	-	-	+	++
B	+	-	+	+	++	-	-	-	++++	-	+
A_3	-	-	+	+	-	-	-	-	-	+	+
A_4	-	-	+	+	+	-	-	-	++++	+	+

- Absence of a particular cyathin fraction
+ Amount of a cyathin fraction present based on the intensity and brightness of the spot

TABLE XXII

Evaluation of Chromatographic Spectrum of the Culture Broth from Various Carbon and Nitrogen Sources at the 35th-day

Cyathin Fractions	Starch	Sucrose	$(NH_4)_2SO_4$	$NaNO_3$	NH_4NO_3	Mannitol	Maltose	Laevulose	KNO_3	Glycine	Dextrose
No. 1	+	-	+	+	-	+	++	+	-	-	+
No. 2	+	-	-	-	-	-	-	+	-	-	+
A_5	+	-	+	++	+	-	++	+	-	+	+
B	+	-	+	+	+	-	-	++	+++++	-	-
A_3	+	-	+	+	-	-	-	+	-	-	+
A_4	+	-	-	+	+	-	-	++	+++	+	+++

- Absence of a particular cyathin fraction
+ Amount of a cyathin fraction present based on the intensity and brightness of the spot

TABLE XXIII

EValuation of Chromatographic Spectrum of the Culture Broth from Various Carbon and Nitrogen Sources at the 40th-day

Cyathin Fractions	Starch	Sucrose	$(NH_4)_2SO_4$	$NaNO_3$	NH_4NO_3	Mannitol	Maltose	Laevulose	KNO_3	Glycine	Dextrose
No. 1	+	-	+	+	-	+	+	+	-	-	+
No. 2	-	-	+	+	-	-	+	+	-	-	-
A_5	++	-	+	+	-	+	+	+	-	+	+
B	+	-	-	-	++	-	-	++	+++	+	-
A_3	+	-	+	+	-	-	+	+	+	+	-
A_4	-	-	-	-	-	-	-	+	+	-	+

- Absence of a particular cyathin fraction
+ Amount of a cyathin fraction present based on the intensity and brightness of the spot

although the spot was not obtained on chromatograms made at the 30th day. Starch, mannitol, potassium nitrate and glycine apparently did not bring about the production of this fraction.

Cyathin A_5

Attention was given to this fraction early in the research because this was the first component of the cyathin complex to be crystallized. The yield, however, was very low and the most serious problem had been the inconsistency in the appearance of the spot characteristic for cyathin A_5. It has been observed that even when the nutrient source was constant this fraction sometimes appears, while at others it disappears. For this reason, after the first few milligrams of crystalline material had been obtained no further amounts have been purified. The first lot was sufficient only to permit determination of its empirical formula ($C_{20}H_{26}O_5$). From the chromatographic work it becomes quite clear that cyathin A_5 apparently disappears just after the 25th day. However, up to 25 days it appeared in appreciable amounts from media containing sucrose, sodium nitrate, ammonium nitrate, maltose, laevulose and dextrose. Except for mannitol and potassium nitrate all other nutrients permitted the production of this fraction at one time or another. The maximum amounts were produced on a maltose-containing medium. Maltose appears to be a suitable source of carbon for more or less all the non-polar fractions of cyathin (No. 1, No. 2 and A_5).

Cyathin B ($C_7H_6O_4$)

This fraction of the cyathin complex has been the most completely analyzed. The simplicity of its structure (2,4,5-trihydroxybenzaldehyde) resulted in its chemical synthesis in a short time. Thus its production on culture medium was not of much interest. Surprisingly enough, great amounts of this fraction were produced from medium containing potassium nitrate. On a per cent basis the yield would make up at least 75% of the total cyathin produced on this nitrogen source. It would be interesting to follow the biosynthesis of this fraction and especially the role of KNO_3 in the process.

Cyathin A_3 ($C_{20}H_{30}O_3$)

This fraction of cyathin has an Rf value of 0.27. In those cases where amounts of cyathin B are large (*e.g.* KNO_3), sometimes the spot corresponding to cyathin A_3 becomes masked. Except for the medium containing ammonium nitrate, which produced slightly greater amounts of cyathin A_3, the rest of the sources also produced this fraction (mannitol is an exception) but not in a striking fashion. Even for ammonium nitrate, the spot corresponding to cyathin A_3 disappeared between the 25th day and the 30th day.

Cyathin A_4

Cyathin A_4 falls in the same series as that of cyathin A_3 and it differs from the latter only in the possession of an extra oxygen atom. Cyathin A_4 has the empirical formula $C_{20}H_{30}O_4$. However, as compared with

cyathin A_3, it is very polar in the solvent system used and tends to remain at the point or origin. The demarcation of the spot from the rest of the origin point, however, is clear-cut and therefore, attempts to use other solvent systems have not been considered. At the 25th day only KNO_3 and dextrose produced this compound. A medium containing potassium nitrate yielded the maximum amount, production reaching its peak at the 30th day. Laevulose is one of the sources on which cyathin A_4 did not appear till the 35th day. Maltose, mannitol and sucrose did not produce any detectable amounts of this fraction throughout the 40 day period.

Evaluation of the various carbon and nitrogen sources on the basis of this qualitative scheme (refer to p. 99) does not explain much except that cyathin components are very variable and that even a small change in the composition of the medium can bring about a drastic change in the pattern of the complex. The present study does indicate the best combination of carbon and nitrogen sources which would aid production of the whole cyathin complex or just of one particular fraction. Last but not least important, this chromatographic analysis of the culture broth also indicates the best time of harvesting the cultures when a particular fraction of the cyathin complex would be present in largest amount.

The Effect of the Cyathin Complex on Seed Germination and Growth of Seedlings

Seed Germination

Cyathin impeded the germination of seeds of all the four varieties of plants (timothy, tomato, radish and pea). The results of this study are presented in Table XXIV. In the case of timothy and tomato there was no germination, while in the case of pea, the seeds recovered from the action of cyathin (360 µg/ml) after 4 days, and at 10 days there was 100% germination. Recovery at a concentration of 180 µg/ml was even faster; 80% of the seeds germinated after 4 days. In the case of radish, recovery at a concentration of 180 µg/ml was slow and even after 10 days only 31% germination was recorded. Increasing the cyathin concentration to 360 µg/ml resulted in a complete check of germination. In this experiment seeds were placed on filter paper soaked with antibiotic solution. In the second experiment seeds of all the four varieties were soaked for 24 hours in a cyathin solution of a concentration of 90 µg/ml. Seeds were washed thoroughly afterwards with distilled water and allowed to germinate. Except for tomato, remaining three kinds of seeds recovered after two days and showed 85, 92, and 100% germination for timothy, radish, and pea respectively (Table XXV). It is difficult to seek an explanation for the results of the above two experiments; in the latter experiment it would be expected that the effect of cyathin should be more severe. However,

TABLE XXIV

Germination of Seeds in the Presence of Cyathin (%)

Days	RADISH			PEA			TIMOTHY			TOMATO		
	Control	Cyathin (µg) 360	720	Control	Cyathin (µg) 360	720	Control	Cyathin (µg) 360	720	Control	Cyathin (µg) 360	720
1	95	0	0	0	0	0	0	0	0	0	0	0
2	98	0	0	0	0	0	0	0	0	0	0	0
3	100	9	0	100	5	0	75	0	0	40	0	0
4	100	9	0	100	80	0	100	0	0	50	0	0
5	100	13	0	100	100	20	100	0	0	55	0	0
6	100	13	0	100	100	20	100	0	0	68	0	0
10	100	31	0	100	100	100	100	0	0	70	0	0

TABLE XXV

Effect of Presoaking in Cyathin* on the Subsequent Germination of Seeds (%)

Days	RADISH		PEA		TIMOTHY		TOMATO	
	Control	Cyathin Treated	Control	Cyathin Treated	Control	Cyathin Treated	Control	Cyathin Treated
2	98	80	100	90	75	12	0	0
3	100	92	100	100	90	85	40	0
4	100	92	100	100	100	95	50	0
10	100	100	100	100	100	100	70	8

*Seeds were soaked for 24 hr

it is possible that inhibitors of seed germination leached out during the soaking period and counteracted the effect of cyathin on seeds. As was noted, radish seeds germinated almost 100%. The effect of presoaking these seeds using three different cyathin concentrations was also studied on seed germination and growth of seedlings. The results are shown in Table XXVI. Increasing the concentration of cyathin from 50 µg/ml (MIC for *Staphylococcus aureus*) to 360 µg/ml, had a pronounced effect. In the highest concentration only 5% of the seeds germinated, even after 8 days of observation. The controls showed 98% germination by this time. It was also observed that, even at the lowest concentration (50 µg/ml), growth of the seedlings was very slow. Also root hairs, cotyledons and primary leaves did not develop normally.

Similar retardation of the growth was also observed when 3-day old healthy seedlings of radish were transferred to a Petri plate lined with cyathin-soaked absorbent cotton. The effect of cyathin was always more pronounced on the root system than on the shoot system. The formation of lateral roots was completely checked and the root hairs were killed within a 24 hr period; an overall effect was injury to the seedlings, resulting in complete cessation of growth.

Growth of Seedlings

To avoid a direct contact of cyathin with the entire plant, in another experiment, the antibiotic was added to nutrient Hoagland's solution. Some 6-day old

TABLE XXVI

Effect on Germination of Presoaking Radish Seeds in Various Concentrations of Cyathin

Days	Control (Germ. %)	50 µg/ml		100 µg/ml		360 µg/ml	
		(Germ. %)	(Inhibition %)	(Germ. %)	(Inhibition %)	(Germ.%)	(Inhibition %)
2	76	63	10	30	46	0	76
4	89	80	9	55	34	2	87
6	98	90	8	73	25	5	93
8	98	90	8	73	25	5	93

% Inhibition of germination has been expressed relative to the germination of seeds in control batch at a particular day interval

seedlings of radish, pea and tomato were placed so that their root systems only were in contact with the nutrient solution. Five different concentrations of cyathin were used: 10 µg/ml; 18 µg/ml; 38 µg/ml; 45 µg/ml and 340 µg/ml. For each concentration of cyathin, five plants were used. During this experiment it was observed that even a 1:10 proportion of methanol to Hoagland's solution was toxic. The control plants died. Addition of methanol was made to make sure that the effect observed on seedlings was that of cyathin and not of methanol (as methanol was used in preparing cyathin solutions of 38 µg/ml and 340 µg/ml concentrations). In the following results, these two concentrations of cyathin and their effects on growth of seedlings have been omitted because of the toxicity of methanol.

Radish

The seedlings at a concentration of 45 µg/ml died within 16 hr. However, at 18 µg/ml concentration, both root and shoot system appeared healthy, although there was almost a complete lack of root hairs. At the 10 µg/ml concentration also, except for a decrease in the number of root hairs, treated seedlings appeared healthy as compared with untreated seedlings.

On the seventh day, the plants were still alive in the above mentioned two concentrations, although there was an overall effect of retardation of growth as compared with control plants. The effect appeared to be more severe on the root system, which showed a complete lack of laterals

and also root hairs. Also a slight brown coloration of the root tip could be seen. At 14 days it was observed that leaves were also smaller than normal and the primary leaves showed a yellow coloration and black spots on the surface.

Pea

Twenty four hours after transfer of the seedlings to the cyathin solution, they appeared healthy; both root and shoot system appeared to be comparable to those of control plants. Even at the highest concentration, root hairs were present. On the seventh day, seedlings in 10 µg/ml and 18 µg/ml concentrations still survived, although there was a general effect on the plants; growth was slow, and the plants were less developed than control plants. The root system showed a slight brown coloration. An interesting effect on the leaves was mottling; light yellow patches were present all over the surface. By the 14th day growth of each whole plant was slow and secondary roots did not develop. Leaves showed a marked chlorotic effect with yellow patches all over the leaf surface.

Tomato

At 24 hours, seedlings appeared healthy and comparable with control plants, both in their root system and shoot system. There was no deformation or coloration of the tissues. On the 7th day, the effect on root and shoot system was not as severe as was noted for radish and pea. However, slight brown coloration of the roots was observed even at a concentration of 10 µg/ml. No other abnormalities

were noted.

In 14 days general growth of the plants slowed down. Root systems did not develop any secondary laterals. The formation of leaves was also checked, as only the first two leaves (in cyathin treated plants) could be seen, whereas control plants had by then developed at least four leaves. The leaves of treated plants showed a slight curling of the tips and some reduction in the size. Root hairs were almost lacking.

The tests of the effect of cyathin on seedlings of radish, pea and tomato clearly demonstrated that cyathin is toxic to plants and that the root system appears to be adversely affected. A further test of the effect of cyathin on the root system was made using excised tomato roots. It was noted that concentrations of cyathin as low as 6 µg/ml were sufficient to bring about a 70% inhibition of root growth (Table XXVII). Increase in cyathin concentration to 12 µg/ml, 24 µg/ml and 96 µg/ml, resulted in root inhibition by 80, 83, and 90% respectively. Moreover, in all the concentrations, cyathin-treated roots developed few secondary roots or root hairs. A slight brown coloration was noticeable at the highest concentration used in this experiment. The implications of the effect of cyathin, especially on root hairs are discussed elsewhere.

To test further the toxicity of cyathin, 15-day old Broad Bean plants were injected with various concentrations of cyathin solutions. Plants were kept in a controlled

TABLE XXVII

Effect of Various Concentrations of Cyathin on Growth of Tomato Roots *In Vitro*

Cyathin (µg/ml)	Root Growth* (cm)	Inhibition of** Growth (%)
Control (no cyathin)	18.5	0
6	4.45	70.6
12	3.45	80.2
24	3.1	83.3
96	1.8	90.3

* Average of five measurements

**Control taken as 100% growth

growth chamber and grown on Hoagland's nutrient solution. The plants remained normal and healthy even after 3-week's growth, although the root system showed a brown-black coloration. Similar results were obtained by injecting into Red Kidney Bean plants (15-day old) different concentrations of chemically synthesized cyathin B (500 µg, 750 µg and 1.25 mg per plant). Plants in triplicate were injected hypodermically along with control plants which were injected with an equal volume of 50% acetone water [cyathin B solution was made up in acetone:water (1:1)]. Plants were grown on a U.C. mixture in earthenware pots. The plants were kept under a 16 hr day length. Temperature varied between 70-60°F during day and night.

Control plants as well as cyathin B-treated plants at all the concentrations appeared to be normal and healthy even after three weeks of treatment.

DISCUSSION

The activity of cyathin against actinomycetes, bacteria, and fungi leaves no doubt that it is an antibiotic having a broad action-spectrum. All actinomycetes examined proved to be sensitive. Among the bacteria tested, sensitivity was most marked for gram-positive organisms; gram-negative bacteria responded weakly even at a high concentration of cyathin. A selective action against bacteria might be accounted for by the differences in the structure of the bacterial cell membranes. Salton (1967) recently reviewed the structure and function of bacterial cell membranes and pointed out that, in gram positive bacteria, cell membranes are composed of a plasma membrane and the mesosome, whereas gram-negative bacteria possess an additional external layer which can prevent certain compounds of low molecular weight from penetrating the cell. As far as is known, all the four purified cyathin fractions have a low molecular weight. Thus the possibility exists that the gram-negative bacteria do offer some barrier that prevents cyathin from entering some bacterial cells.

Among the fungi tested, most of the sensitive members belong to the class Deuteromycetes (Alexopoulos, 1961). None of the Phycomycetes and yeasts exhibited any noticeable sensitivity. The resistance of yeasts may be explainable on the basis of the chemical constitution of the cell wall, which in yeasts is composed mainly of glucan, mannan and proteins (Aronson, 1965). Chitin is present only in

very small proportion. In contrast, members of the order Mucorales have chitosan-chitin as the main cell-wall component, and Deuteromycetes have chitin-glucan (Bartniki-Garcia, 1968). The resistance of species of the order Mucorales is difficult to explain on the basis of cell-wall structure as the latter is comparable with that of the Deuteromycetes; there may exist some 'blocking' mechanism for the action of antibiotics in the metabolism of the cell contents. No tests of the activity of cyathin against protozoa or tumors have been made so far; it would be desirable to make such tests in order to assess the possible practical value of cyathin. The latter would depend upon its toxicity as, in all the antimicrobial tests, a very high concentration of the antibiotic was employed as compared with the concentration of known antibiotics usually employed. It has been established in this study that cyathin is phytotoxic even at very low concentrations, at least as far as root systems are concerned.

Comparison of the production of cyathin by different strains of *Cyathus helenae* was made using only one medium. Some strains did produce the antibiotic, others did not. The absence of cyathin production in some strains points to physiological differences among strains of the fungus, and one might suppose that these strains lack some enzyme(s) catalysing the reactions necessary for the synthesis of cyathin. It is probable also that different strains require different nutrition for the activation of

the system which would result in cyathin production. In the limited study reported herein no efforts were made to find the answer to the problem posed above.

Although Brodie (1966) suggested the relationships between *Cyathus helenae* and *C. striatus* (later confirmed by Olchowecki and Brodie, 1968), the production of cyathin by both these species and a comparison chromatographically of the products did not prove anything as to species relationship beyond what the above workers had already revealed. As the present work was not directed towards the solution of this problem, no further study was made. In the writer's opinion, however, it would be rewarding not only to compare *C. helenae* and *C. striatus* but also other species in greater detail to establish their relationship on a biochemical basis, using antibiotic production and chromatographic spectra.

A comparative analysis of sectors produced by mycelium of *C. helenae* (Olchowecki and Brodie, 1968) was made regarding their exogenous and endogenous cyathin production as well as the total titres of cyathin obtained at various time intervals. The chromatographic analysis left no doubt that all sectors produce the same complex with all its components present. Any difference in the total titres of cyathin from various sectors is probably determined by the amounts of nutrients reaching the cyathin-producing machinery.

Reports of synergism and antagonism are frequent

in research literature concerning drugs. The latter phenomenon has also been observed for cyathin. This is well illustrated by the zones of inhibition produced by the cyathin complex and by its individual components. Whereas cyathin produced 10 mm inhibition zones at a concentration of 500 µg per disc the most active fraction, A_{51}, also produced the zones of the same size. However, it might be expected that when all the fractions were present together (as in cyathin) they should be more effective than when present separately. Since this was not found to be true, one must conclude that when the fractions are present together, one fraction antagonizes the effect of another or of the others.

Even the most active fraction of cyathin, cyathin A_{51}, when compared with aureomycin, novobiocin and tetracycline, proved to be at least fifty times less effective in terms of concentration.

Yousef *et al.* (1967) have clearly demonstrated that the inhibition zones of an antibiotic produced on an agar medium depend on the extent to which it may be 'bound': the tighter the binding is, the smaller the inhibition zones. It is quite possible that cyathin happens to fall in this category of compounds, with the result that small inhibition zones are produced, even at very high concentrations. Moreover, the possible existence of inhibitors of antibiotic action, such as might be present in the bacteriologic agar, must be recognized. Kunin

and Edmondson (1968) recently showed that the smaller zones of inhibition produced by the polypeptide antibiotics, polymyxin and colistin, result from the presence of sulfate groups in the agar. Protamine sulfate and toluidine blue were found to be effective in increasing the zone sizes. In the writer's opinion, such an observation cannot be taken as being universally applicable, because both toluidine blue and protamine sulfate proved to be inhibitory to the strain of *Staphylococcus* used for cyathin testing. This, however, does not exclude the possibility of inhibitors restricting the action of cyathin.

Hustavova *et al.* (1965) utilized a simple technique involving paper strips in evaluating the effects of substances influencing the oxidation-reduction potential of the medium on the action of oxytetracycline (OTC). The pronounced resistance of resistant strains of staphylococci to the action of OTC, especially the reducing activity (*viz.* the reduction of triphenyltetrazolium chloride) indicated its implication as the site of action in those bacteria that are sensitive. The purpose of testing the action of various substances on cyathin was also to explore the possibility of such an effect; the results might indicate the mechanism or site of action of cyathin. As mentioned in the Results section, l-cysteine inhibited the action of cyathin, thus indicating that action of cyathin might involve thiol inactivation. Molnar (1967), in an account of the mechanism of action

of raphanin, mentioned that thiol inactivation is fairly widespread among antibiotics. Several antibiotics of different chemical composition and having different mechanisms of action (such as penicillin and streptomycin) are known to inactivate the thiol compounds. Molnar has shown that raphanin has a non-competitive inhibitory action on the -SH enzymes urease and succino dehydrogenase. Raphanin was found to have no effect on enzymes lacking -SH groups, indicating that the antibacterial action of raphanin may be due to inactivation of -SH enzymes. In Molnar's experiments, the urease inactivating effect of 200 µg/ml of raphanin was almost completely prevented by 100 µg/ml of l-cysteine. In the present study also, 90% inhibition of cyathin action at 400 µg concentration was brought about by the addition of 0.1 ml of a 0.1 M l-cysteine solution. It would be interesting to follow this to see whether or not cyathin also 'distinguishes' between enzymes having or lacking -SH groups. A recent report by Vincent and Sisler (1968) also attributes the antifungal action of 2,4,5,6-tetrachloroisophthalonitrile (TCIN) to thiol inactivation. These authors suggest that TCIN probably causes simultaneous inhibition of a number of thiol-dependent reactions in fungal cells. Komatsu and Tanaka (1968) have recently suggested that the inhibitory action of showdomycin (2-β-D-ribofuranosyl maleimide) on the incorporation of amino acids and purine and pyrimidine bases into macromolecules in *E. coli* K-12 cells, could

be reversed by the addition of a nucleoside or a sulphydryl compound. The substances l-cysteine and 2-mercaptoethanol reversed the inhibitory effect; time-course studies showed that the earlier these compounds were added, the greater the reversing effect during a short period of incubation. Although -SH compounds had a reversing effect, these workers were not able to locate the site of action of the antibiotic; therefore, it is difficult to decide the role of -SH compounds. However these reports do not exclude cyathin from being an inhibitor of -SH-containing-enzymes or from having an action on the synthesis of macromolecules. Further work with cyathin in this respect would be very interesting and rewarding.

A note may be made here of cyathin B, which seems to have a different mode of action from cyathin: l-cysteine did not have a very pronounced reversing effect. Caution is needed when working with the separate components of cyathin for it is quite possible that each component has a separate mode of action.

The use of 'still' cultures as opposed to the shake culture process in the production of antibiotic substances by fungi is quite common. Cyathin also was found to be produced in higher titres on a 'still' culture. In the shake culture the fungus produced globular, clumped mycelial structures and grew unevenly. Probably this would result in reduced cellular activity, although shaking does provide the best utilization of dissolved

oxygen. Moreover, release of an antibiotic from the mycelium into the medium could be retarded. The different titres of cyathin obtained when various types of media were utilized, needs no discussion, as it is self evident that the fungus might grow better under some conditions than under others. For antibiotic production, usually the criterion is not the amount or weight of fungal growth, but of high antibiotic titre irrespective of the development of the organism. To achieve this on a commercial scale, various cheap sources of nutrients are examined. One such trial for cyathin production was the use of corn-steep liquor and of soil extract. The former product proved to be of no value, as no indication of the presence of cyathin could be found. The use of soil extract did not result in an increase in the yield of cyathin. Similarly, yeast extract was of no use, whereas on peptone, cyathin titres were comparable to those produced on basal medium. Owing to the difficulty of obtaining such natural products (which are quite often waste products of various processes), a detailed survey of various media is still required for the commercial production of cyathin.

Regarding the possible effect of micronutrients, only zinc increased the yields of cyathin. It is not surprising to find such an effect because zinc appears to be essential for all fungi (Lilly, 1965). Zinc is a constituent of or an activator of a number of enzymes and, therefore, an increase in cyathin titres could be accounted

for by the proper functioning of the machinery involved in the activity of fungal cells. The low titres of cyathin in the presence of copper and iron can be explained on the basis of the concentrations used, although fungal growth under these conditions appeared to be quite normal. Very often these ions, if present alone, become inhibitory, while the presence of another ion may activate the action. This mutual synergism has been reported (Lilly, 1965) but to draw any conclusions on the inhibitory action of copper or iron on the basis of present study may not be justified.

The optimum temperature for cyathin production was found to be 22°C ±1, although fungal growth appeared to be better at 25°C. Deverall (1965), in his review of the effects of temperature on metabolism, mentions a report on the submerged culture of *Claviceps paspalis*, where the concentration of a lysergic acid derivative (after 9 days) was 530 µg/ml at 21°C, but only 20 µg/ml at 30°C, although the dry weight of mycelium differed but little over this temperature range. That is, the best temperature for fungal growth may not necessarily be the best for the production of a secondary metabolite.

The investigation of the effect of the pH of the medium on the titres of cyathin shows that, regardless of pH, cyathin production continues. However, the maximum production appeared at a pH close to 5; this value beyond doubt was not optimal for fungal growth, which occurs at approximately pH 6. A slightly reduced growth

would result in a slow use of nutrients thus allowing sufficient time for the release of secondary metabolites.

Carbon and nitrogen sources are the major nutritional factors affecting the growth of fungi as well as the production of secondary metabolites. Evans and White (1966) studying the effect of carbon sources on the yield of radicicolin and radicicol (produced by *Cylindrocarpon radicicola*) reported that lactose and mannitol proved to be poor sources of carbon whereas maltose, dextrose, sucrose, starch and glycerol gave high titres of radicicolin and radicicol. *Cyathus helenae* grew poorly on sucrose and on mannitol. Dextrose was the best source for obtaining a high titre of cyathin, probably because the fungus did not utilize dextrose too rapidly; the latter condition might create a 'semistarvation' period. Perlman (1965) has recorded that such a 'semistarvation' is required for highest level of penicillin production. If too much sugar is present, or one that is utilized very rapidly, more of the sugar is converted into mycelium, resulting in lowered penicillin production. For cyathin production, maltose, d-fructose (laevulose) as well as soluble starch are all good carbon sources; however they are utilized fairly rapidly and the result is that yields are low. Recently Fulton and Bollenbacher (1968) reported the effect of various carbon sources on the production of a phytotoxic principle produced by *Alternaria tenuis*. Of twelve sugars tested, sucrose gave the best yields, while d-xylose,

d-fructose, l-sorbose, maltose, and cellobiose produced no toxin; d-glucose, d-galactose, d-mannose, lactose and raffinose gave intermediate yields. These reports show that every fungus has a different pattern and the production of secondary metabolites from one carbon for a particular fungus may be of no value for another fungus, owing to the different systems operating for the utilization of sources.

Westlake *et al.* (1968) studied the influence of nitrogen source on the formation of chloramphenicol by *Streptomyces* sp. (strain 3022a). The compounds tested included common amino acids and some inorganic nitrogen sources. They reported that sources such as peptone and nitrate supported rapid growth, but that the high initial rate of antibiotic production was not sustained. On the other hand, nitrogen sources which resulted in slow, controlled growth provided the highest yields of chloramphenicol. The substitution of part of the nitrogen (supplied as DL-serine) by nitrate (*e.g.* up to 2% of the total nitrogen) decreased the fermentation time required without affecting the total amount of chloramphenicol produced. The same is true for cyathin. When calcium nitrate was added to asparagine it increased the production of cyathin without affecting mycelial growth. Other nitrates had a similar effect (except KNO_3); slow and controlled growth was maintained only on calcium nitrate. Therefore, for a good yield of cyathin, a combination of

carbon and nitrogen sources allowing slow and controlled growth would be expected to result in better yields.

The medium can affect the character of chromatograms obtained from the fermentation broth. Plocieniuk and Kowezyk-Gindifer (1968) reported that the chromatograms of broth cultures of *Streptomyces vinaceus* (which produces viomycin), changed during fermentation. In their study, glucose caused the disappearance of the inhibition zone in the position characteristic of viomycin. Similar chromatographic changes in the cyathin complex have been noted when carbon or nitrogen sources are altered. These changes would be more pronounced in a complex of antibiotic (as for cyathin) than for a single component antibiotic; moreso if the synthesis of different components occurs via different metabolic routes. For cyathin, biosynthetic routes have not been worked out so far; therefore, the changes in the chromatographic pattern obtained from the culture broth are difficult to interpret at this time. All the components of the cyathin complex, purified to date, contain only carbon, hydrogen and oxygen; three of them, *viz.* cyathin A_3, A_4, and A_5 seem to belong to one series; cyathin B ($C_7H_6O_4$) does not fit into the same scheme. Whether or not all four cyathin components mentioned above are synthesized through one metabolic route is yet to be elucidated.

As mentioned in the Results section, cyathin appears to be more toxic to plant root systems than to

shoot systems. Root hairs, especially, seem to be adversely affected. Stein (1967), studying the effect of actinomycin D on the development of epidermal hairs on seedlings of *Sinapsis alba* L., reported that the application of 0.75 µg of antibiotic reduced hair density from 70 to 15 per 10 mm length of seedling. Hair suppression was irreversible and was ascribed to the inhibition of mRNA by actinomycin D. Whether such a possibility exists for cyathin, cannot be decided on the basis of the present study. Root hair formation is a morphogenetic process within a single cell and, therefore, a study of the process regarding the effect of cyathin would afford an opportunity of tracing the developmental sequence from gene activation through mRNA synthesis and enzymatic changes to polar cell elongation.

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APPENDIX

Chemistry of the Cyathin Complex

Thin-layer chromatography, both analytical and preparative, was carried out on silica gel G (0.5 mm), containing 1% electronic phosphor. The materials were detected either by spraying with 30% sulphuric acid and charring, or in the case of the preparative work, by viewing under ultra-violet light.

Preparative TLC

A solution of the crude extract (700 mgm) in ethyl acetate (5 ml) was applied to a one meter preparative TLC plate. The plate was developed with benzene: dioxan:acetic acid (100:25:1). Under the ultra-violet light several bands could be seen.

(i) The silica gel corresponding to material with Rf 0.51 (which could also be seen in visible light), was scraped from the plate and extracted with ether. After filtration and evaporation, the etheral solution gave 200 mgm of a brown red solid. This was further purified by TLC and from acetone/heptane gave reddish brown crystals (57 mgm), mp 240-242°.

γmax (Nujol): 3370, 3160, 1630, 1610, 1590, 1520, 1290, 1190, 1130, 860, 840, 795.

λmax (CH_3OH): 215 mμ (ξ-8,800), 242 mμ (ξ-13,200), 284 mμ (ξ-11,000) and 347 mμ (ξ-6,500)

N.m.r. (100 Mcs: acetone d_6) - 1.1 (multiplet): 0.30 (singlet), 2.9 (singlet), 3.6 (singlet), 6.5 (broad band).

Addition of D_2O caused absorption at -1.1 and 6.5 to disappear.

Mass Spectrum. Parent peak at 153, exact mass equivalent to formula $C_7H_6O_4$.

The above spectral data indicated that the compound was 2,4,5-trihydroxybenzaldehyde. This was confirmed by synthesis according to the method of Gatterman (1899)*. It must be noted that the material which crystallized constituted about one third of the total amount, the remainder being an oil which showed several spots on TLC.

(ii) The silica gel corresponding to material with Rf 0.54 was removed from the plate and extracted with ether After filtration, evaporation of the ether gave a colorless oil (217 mgm). After further purification by TLC, white crystals (40 mgm) were obtained from ether pentane; mp 225-226°.

γmax: 3400, 3200, 1695, 1675, 1235, 1220

λmax: (CH_3OH): 215 mμ (ξ-11,000) and 220 mμ (ξ-13,000)

N.m.r.: (100 Mcs; acetone d_6), showed bands at 0.1 (singlet), 2.73 (singlet), 5.16 (multiplet), 6.3 (quartet), 6.6-6.8 (multiplet), 8.85 (singlet), 8.91 (singlet), 9.05 (singlet) and 9.15 (singlet)

Mass spectrum: Showed parent peak at 346, the exact mass of which is equivalent to $C_{20}H_{26}O_5$. For this compound also, crystalline material was only part of total extract, remainder was an oil.

* Ber. 32, 282 (1899)

The ethyl acetate extract (3.0 g) in methanol (10 ml) was added to 10% KOH solution (100 ml). The resulting solution was extracted with ethyl acetate. The ethyl acetate was dried and solvent removed to yield a yellow oil (1.16 g). TLC showed several spots, two major materials being at Rf 0.27 and Rf 0.06. These two materials were isolated by means of preparative TLC.

(i) The material with Rf 0.27 was obtained as a clear colorless oil (0.21 g), which could not be crystallized.

γmax ($CHCl_3$): 3600, 3400, 1645, 1450, 1370

λmax (CH_3OH): 214 mμ (ξ-3800) and 220 mμ (ξ-4500).

N.m.r. ($CDCl_3$): Peaks at 3.75 (doublet), 4.05 (narrow band), 5.55 (broad band), 5.75 (narrow band), 6.15 (broad band), 6.45 (narrow band), 7.15 (triplet), 7.8 (triplet), 8.6 (singlet), 9.02, 9.04, 9.08, 9.16 (singlets).

Mass spectrum: parent peak at 318--corrsponds to formula $C_{20}H_{30}O_3$. Other prominent peaks were at 303, 191, 189, 175, 149, 127, 121, 119, 105, 101, 91, 55, 41.

(ii) The material with Rf 0.06 was also obtained as an oil (0.32 g) and again crystallization could not be achieved.

γmax ($CHCl_3$): 3620, 3400, 1650, 1455, 1375

λmax (CH_3OH): 214 mμ (ξ-2400) and 220 mμ (ξ-3200)

N.m.r. ($CDCl_3$) had absorption at 5.05 (narrow band), 6.8 broad band), 7.7 (broad band), 8.1 (broad band),

8.85 (broad band), 9.6 (broad band), 10.1 (singlet).

Mass Spectrum: parent peak at 334--corresponds to formula $C_{20}H_{30}O_4$. Other prominent peaks were at 316, 203, 189, 175, 161, 149, 133, 121, 119, 109, 107, 105, 95, 93, 91, 81, 79, 55, 43, 41.

The I.R. Spectra of the above mentioned four fractions of cyathin (B, A_5, A_3 and A_4) are given in Plates A and B.

PLATE A Infrared Absorption Spectrum of the Cyathin Components (Chloroform)

Fig. A1. Cyathin A_3 ($C_{20}H_{30}O_3$)

Fig. A_2. Cyathin A_4 ($C_{20}H_{30}O_4$)

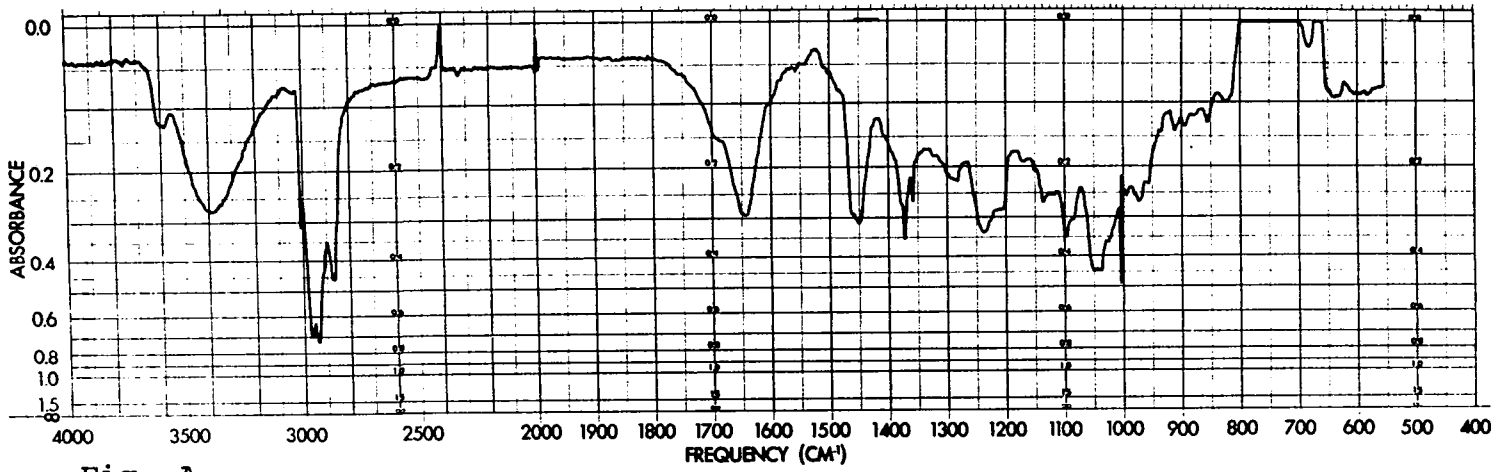

Fig. A_1

PLATE A

Fig. A_2

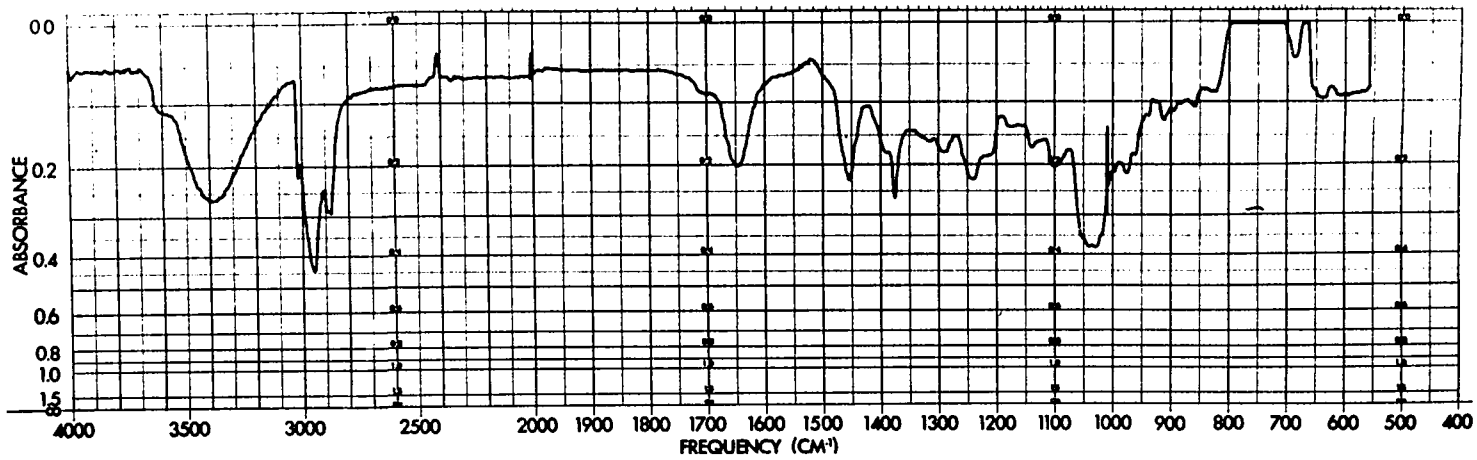

PLATE B Infrared Absorption Spectrum of the Cyathin Components (Nujol)

Fig. B_1. Cyathin B ($C_7H_6O_4$)--2,4,5-trihydroxybenzaldehyde

Fig. B_2. Cyathin A_5 ($C_{20}H_{20}O_5$)

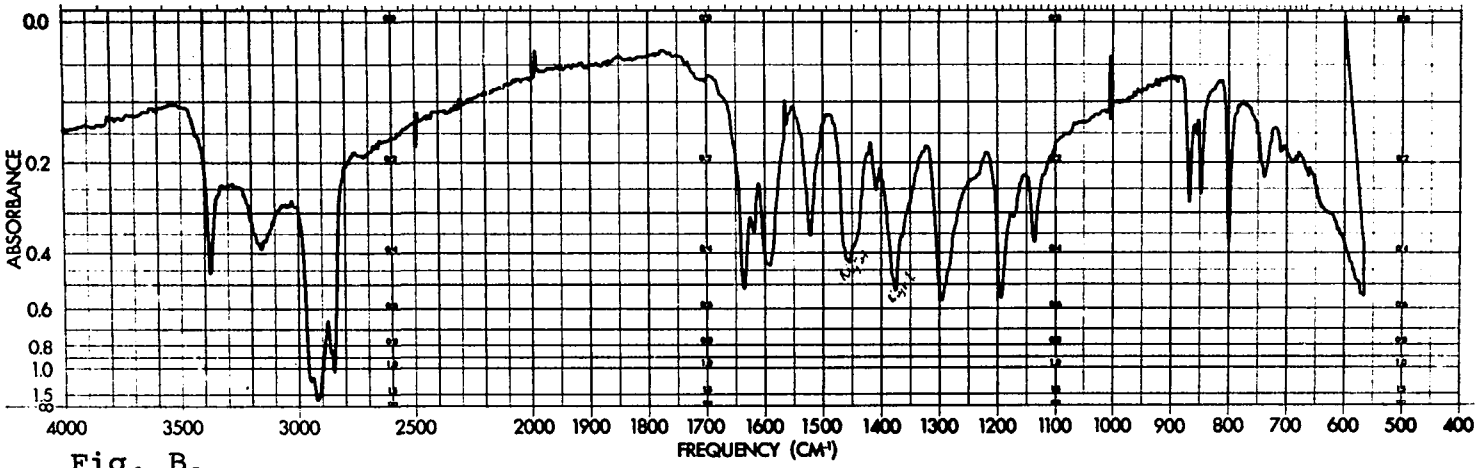

Fig. B_1

PLATE B

Fig. B_2