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BIOLOGICALLY ACTIVE PIGMENTS FROM EPICOCCUM NIGRUM

WILLIAM R. BURGE

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BIOLOGICALLY ACTIVE PIGMENTS FROM *EPICOCCUM NIGRUM*

BY

WILLIAM R. BURGE

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M.S., University of New Hampshire, 1969

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TABLE OF CONTENTS

LIST OF TABLES vi

LIST OF ILLUSTRATIONS viii

ABSTRACT x

INTRODUCTION 1

MATERIALS AND METHODS 7

1. Procedure for Assaying Activity of Solution against B. megaterium spores 7
2. Procedure for Assaying Position of Activity on TLC Plates 8
3. Procedure for Screening Fungi for Activity against B. megaterium spores 8
4. Procedure for Stock Culturing Fungi 9
5. Procedure for Growth of E. nigrum and Isolation of Flavipin 10
6. Procedure for Assaying the Biological Activity of Flavipin 13
7. Infrared Spectrum of Flavipin 14
8. Visible and Ultraviolet Spectrum of Flavipin 15
9. Elemental Analysis of Flavipin 15
10. Procedure for Growth of E. nigrum and Isolation of the Humic Acid 15
11. The Molecular Weight Determination of the Humic Acid 16
12. Infrared Spectrum of the Humic Acid 17
13. Visible and Ultraviolet Spectrum of the Humic Acid 17
14. Elemental Analysis of the Humic Acid 17

TABLE OF CONTENTS (cont'd)

MATERIALS AND METHODS (cont'd)

15. Procedure for Assaying the Biological Activity of the Humic Acid 17

16. Procedure for Growth of E. nigrum and Isolation of Epirodin A 19

17. Infrared Spectra of the Epirodins 23

18. Visible and Ultraviolet Spectra of the Epirodins 23

19. Elemental Analysis of the Epirodins 25

20. Melting Points of the Epirodins 25

21. Mass Spectra of the Epirodins and Other Pigments 25

RESULTS AND DISCUSSION 26

1. Isolation of E. nigrum 26

2. Flavipin 26

3. Humic Acid 43

4. Epirodins 54

5. Comparison of the Humic Acid and Flavipin with Epirodin A 115

6. Fusarium Pigments 118

7. RNA Polymerase Inhibitors 123

SUMMARY 132

BIBLIOGRAPHY 135

APPENDIX 138

LIST OF TABLES

Table 1. Column sizes, support materials and solvents used in the isolation of Epirodin A.

Table 2. Absorption wavelengths in the infrared spectrum of flavipin.

Table 3. Molar absorptivity of flavipin.

Table 4. Elemental analysis of flavipin.

Table 5. Melting point of flavipin.

Table 6. Antibacterial activity of flavipin against B. megaterium spores.

Table 7. Activity of flavipin against Chlorella species.

Table 8. Results of thin layer gel filtration on the humic acid.

Table 9. Absorption wavelengths in the infrared spectrum of the humic acid.

Table 10. Comparison of the humic acids isolated by various researchers by means of the reported elemental analysis data.

Table 11. Activity of fractions from Column A against B. megaterium.

Table 12. Weight of fractions from Column A.

Table 13. Extinction coefficients of Epirodin A and Epirodin B mix.

Table 14. Decrease in absorbance of Epirodin A in distilled water at pH 12 with time at 25 C.

Table 15. Decrease in absorbance of Epirodin A in distilled water at pH 12 with time at 110 C.

Table 16. Decrease in absorbance of Epirodin A in distilled water at pH 2 with time at 25 C.

Table 17. Absorption wavelengths in the infrared spectra of Epirodin A and Epirodin B mix.

Table 18. Elemental analysis data on Epirodin A and Epirodin B mix.

Table 19. Results of thin layer gel filtration on Epirodin A.

LIST OF TABLES (cont'd)

Table 20. Activity of Epirodin A against B. megaterium and B. subtilis.

Table 21. Formula for determining rings plus double bonds.

Table 22. Visible maxima and molar absorptivity of several Fusarium pigments.

Table 23. Substitutions on rifamycin and streptovaricin structure in order to obtain a particular rifamycin or streptovaricin.

LIST OF ILLUSTRATIONS

Diagram 1.	Extraction of flavipin.
Diagram 2.	Thin layer gel filtration apparatus.
Diagram 3.	Extraction of Epirodin A from cultures.
Diagram 4.	Acid-base extraction procedure.
Figure 1.	Structure of flavipin.
Figures 2 and 3.	Infrared spectrum of flavipin.
Figure 4.	Visible and ultraviolet spectrum of flavipin.
Figure 5.	Molecular weight of Epirodin A by gel filtration.
Figures 6 and 7.	Infrared spectrum of the humic acid.
Figure 8.	Visible and ultraviolet spectrum of the humic acid.
Figure 9.	Activity of fractions from Column A, zones obtained at 0.25 mg/ml concentration.
Figure 10.	Weight of fractions from Column A.
Figure 11.	TLC pattern of crude 1st extract on Eastman plates in acetone-methanol (1:1) solvent system.
Figure 12.	Appearance of Column A during chromatography.
Figure 13.	Appearance of Column B during chromatography.
Figure 14.	TLC pattern of fractions 1-F, 2-F, 3-F and pigment P-1A on Eastman prepared plates in acetone-methanol (1:1) solvent system.
Figure 15.	TLC pattern of fractions 1-F and 2-F, and pigment P-1A on Brinkman prepared plates in acetone-methanol (1:1) solvent system.
Figure 16.	Appearance of Column C of 1-F material during chromatography.
Figure 17.	The TLC pattern of fractions from Column C using column 5 on Eastman prepared plates using acetone-methanol (1:1) solvent system.

LIST OF ILLUSTRATIONS (cont'd)

Figure 18.	Appearance of Column C of 2-F material during chromatography.
Figure 19.	Appearance of Column D during chromatography.
Figure 20.	Visible and ultraviolet spectrum of Epirodin A.
Figure 21.	Visible and ultraviolet spectrum of Epirodin B mix.
Figure 22.	Stability of Epirodin A in basic solution at 25° C.
Figure 23.	Stability of Epirodin A in basic solution at 110° C.
Figure 24.	Stability of Epirodin A in acidic solution at 25° C.
Figure 25.	Recorded visible spectrum of Epirodin A acidic solution (1), basic solution (2) and distilled water (3).
Figure 26.	Structures of 4-hydroxypyridine-1-oxide and indicator yellow.
Figures 27 and 28.	Infrared spectrum of Epirodin A.
Figures 29 and 30.	Infrared spectrum of Epirodin B mix.
Figure 31.	Isotope cluster in 602 molecular weight pigment.
Figure 32.	Molecular weight of Epirodin A by gel filtration.
Figure 33.	Structures of Fusarium pigments.
Figure 34.	Structures of Rifamycins and Streptovaricins.
Figure 35.	Structures of Geldanamycin and Tolypomycin.
Figure 36.	Structures of Maytansine and Streptolydigin.

ABSTRACT

BIOLOGICALLY ACTIVE PIGMENTS

FROM EPICOCCUM NIGRUM

BY

WILLIAM R. BURGE

A strain of Epicoccum nigrum was isolated from a moldy feed sample and shown to produce several pigments which had antibiotic activity. These pigments were named Epirodins. Epirodin A was the only one isolated and shown to be chromatographically pure. The pigment along with a mixture of Epirodin pigments referred to as Epirodin B_{mix} were isolated using a series of column chromatographic steps with the final purification of Epirodin A being done by thin layer chromatography. The infrared spectra, the visible and ultraviolet spectra, the elemental analysis data and the melting point data on Epirodin A and Epirodin B_{mix} are included in this thesis. The molecular weight of Epirodin A as determined by thin layer gel filtration and the antibiotic activity against Bacillus megaterium spores and Bacillus subtilis spores are also reported. Epirodin A was shown to inhibit DNA-dependent RNA polymerase from E. coli.

Several other pigments previously reported to be produced by E. nigrum were isolated from cultures of our strain. Flavipin was shown to have very little activity against B. megaterium but was shown to have considerable phytotoxic activity against Chlorella species. The phytotoxic activity of flavipin had not been previously reported. The humic acid isolated from our strain did not show any activity against B. megaterium or Chlorella species. The infrared spectra, the visible and ultraviolet spectra, and the elemental analysis data on these two pigments are included in this thesis. The melting point data on flavipin is also included. These pigments along with several pigments from Fusarium species were shown to be quite different from Epirodin A. From the data presented in this thesis, it is concluded that Epirodin A and Epirodin B_{mix} represent a new class of pigments from E. nigrum. Epirodin A and Epirodin B_{mix} also represent a new class of DNA-dependent RNA polymerase inhibitors. The fact that Epirodin A inhibits RNA polymerase also suggests that this pigment might possess antiviral and anticancer activity.

INTRODUCTION

Epicoccum nigrum appeared first in the mycological literature in 1816 when Link[1] described the dark red mold. *E. nigrum* has not been reported previously to be a phytopathogen even though it has been shown during the course of this research to produce a compound that is phytotoxic to *Chlorella* species. *E. nigrum* has been shown by Cambell[2] to produce an antibiotic that inhibits the growth of the fungus *Helminthosporium sativum*. *E. nigrum* was also reported to be a pathogen of *H. sativum*. Cambell was able to conclude that antibiosis and direct parasitism were responsible for the disorganization of the mycelium of *H. sativum*. *E. nigrum* has been shown to grow on paper, wood, potatoes, wheat grain, rice, cotton seed and soil.[3] *E. nigrum* is capable of producing several types of degradative enzymes which enable it to grow on the substrates mentioned.[3]

Since *E. nigrum* is a pigmented mold, several researchers have investigated the pigments produced by the mold when grown under laboratory conditions. The research reported in this thesis is an attempt by the author to isolate and identify the biologically active pigments produced by the mold.

In a review article by Foppen[3] most of the pigments produced by *E. nigrum* are discussed. The article describes in detail two of the classes of pigments (carotenoids and humic acids) produced by the fungus.

The pigment flavipin is also mentioned but is not discussed at any length even though it is the only one that had been shown to have any biological activity. This was probably due to the fact that Foppen was unable to isolate this compound, and he was unable to show that E. nigrum had significant antibiotic or antifungal activity against fifteen different micro-organisms selected from among bacteria, yeast, fungi and algae. He was able to show that the fungus was capable of producing several carotenoids, which were identified, and a humic acid which was partially studied. The amino acids and several of the phenols composing the humic acid were reported, but no structure was proposed for the humic acid. Other papers have reported investigations on the humic acids from E. nigrum and other sources. Martin et al.[4] also studied E. nigrum,identifying some phenols that are incorporated into the humic acid that Foppen[3] had not detected. Eka[5] reported that his findings tended to support the presence of flavipin-like substances in the crude extracts of E. nigrum cultures. Eka also pointed out that there was a possibility that carotenoids, flavinoids and humic acids were elaborated by the fungus. A later paper by Eka[6] reported on a crude pigment which had a pH-dependent fungistatic activity against Botrytis allii. The pigment was reported to be golden yellow in color.

Thus at the beginning of this research there were two classes of pigments (carotenoids and humic acids) and one other pigment (flavipin) which had been reported to be elaborated by E. nigrum. Only one of them, flavipin, had been reported to have antifungal activity and hardly any antibacterial activity.

Using a screening technique described in this thesis, the author was able to isolate a strain of E. nigrum from a moldy animal feed sample that had considerable activity against Bacillus megaterium spores. The activity was shown to reside with the yellow pigments when assayed on Eastman TLC plates, and it was shown that the active pigments were not carotenoids or flavipin. The pigments of E. nigrum were separated by a series of column chromatographic steps using different support materials and different solvent systems. Thin layer chromatography was also employed in the separation procedures.

Since Foppen[3] was unable to get his strain of E. nigrum to produce flavipin, it seemed at the time that not all strains would be capable of producing this compound. An attempt at the isolation of flavipin using Bamford's procedure was carried out successfully with the author's strain. Flavipin was shown to have no activity against B. megaterium spores at a concentration of 0.5 mg/ml, but had considerable activity against the green alga Chlorella pyrenoidosa.

The first effort to identify the biologically active pigments of E. nigrum was carried out by Bamford et al.[7] They reported on the isolation and characterization of flavipin. Their verification of the structure of flavipin came mainly from the data that was contained in a paper by Raistrick and Rudman.[8] The workers had isolated flavipin from Aspergillus flavipes and Aspergillus terreus. Both Bamford's and Raistrick's groups showed that flavipin had antifungal activity against Botrytis allii, but had hardly any antibacterial activity. The structure of flavipin is shown in Figure 1.

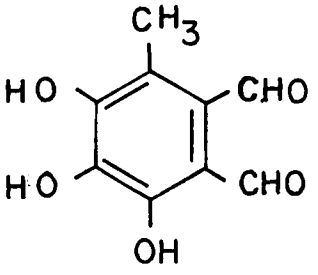


Figure 1

Structure of Flavipin

Besides flavipin, the fungus appeared to produce a complex series of pigments, which will be referred to in this thesis as Epirodins. These are not carotenoids or humic acids and therefore represent a new class of Epicoccum pigments.

A possible mode of action of the Epirodins was revealed when Epirodin A was incubated in an in vitro system set up to assay for E. coli DNA-dependent RNA polymerase activity. It was shown that Epirodin A inhibited the activity by binding to the RNA polymerase and not to DNA, and that it seemed to inhibit both chain initiation and chain elongation. (See Appendix)

To date there have been five compounds that have been shown to bind directly to RNA polymerase. Four of these belong to a class of antibiotics known as ansamycins.[9,10] They are the rifamycins, the tolypomycins, the streptovaricins, and geldanamycin. The fifth is streptolydigin. Geldanamycin differs from the other ansamycins in that its principal activity is against protozoa rather than bacteria.[9] Several of the ansamycins have been shown to act by inhibiting RNA polymerase from procaryotic organisms.[9,10] The rifamycin series inhibits before RNA chain initiation and is without apparent effect during polymerization.[9] The streptovaricins have similar activity.[9] Streptolydigin, however, inhibits both initiation and elongation.[11] It has also been shown to stabilize the enzyme template complex. These compounds

mentioned are of considerable interest also because of their marked antiviral activity. Some of the viruses responsible for tumors are inhibited by members of this group.[9,10] Formyl rifamycin SV derivatives have been shown to have considerable antiviral activity.[9,10] The streptovaricin complex has also been shown to inhibit RNA-dependent DNA polymerase present in an oncogenic RNA virus.[12]

Reported in this thesis is the finding of a new inhibitor of RNA polymerase isolated from a species belonging to the Deuteromycetes (Fungi Imperfecti). All other inhibitors of RNA polymerase to date have been isolated from species belonging to the Actinomycetes. Isolation and partial characterization of the inhibitory pigments of E. nigrum are presented along with a comparison with pigments that have been isolated from Fusarium, a genus closely related to Epicoccum. Epirodin A has also been compared with other related biologically-active pigments found to be elaborated by E. nigrum.

MATERIALS AND METHODS

Procedure for Assaying Activity of Solution against B. megaterium spores

The standard procedure for assaying activity of the Epirodins involved using the paper disc technique described by Jayaraman et al.[13] Varying concentrations of the antibiotic were assayed using this technique to obtain the minimal inhibitory concentration (M.I.C.).

The medium used consisted of the following:

Tryptone	5.0 g
Yeast Extract	2.5 g
Glucose	10.0 g
Agar	10.0 g
Water	1000 ml

The medium was dispensed in 100-ml volumes in 250-ml Erlenmeyer flasks and autoclaved. For seeding the agar medium with the test organism the medium was liquefied and placed in a water bath at 80° C. Each 100-ml portion of the medium, after 15 minutes' equilibration time in the water bath, was then seeded with 1 ml of a B. megaterium (UNH strain) spore suspension containing 1×10^8 spores per ml. The spore medium was incubated in the 80° C bath for an additional 20 minutes to activate the spores. At the end of this period 10-ml aliquots of the medium were dispensed into 10 disposable Petri plates (Fisher Scientific Co.) and allowed to solidify. Solutions

(20 ul) of the antibiotic in ethanol were absorbed onto 0.7 cm concentration discs (Difco) which were then placed onto the surface of the seeded agar medium in each plate. Usually a total of three discs along with an ethanol blank were assayed on each plate. The plates were then incubated at 28° C for 16 hours. At the end of this period the zones of inhibition were measured and recorded as net zones of inhibition (total diameter of inhibition zone minus 0.7 disc diameter).

Procedure for Assaying Position of Activity on TLC Plates

The preparation of the seeded B. megaterium medium is the same as described above. Prepared TLC plates (Eastman No. 6061) were used because of the ease with which these plastic plates could be cut up into small strips. A TLC plate was developed in the desired solvent system, dried and cut up into strips which were then divided into sections containing the top, middle and bottom regions of the plate, respectively. These sections were then placed inside disposable Petri plates and overlaid with the seeded spore medium. After incubation the positions of the active components could be ascertained by the clear zones of inhibition of growth.

Procedure for Screening Fungi for Activity against B. megaterium spores

In order to test fungal cultures for antibiotic activity, malt extract agar medium (see next section)

was dispensed into Petri dishes and allowed to cool. The plates were inoculated with the fungi, and after 4-5 days of growth at room temperature the fungus-containing plates were overlaid with the same medium containing B. megaterium spores. The plates were incubated at 28° C for 16 hours and the activity at the end of this period recorded. Where antibiotic substances were being produced a clear zone of inhibition could be discerned around the fungal colony.

Procedure for Stock Culturing Fungi

The slant medium used for storing the fungal cultures was Malt Extract Agar and consisted of the following:

Malt Extract	20.0	g
Peptone	1.0	g
Glucose	20.0	g
Agar	20.0	g
Water	1000	ml

The medium was autoclaved in 15-ml aliquots dispensed in screw-capped test tubes and the tubes cooled in an inclined position. The slants were inoculated using mycelia from plate cultures. The slant cultures were allowed to grow at room temperature for 3-5 days depending on how fast the surface of the medium became covered with mycelia. The cultures were then stored

in the refrigerator at 4° C to be used later as seeding material for growth of the fungus on rice.

Procedure for Growth of E. nigrum and Isolation of Flavipin

Flavipin was isolated using the procedure of Bamford et al.[7] The medium was changed slightly and consisted of the following:

Glucose	50.0	g
Potassium dihydrogen phosphate	1.0	g
Magnesium sulfate 7 H_2O	0.5	g
Bacto-Tryptone	2.5	g
Minor Element Solution	1.0	ml
Water	1000	ml

Minor Element Solution

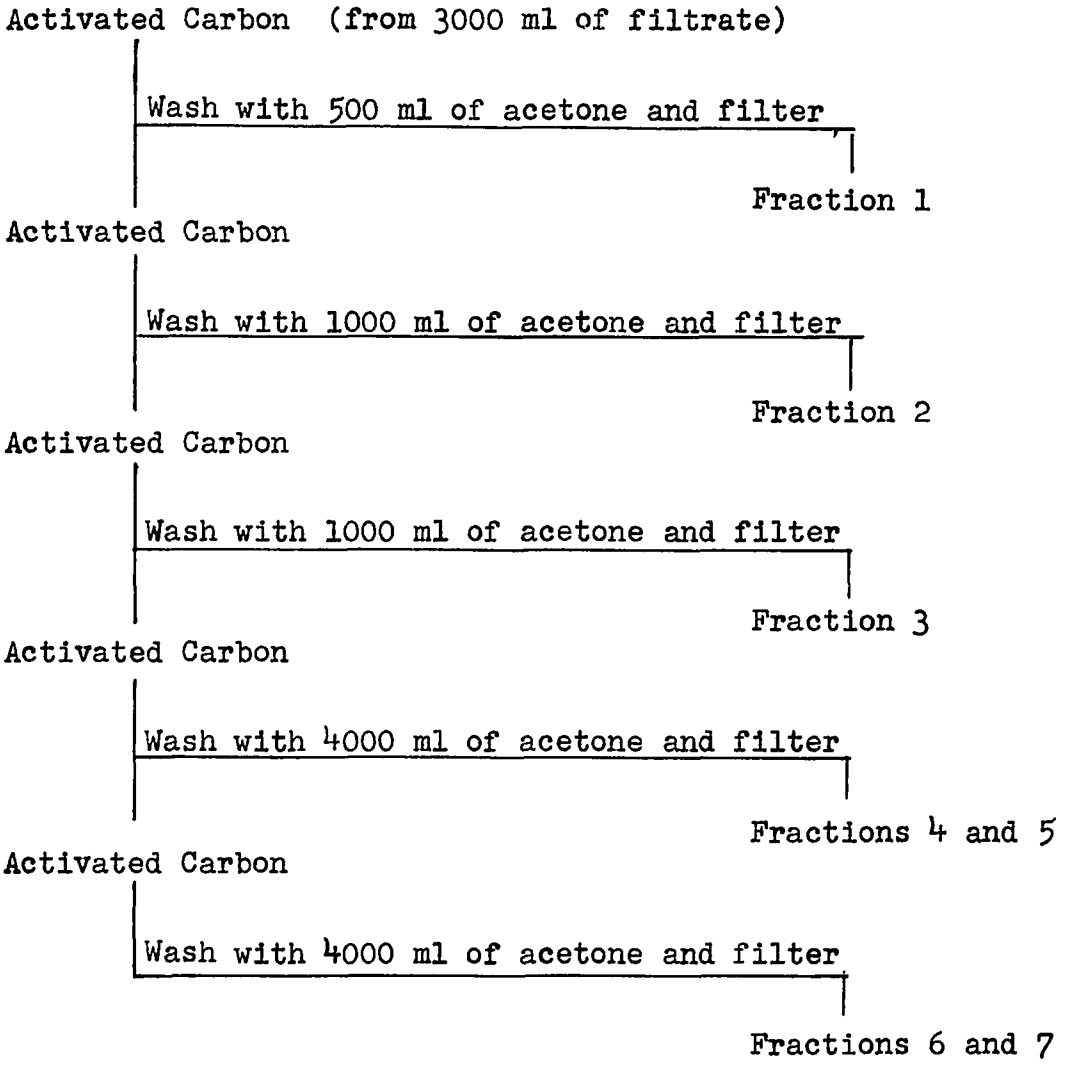
Ferrous sulfate 7 H_2O	0.100	g
Cupric sulfate 5 H_2O	0.015	g
Zinc sulfate 7 H_2O	0.100	g
Manganese chloride 4 H_2O	0.010	g
Ammonium molybdate 4 H_2O	0.010	g
Water	100	ml

The minor element solution was acidified with 6 N HCl just enough to dissolve the precipitate.

The medium was autoclaved for 20 minutes after being dispensed into 125-ml Erlenmeyer growing flasks and into test tubes (5 ml per tube and 75 ml per Erlenmeyer flask). The test tubes were inoculated with mycelia from slant cultures of E. nigrum. The tubes were placed

under light at 24-27° C for 5 days, and at the end of the growing period these tubes were used to inoculate the medium in the 125-ml Erlenmeyer growing flasks. The inoculated growing flasks were placed under light for 28 days. At the end of the growing period the culture medium was filtered through Whatman No. 1 filter paper. The mycelial mat was autoclaved and discarded. The filtrate was acidified with concentrated HCl to pH 2 and activated carbon (Darco G-60, 15 g per liter of filtrate) was added and the mixture stirred for 15 minutes. The solution was filtered using suction and the carbon dried under vacuum for 4.5 hours. The dried carbon was treated according to the procedure shown in Diagram 1.

Repeated washings of the dried carbon yielded wash fractions 1-7. Fractions 4-7 were combined and evaporated to dryness *in vacuo*, and the residue extracted four times with 150-ml portions of chloroform. The chloroform extracts were evaporated to dryness and dissolved in 5 ml of ethanol. Water was added to bring the volume up to 200 ml, and the resulting solution was lyophilized and the residue weighed. The material was then taken up in acetone and recrystallized. The crystallized product was sublimed at 140° C to give pure material used to obtain the infrared, visible and ultraviolet spectra, the elemental analysis data and the biological data reported for flavipin in this thesis.



(Washing was done by adding the acetone in a 4000 ml beaker and stirring for 5 minutes).

Diagram 1

Extraction of Flavipin

Procedure for Assaying the Biological Activity of Flavipin

The procedure for assaying the inhibitory action of flavipin against B. megaterium was the same as that described earlier in this section.

The procedure using Chlorella as the assay organism involved using the method described by Ikawa et al.[14] Two strains of Chlorella pyrenoidosa (UNH strain and strain 15-2070 from the Carolina Biological Supply Co., Burlington, N.C.) and one of Chlorella vulgaris (strain 15-2075 from the Carolina Biological Supply Co.) were employed in the assay. The medium used consisted of the following:

Potassium nitrate	1.00 g
Magnesium sulfate 7 H_2O	0.25 g
Potassium dihydrogen phosphate	0.125 g
Potassium monohydrogen phosphate	0.125 g
Glucose	10.00 g
Trace Element Solution	0.8 ml
Water	1000 ml

Trace Element Solution

Calcium chloride 2 H_2O	5.00 g
Ferrous sulfate 7 H_2O	1.00 g
Zinc sulfate 7 H_2O	1.00 g
Manganese chloride 4 H_2O	0.10 g
Boric acid	0.10 g
Ammonium vanadate	0.10 g

Trace Element Solution (cont'd)

Ammonium molybdate 4 H_2O	0.10 g
Cobaltous chloride 6 H_2O	0.10 g
Cupric sulfate 5 H_2O	0.10 g
Sodium citrate	20.00 g
Water	1000 ml

The medium was divided into equal portions and one portion was made 2 per cent in agar. Both the non-agar and agar solutions were dispensed into 250-ml Erlenmeyer flasks and autoclaved. The assay involved inoculation of a non-agar portion with a thick growing Chlorella stock culture grown in the same non-agar medium. A flask containing an equal volume of 2 per cent agar medium that had previously been liquefied and cooled to 45-50° C was then added aseptically to the inoculated non-agar solution. Ten ml aliquots were then taken and placed into disposable Petri plates and the plates were allowed to solidify. Various concentrations of flavipin in ethanol were absorbed onto 0.7 cm discs and placed on the surface of the solidified agar medium. The plates were incubated at 24° C under fluorescent lighting for 5 days (7 days in the case of C. vulgaris) at the end of which the net zones of inhibition were recorded.

Infrared Spectrum of Flavipin

One mg of flavipin was mixed with 100 mg of potassium

bromide and dissolved in 50 ml of water and lyophilized. Pellets were then made of the lyophilized material using a Mini-Press (Wilks Scientific Corp., South Norwalk, Conn.). The spectrum was taken using a Perkin-Elmer 337 infrared spectrophotometer.

Visible and Ultraviolet Spectrum of Flavipin

One mg of flavipin was dissolved in ethanol and the solution diluted with ethanol to give a 10 ug/ml solution. The spectrum of this solution was taken using a Beckman DBG recording spectrophotometer. A 10 ug/ml solution was also read in a Perkin-Elmer double beam spectrophotometer set at wavelengths 347 nm and 265 nm to obtain the absorptivity data at these wavelengths.

Elemental Analysis of Flavipin

Two mg of sublimed flavipin was subjected to combustion analysis for carbon, hydrogen and nitrogen in the Chemistry Department (UNH) by L. Heavner. The runs were made in duplicate.

Procedure for Growth of E. nigrum and Isolation of the Humic Acid

The fungus was grown up on the following medium:

Glucose	10.0 g
Yeast autolysate	5.0 g
Water	1000 ml

The medium was dispensed in test tubes and 500-ml

Erlenmeyer flasks (5 ml per tube and 100 ml per Erlenmeyer flask). The test tubes were inoculated with mycelia from slant cultures and allowed to grow at 25° C under fluorescent lighting for 5 days. This inoculum was used to inoculate the medium in the Erlenmeyer flasks. These Erlenmeyer flasks were then incubated under fluorescent lighting at 25° C in a rotatory shaker (Psycrotherm, model G27, New Brunswick Scientific Co., New Brunswick, N.J.) running at 220 rev./min. for 3 days. At the end of the growth period the culture was filtered through Whatman No. 1 filter paper. The filtrate was collected and lyophilized to dryness. The residue was taken up in 50 mM Tris buffer pH 7.5. The resulting solution was dialysed against 3000 ml of the same buffer for 72 hours. The nondialysable material was placed on a Sephadex G-25 (Sigma Chemical Co.) column. The brown band from this column was collected and concentrated to dryness by lyophilization. The residue was taken up in 10 ml of distilled water and dialysed against distilled water for 72 hours. The nondialysable material was used to obtain the spectral and biological properties, and the elemental analysis data reported in this thesis on the humic acid of E. nigrum.

The Molecular Weight Determination of the Humic Acid

The molecular weight was determined by thin layer gel filtration using Sephadex G-50 fine. The standards

used were Vitamin B_{12} (Calbiochem), myoglobin (Schwarz/Mann) and cytochrome c (Schwarz/Mann). The apparatus used is shown in Diagram 2.

Infrared Spectrum of the Humic Acid

One mg of the humic acid was mixed with 100 mg of potassium bromide and dissolved in 50 ml of water and lyophilized. Pellets were then made of the lyophilized materials using a Mini-Press. The spectrum was taken on a Perkin-Elmer 337 infrared spectrophotometer.

Visible and Ultraviolet Spectrum of the Humic Acid

One mg of the humic acid was dissolved in distilled water and diluted with distilled water to give a 50 ug/ml solution. This solution was used to obtain the visible and ultraviolet spectrum on a Cary 15 recording spectrophotometer.

Elemental Analysis of the Humic Acid

Two mg of lyophilized humic acid was subjected to combustion analysis for carbon, hydrogen and nitrogen in the Chemistry Department (UNH) by L. Heavner. The runs were made in duplicate.

Procedure for Assaying the Biological Activity of the Humic Acid

The procedures described above for the assay of the activity of flavipin against B. megaterium spores and

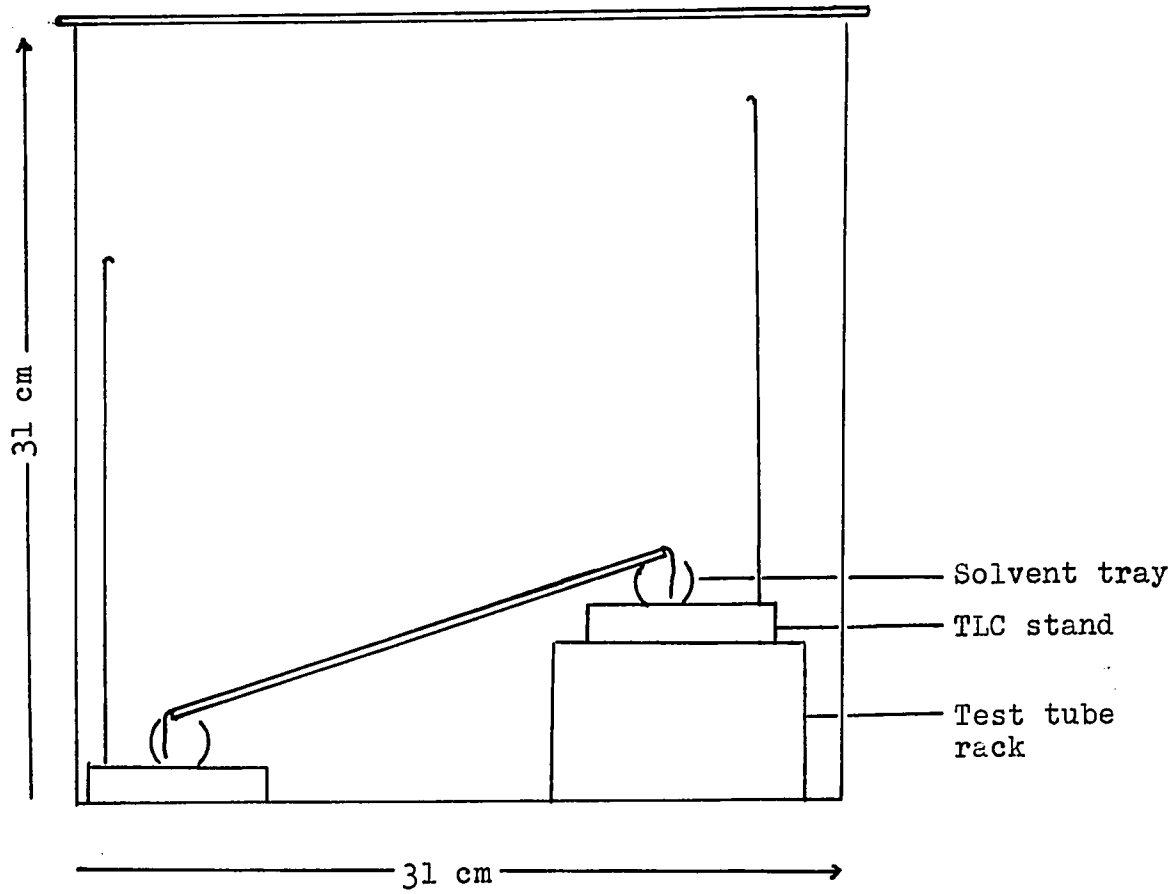


Diagram 2

Thin layer gel filtration apparatus.

Chlorella species are the same employed in determining the activity of the humic acid.

Procedure for Growth of E. nigrum and Isolation of Epirodin A

One hundred g of cooked rice in a 1-L Erlenmeyer flask were autoclaved and inoculated by streaking mycelia from a slant culture of E. nigrum over the surface of the rice. The fungus was allowed to grow at 25° C for 16 days under fluorescent lighting. At the end of the 16-day period the rice culture was extracted in a blendor with ethanol. Diagram 3 illustrates the procedure when two flasks were extracted.

Extracts 2, 3, 4 and 5 were combined and concentrated to dryness in vacuo. Extract 1 was concentrated to dryness and the residue reextracted with ethanol. The The ethanol solution was filtered and concentrated to dryness. The residue was combined with the residue from extracts 2-5. The combined residues were dissolved in a minimal amount of ethanol and the ethanol solution was diluted ten-fold by adding the appropriate amount of water. The resulting suspension was lyophilized. The residue was weighed and dissolved in 5 per cent methanol in chloroform. The solution was filtered and placed inside the freezer until it was ready for column chromatography. The material that did not dissolve in the 5 per cent methanol in chloroform solution was collected from the filter paper and dissolved in 50 mM

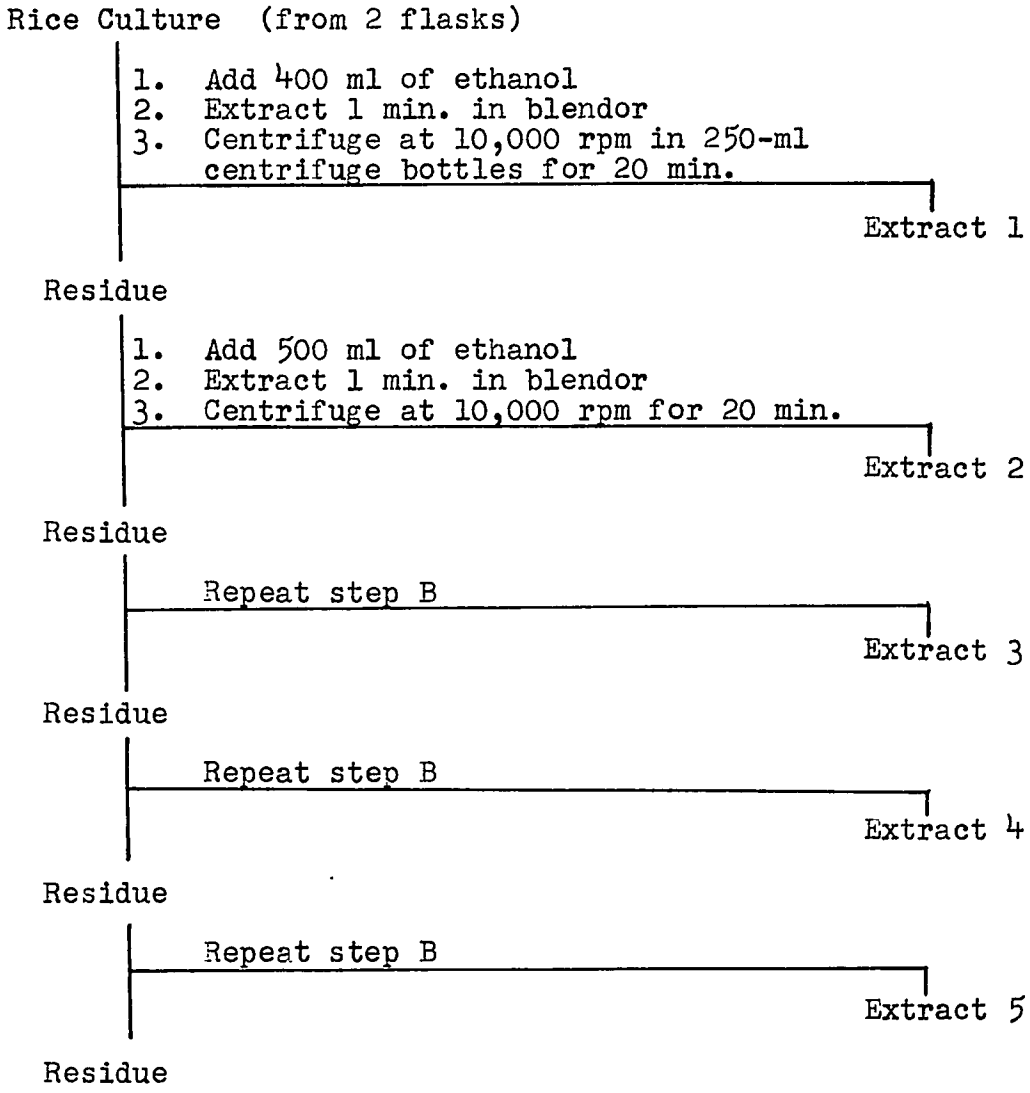


Diagram 3

Extraction of Epirodin A from cultures.

Tris buffer pH 7.5. This solution was frozen and stored in the freezer.

Several columns were employed in the isolation of Epirodin A and the other pigments, the sizes of which are given in Table 1. Column sizes 1 and 2 were used in the first stages of column chromatography. A solution of the crude extract (material dissolved in 5 per cent methanol in chloroform) containing approximately 2.5 g was placed on a column containing 100 g of silicic acid (Fisher Scientific Co.) and the column was developed with 5 per cent methanol in chloroform. This column was designated Column A and is shown in Figure 12.

The most active material from Column A was rechromatographed on a column containing 50 g of Silica Gel H (E. Merck Reagent) as support using acetone-methanol (1:1) as solvent. This column was designated Column B and is shown in Figure 13. The 1-F fraction from Column B was concentrated and rechromatographed on a column containing 50 g of Silica Gel G (E. Merck Reagent) using ethanol as solvent. This column was designated Column C and is shown in Figure 16. Fraction 2-G from Column C was collected and concentrated to dryness, dissolved in acetone-methanol (1:1) and rechromatographed on a 30 g Silica Gel H column using the acetone-methanol (1:1) solvent system. This column was designated Column D and is shown in Figure 19. The yellow orange band (see Figure

Table 1

Column sizes, support materials and solvents used in the isolation of Epirodin A.

Stage of Separation Used	Dimensions	Columns	Supports	Solvent Systems
Column A	5 by 58 cm	1	Silicic Acid	5% methanol in chloroform
	5 by 46 cm	2		
Column B	5 by 58 cm	1	Silica Gel H	acetone-methanol (1:1)
	5 by 46 cm	2		
Column C	5 by 58 cm	1	Silica Gel G	ethanol (95%)
	5 by 46 cm	2		
	4 by 36 cm	3	Silica Gel H	acetone-methanol (1:1)
	2 by 30 cm	4		

19) coming from this column was assayed for chromatographic purity by running thin layer chromatography plates in different solvent systems. The solvent systems employed were acetone-methanol (1:1); isopropanol-water (74:36); and hexane-acetone (88:12). Two types of TLC plates were used: Eastman Silica Gel plates (No. 6061 w/o fluorescent indicator); and Brinkman Silica Gel (E. Merck Reagent) plates (Silplate-22). The final purification of Epirodin A was done on Eastman TLC plates.

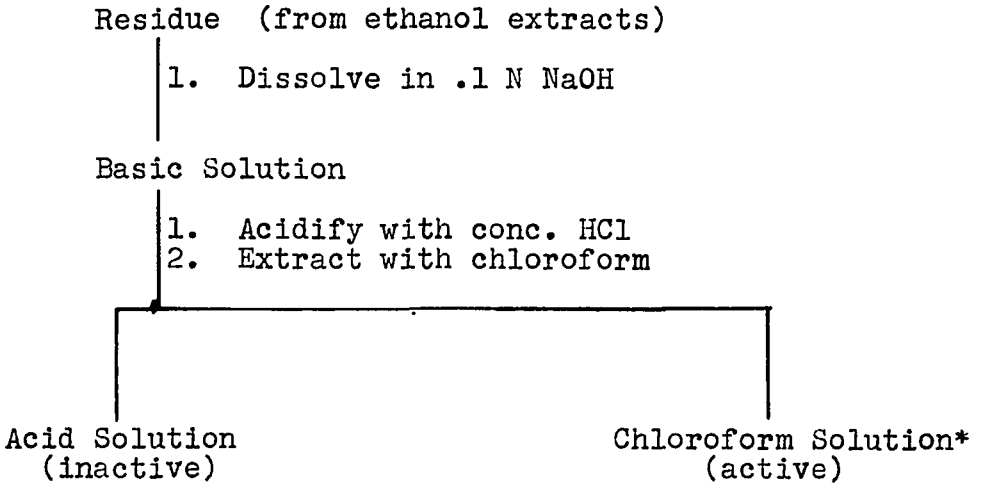
The other active yellow components from Column A were isolated using the same series of column chromatographic separations. In the case of the pigment Epirodin B_{mix} Column D was omitted. Acid-base treatment was employed originally in the isolation of some yellow pigments from E. nigrum. The procedure is shown in Diagram 4. Column chromatography and thin layer chromatography were used to purify those pigments.

Infrared Spectra of the Epirodins

One mg of Epirodin A or Epirodin B_{mix} was mixed with 100 mg of potassium bromide and dissolved in 50 ml of water and lyophilized. Pellets were then made of the lyophilized materials using the Mini-Press. The spectra were taken using a Perkin-Elmer 337 infrared spectrophotometer.

Visible and Ultraviolet Spectra of the Epirodins

One mg of Epirodin A or Epirodin B_{mix} in ethanol was diluted with ethanol to give a solution containing



* Used for column chromatography.

Diagram 4

Acid-base extraction procedure.

10 ug/ml. The spectrum was taken on a Cary 15 recording spectrophotometer. The absorbances at the maxima were determined on a Perkin-Elmer double beam spectrophotometer.

Elemental Analysis of the Epirodins

Two mg of lyophilized Epirodin A and two mg of lyophilized Epirodin B_{mix} were subjected to combustion analysis for carbon, hydrogen and nitrogen in the Chemistry Department (UNH) by L. Heavner. Each sample was run in duplicate.

Melting Points of the Epirodins

The melting points of lyophilized samples were determined on a capillary melting point apparatus (A. Thomas Co., Philadelphia, Pa.).

Mass Spectra of the Epirodins and Other Pigments

Several samples were examined by high resolution mass spectrometry at the RT Center for Mass Spectrometry, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, by the direct inlet method at 180° C. Epirodin A was examined by high resolution mass spectrometry at the Research Triangle Institute, Research Triangle Park, North Carolina, by the direct inlet method. The sample was run at temperatures from 150° C to 350° C.

RESULTS AND DISCUSSION

Isolation of Epicoccum nigrum

At the beginning of this investigation moldy animal feed samples obtained from H.A. Davis, Analytical Services (Feeds and Fertilizers), New Hampshire Agricultural Experiment Station, University of New Hampshire, were plated on malt extract agar and fungal colonies were isolated and tested for activity against B. megaterium spores. One of the most active molds isolated by this screening procedure was a red fungus which was identified as Epicoccum nigrum by J.J. Ellis, Northern Utilization and Development Division, Agricultural Research Service, U.S. Dept. of Agriculture, Peoria, Illinois.

Flavipin

Since E. nigrum has been shown by other workers to produce only two water soluble pigments, flavipin and humic acids, the author attempted to isolate these and compare them with the water soluble Epirodin A.

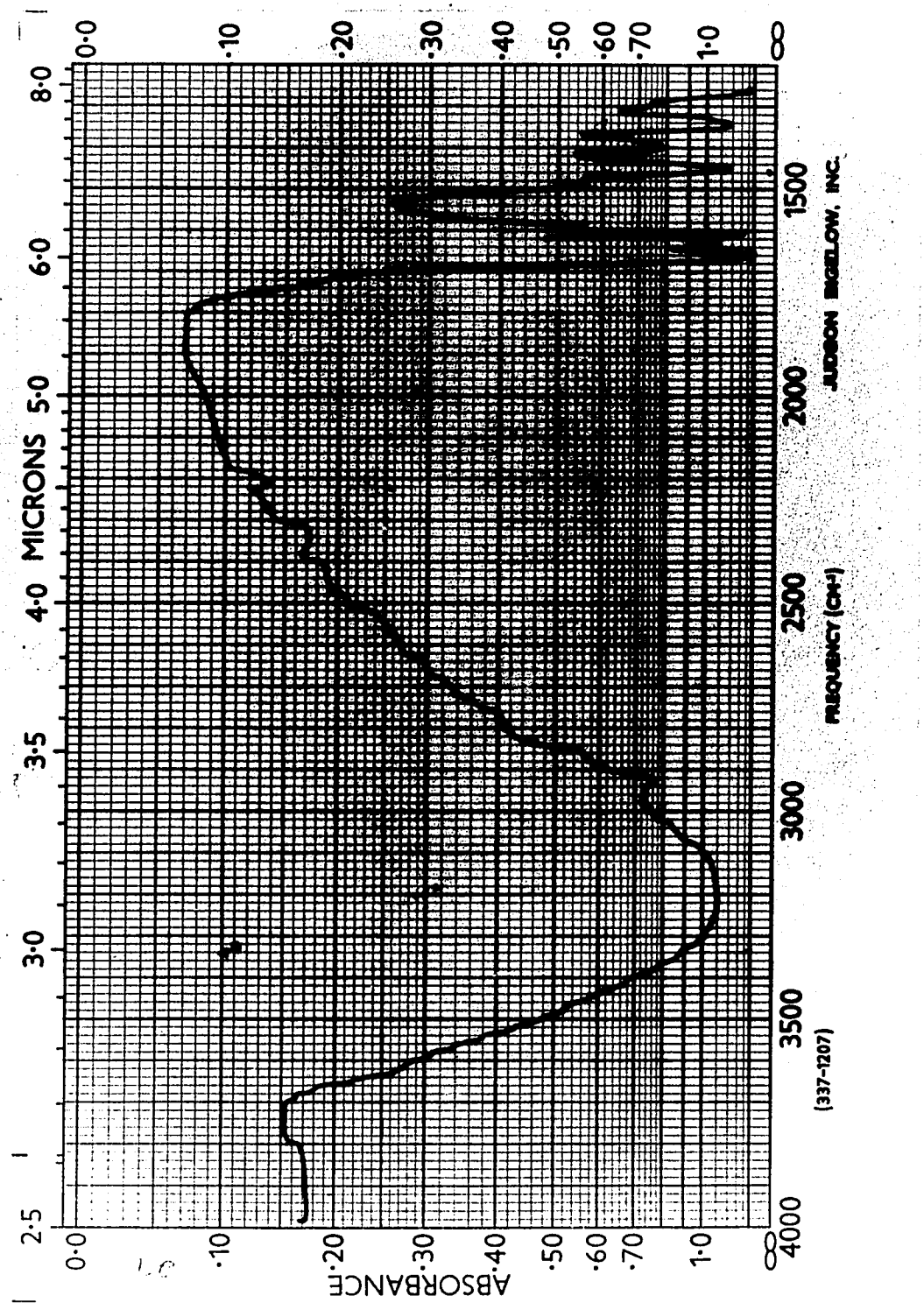
Flavipin was isolated using the procedure described by Bamford et al.[7] From fractions 4, 5, 6 and 7 (Diagram 1), 0.532 g of material was recovered from the 3000 ml of culture filtrate. This was about half of the yield reported by Bamford et al. When 25.8 mg of this material was sublimed, 12.20 mg of yellow flavipin was obtained. A residual greyish-brown material remained in the bottom of the sample tube after sublimation.

One mg of this material was used to obtain the infrared spectrum shown in Figures 2 and 3. A comparison between the infrared absorption bands described by Raistrick and Rudman[8] for flavipin and that obtained in this study is shown in Table 2. The visible and ultraviolet spectrum reported in this thesis was obtained in 95 per cent ethanol while that reported by Raistrick and Rudman[8] was obtained in dioxane. The recorded visible and ultraviolet spectrum is shown in Figure 4, and the absorptivity is shown in Table 3 along with data reported by Raistrick and Rudman.[8] The elemental analysis data is shown in Table 4 along with the data included in the papers by Raistrick and Rudman[8] and Bamford *et al.*[7] Table 5 shows the melting points of these compounds isolated by the various groups and in this study.

Table 2 shows the infrared absorption bands of flavipin reported by Raistrick and Rudman[8] and the absorption bands of the compound isolated by the author. The compound isolated by the author shows similarities in some regions of the spectrum but also differences in other regions. It would have been easier to compare the two spectra if Raistrick and Rudman[8] had included a recorded spectrum in their paper. Bamford *et al.*[7] did not include any data on the infrared spectrum of flavipin. With the differences between the absorption bands found in this study and those reported by Raistrick and Rudman[8], one cannot conclude

Figures 2 and 3

Infrared spectrum of Flavipin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4·0 MICRONS 5·0
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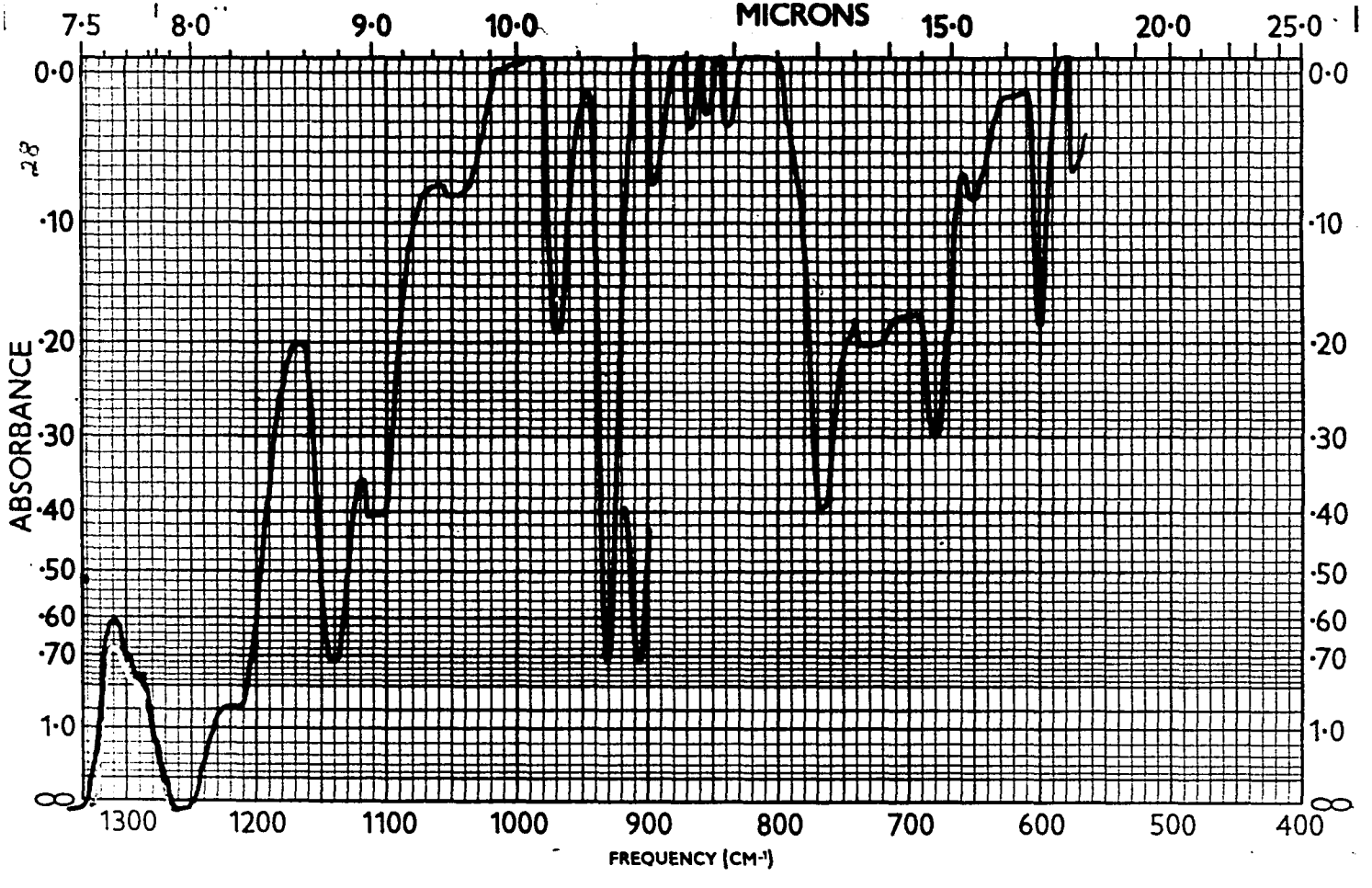
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1000
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700
600
500
400
FREQUENCY (CM⁻¹)

Table 2

Absorption wavelengths in the infrared spectrum of flavipin

Wavelengths (cm^{-1})*	Wavelengths (cm^{-1})[8]	Wavelengths (cm^{-1})*	Wavelengths (cm^{-1})[8]
3200	3226	1140	1145
2995	3067	1109	1115
2860		1050	1060
1680	1658		1039
1620	1615		1016
1600			1002
1490	1489	970	972
1450	1454	930	932
1400	1390	896	
1345	1330	868	
	1303	855	
1255	1264	840	
1215	1217	768	765
		730	

Figure 4

Visible and ultraviolet spectrum of flavipin.

Absorbance
Wavelength (nm)
500
400
300
200

Table 3

Molar absorptivity of flavipin

Wavelengths (nm)	log ε	Author
261	4.31	Raistrick and Rudman[8]
264	4.35	Raistrick and Rudman[8]
330	3.94	Raistrick and Rudman[8]
265	4.22	This study
346	3.81	This study

Table 4

Elemental analysis of flavipin.

Organism	Carbon	Hydrogen	Nitrogen
*E. nigrum**+	54.69	4.22	nil
A. terreus[8]	55.2	4.1	nil
A. flavipes[8+]	54.9	4.1	nil
E. nigrum[7]	55.1	4.1	nil
Calculated Values	55.1	4.1	

* This study.
+ Sublimed samples.

Table 5

Melting point of flavipin

Melting Point (C)	Author
233-234 (decomposed)	Raistrick and Rudman[8]
228-229 (decomposed)	Bamford *et al.*[7]
229-231 (decomposed)	This study

that these are the same compound even though the chances of getting identical spectra using the potassium bromide pellet method are slim at most, even when done by the same researcher.

The ultraviolet data included in Table 3 still does not help in verifying that the compounds are the same. The molar absorptivities as well as the absorption maxima are different. These differences may be due to the different solvent systems used in obtaining the spectra. Interestingly, the values of the absorptivities are about the same and of the same order of magnitude (2.2×10^4 vs. 1.6×10^4).

The elemental analysis data, however, does lend support to the fact that the compounds being the same. The three compounds reported in Table 4 all have the same carbon, hydrogen and oxygen content, and when the molecular formula is computed, $C_9H_8O_5$ is obtained as the formula of flavipin.

The melting point data in Table 5 also lends support to the compounds being the same. In addition to being similar in the above mentioned respects, the compound in each case forms a 2, 4 dinitrophenylhydrazine derivative and gives a steel blue color in ethanol when ethanolic $FeCl_3$ is added.

The evidence taken as a whole suggests that the compounds isolated are indeed the same. It further points out that the strain of *E. nigrum* used in the research

reported in this thesis can produce the same metabolites as other strains used by other researchers. However, Foppen[3] had failed in his attempt to isolate flavipin. The reason for that failure is not clear except that the medium used by Foppen[3] did not contain the same concentrations of all the trace elements employed in the medium of Bamford *et al.*[7] In the first experimental attempt to isolate flavipin, the author was also unable to recover any flavipin using the medium attributed to Bamford *et al.* reported in Foppen's[3] paper.

In the second experiment in which the flavipin was eventually isolated, the procedure for inoculation was different, and the medium was corrected to include the trace elements. Whereas in the first experiment mycelia from slant cultures were used directly to inoculate the growing flasks containing the medium included in Foppen's[3] paper. In the second experiment the mycelia were first transferred to 5 ml of liquid media and allowed to grow for 5 days before transferring to the growing flasks. The most obvious difference between the two experiments came during the end of the growing period of 28 days. The color of the medium was a much darker brown in the second experiment than the yellow brown color in the first experiment.

The cause of the difference in production of flavipin is not clear. The fact that the media were different in trace elements is obvious. Current ideas, however,

suggest that these elements occur in sufficient quantity in tap water to support the growth of micro-organisms, especially bacteria, and so this may not be the deciding factor. The fact that the slant material was grown up for a 5 day period before inoculation of the growing flasks may possibly be the reason for the production. When this is done there is an increase in the amount of inoculum, and, secondly, the fungus is already actively growing at the time of inoculation. Certainly in the case of using the slant culture as inoculum the fungus is growing at a much slower rate at the beginning. From this one might conclude that flavipin could be produced on the medium reported in Foppen's[3] paper by either pregrowing the fungus in test tubes to get inoculum for the growing flasks or by inoculating from slant cultures and allowing the culture to grow for a longer period than the 28 days reported by Bamford et al.[7] Essentially the former procedure was carried out, and the fungus did produce flavipin.

The fact that in the isolation of Epirodin A, the fungus is grown up for 14 days and a solid rice culture rather than a liquid culture is used, might explain why the author was not able to obtain flavipin using this procedure. However, a phenolic compound or compounds were shown to be present by reaction with $FeCl_3$. Since no red solid precipitate was produced with 2,4-dinitrophenylhydrazine, one might conclude that flavipin was not present. Growing

the fungus for longer periods of time on rice was not attempted.

Table 6 shows the activity of flavipin against B. megaterium spores. The activity is small when compared with other inhibitors. The results indicate that flavipin has very minimal activity against B. megaterium spores.

Table 7 shows the activity of flavipin against several species of Chlorella. The compound possesses considerable phytotoxic activity as can be seen by the concentrations at which activity can still be measured. Chlorella vulgaris seems to be more resistent to the phytotoxic effects of flavipin. The Carolina strain of Chlorella pyrenoidosa seems to be the most sensitive. This difference is in keeping with results obtained by Sullivan and Ikawa[15] who have found that strains vary greatly in their susceptibility to various compounds. This activity of flavipin against the Chlorella species might help to explain the bleeching of tobacco leaves infected by the fungus.[16]

Table 6

Antibacterial activity of flavipin against B. megaterium spores.

Conc. (mg/ml)	Avg. net diameter* of zone (cm)	Range of zones (cm)	No. of detns.
2.0	.75	.70-.80	6
1.0	.34	.30-.35	8
0.5	.10+	.10-.15	10

* Net diameter of zone = total diameter of zone minus 0.7 cm disc diameter.

\+ Overgrowth of zone (zone was apparent in an earlier observation but small colonies had begun to appear at the end of the incubation period).

Table 7

Activity of flavipin against *Chlorella* species.

Conc. (mg/ml)	*C. pyrenoidosa* (UNH)		*C. pyrenoidosa* (Carolina)		*C. vulgaris*	
	Avg. net zone of inhib. (cm)	No. of detns.	Avg. net zone of inhib. (cm)	No. of detns.	Avg. net zone of inhib. (cm)	No of detns.
1	1.56 (1.50-1.70)*	9	1.75 (1.65-1.80)	10	.34 (.25-.35)	9
.1	.37 (.30-.45)	12	.50 (.45-.60)	12	no inhibition	9
.05	.14 (.10-.20)	12	.23 (.20-.25)	12		

* Values in parentheses give the range.

Humic Acid

Humic acid is an ill defined polymeric substance generally resulting from the decomposition of organic matter. They are dark brown substances, soluble in alkali and containing phenolic compounds and amino acids. These polymers of phenols and amino acids have relatively high resistance to microbial degradation. The molecular weights as determined on Sephadex gels have ranged from 5000 up to 200,000. The humic acid produced by E. nigrum has been shown to contain 14 phenols among which are orcinol, orsellinic acid, 2,4-dihydroxytoluene, 2,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 2,4,5-trihydroxytoluene, and 3,5-dihydroxytoluene.[4]

Foppen[3], also working with E. nigrum, was able to isolate a humic acid with a molecular weight of about 4,200 ± 200 using Sephadex G-25. The amino acid composition was determined and shown to be as follows: 2 alanine, 2 proline, 2 aspartic acid, 2 glutamic acid, 2 glycine, 2 lysine, 2 serine, 2 threonine and 2 valine. Several of the phenols reported by Martin et al.[4] were also found.

No biological activity had been reported by other workers for the humic acid produced by E. nigrum. The humic acid fraction isolated during this research was not active against B. megaterium spores or Chlorella species. Because of this lack of any activity being

present only a limited amount of additional work was done on this fraction. The molecular weight was determined using Sephadex G-50 thin layer gel filtration. The molecular weight as determined by this procedure was 4,350 ± 350 (Figure 5 and Table 8). The infrared spectrum of the humic acid is shown in Figures 6 and 7, and Table 9. The recorded visible and ultraviolet spectrum is shown in Figure 8. The elemental analysis data is shown in Table 10 along with a comparison with the data from other workers.

Table 8

Results of thin layer gel filtration on the humic acid.

Compound	$1/R^{*}_{hemerythrin}$	
Vitamin B_{12}	2.92 +	1.77 ++
Humic Acid	2.77	1.68
Cytochrome C	2.46	1.62
Myoglobin	2.46	1.57

* 1/R = 1/migration distance of each compound divided by 1/migration distance of the reference compound hemerythrin.

\+ TLG plate at an angle of 25°.

++ TLG plate at an angle of 20°.

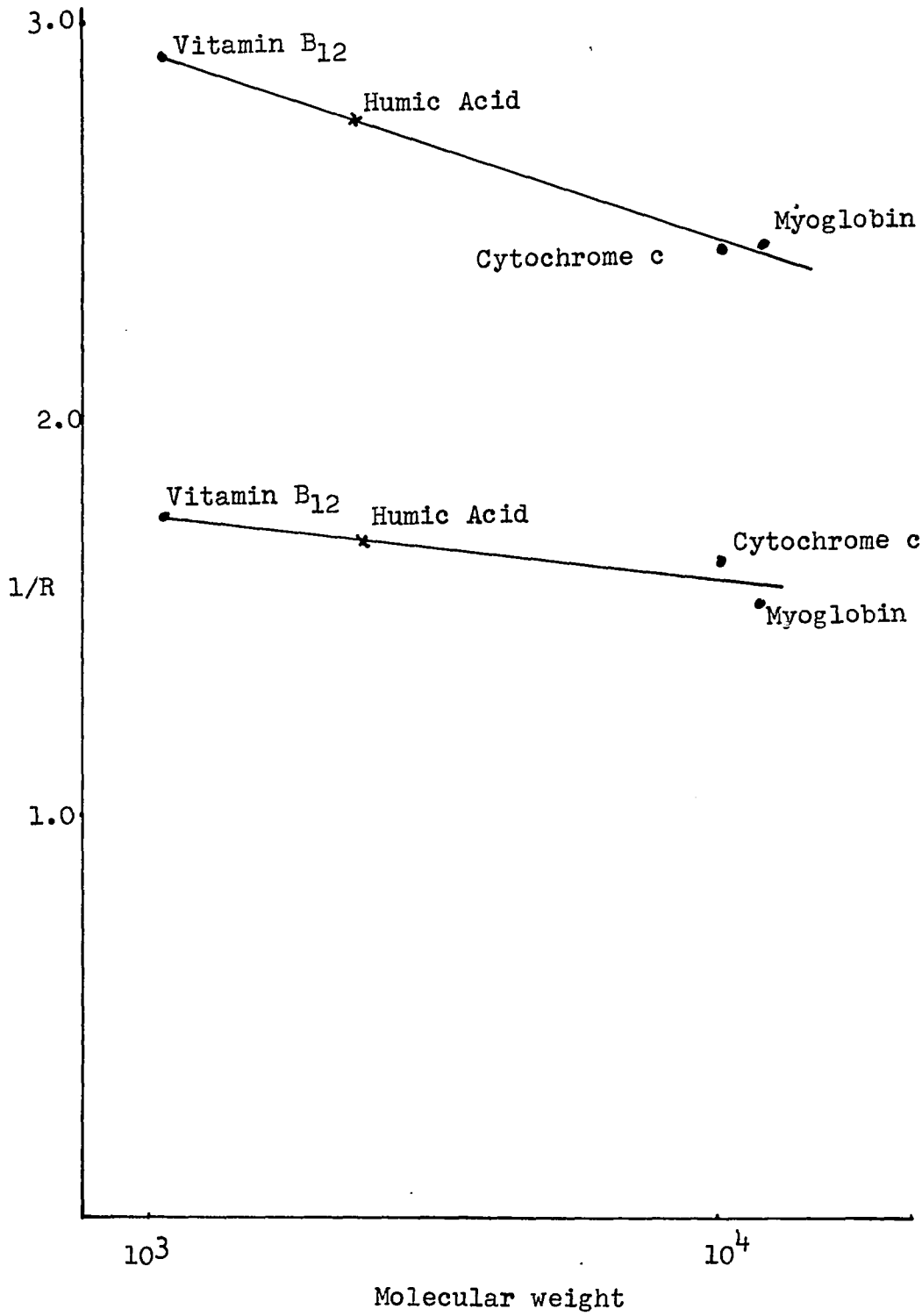
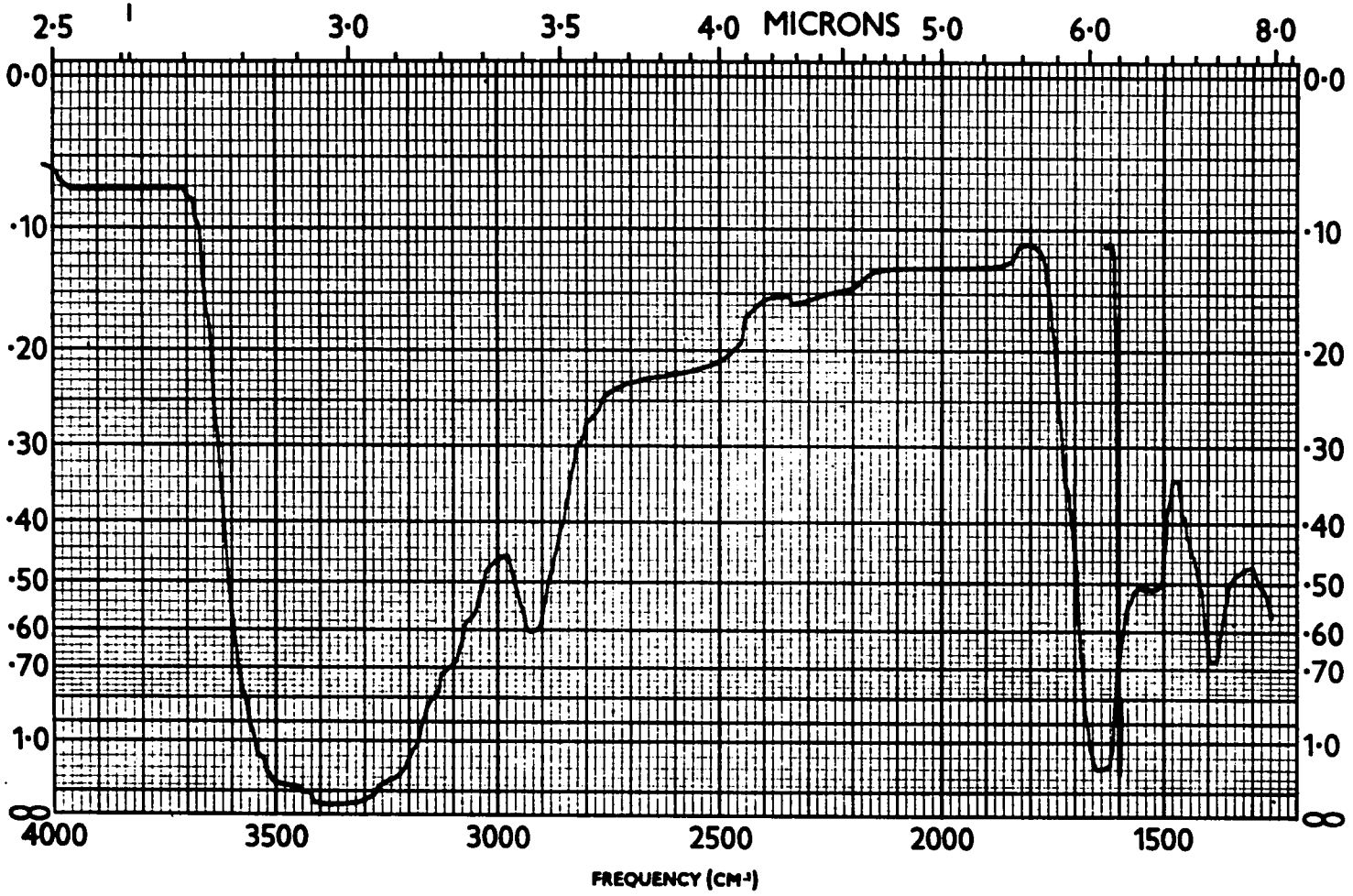


Figure 5

Molecular weight of the humic acid by gel filtration.

Figures 6 and 7

Infrared spectrum of the humic acid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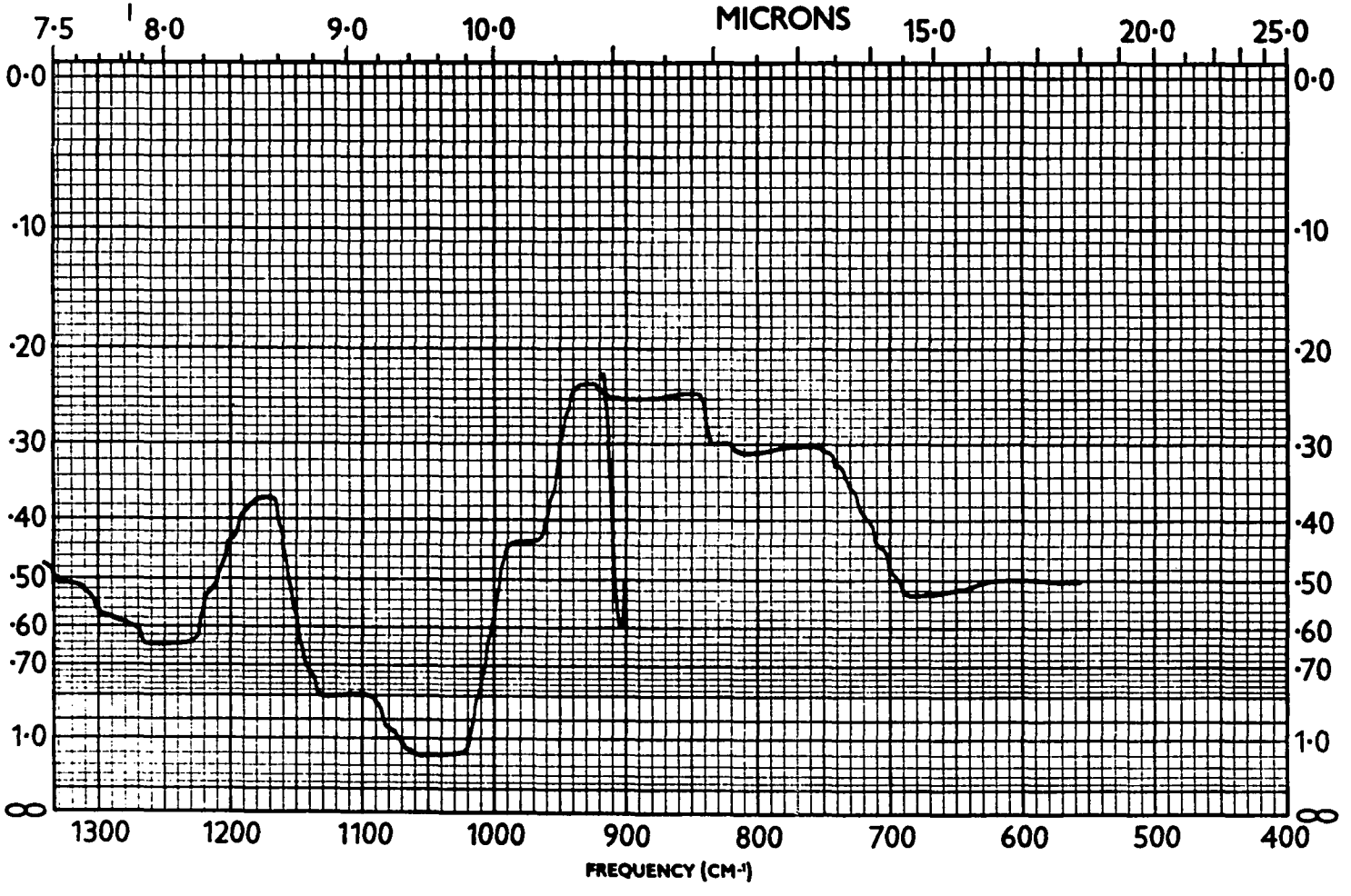
FREQUENCY (CM⁻¹)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FREQUENCY (CM⁻¹)

Table 9

Absorption wavelengths in the infrared spectrum of the humic acid.

Wavelengths (cm^{-1})	Wavelengths (cm^{-1})
3380 (3500-3220)	1125
2935	1040
1650	978
1530	835
1498	800
1245	680

Figure 8

Visible and ultraviolet spectrum of the humic acid.

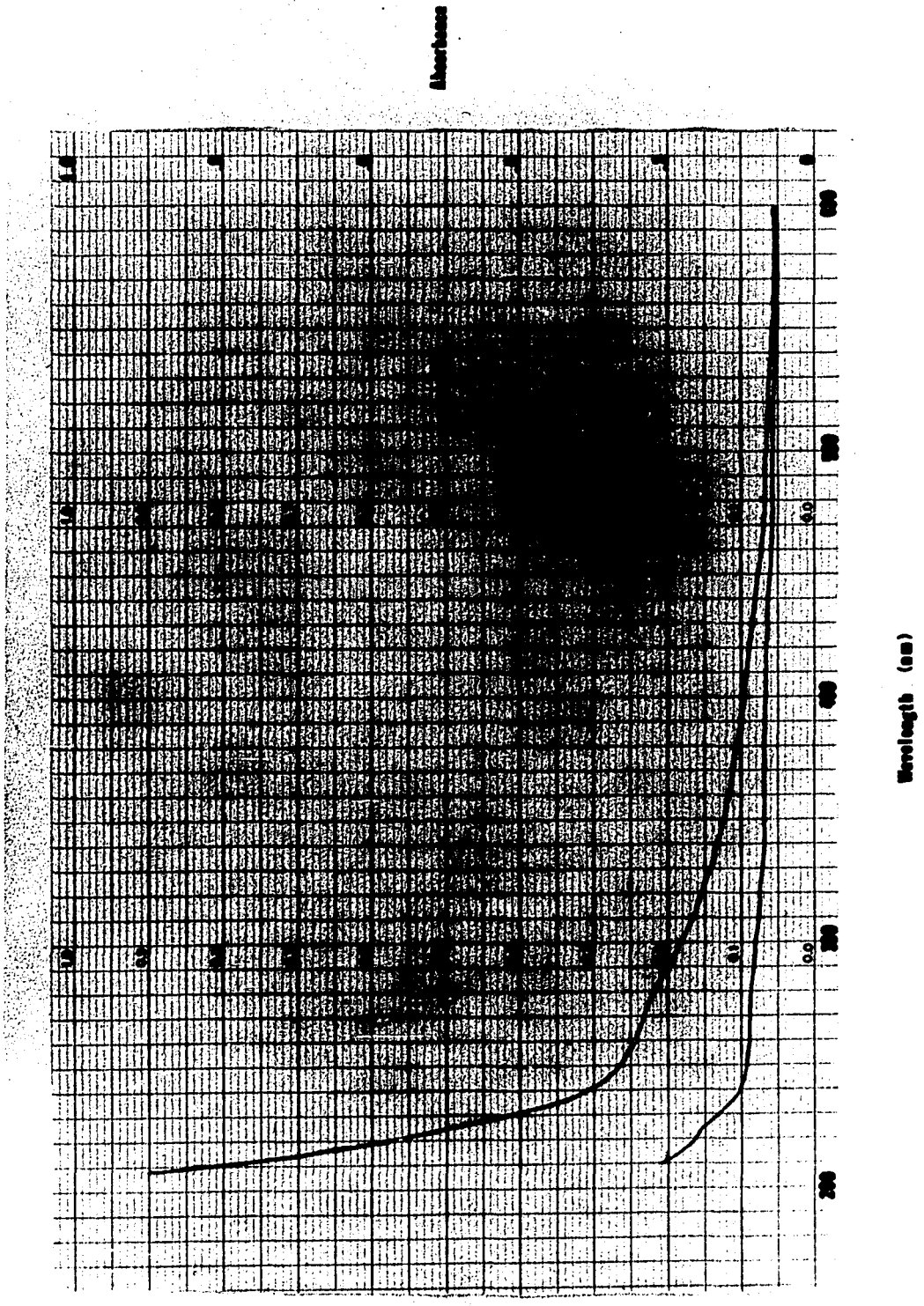

Absorbance
Wavelength (nm)

Table 10

Comparison of the humic acids isolated by various researchers by means of the reported elemental analysis data.

Author	Elemental composition (percentage)		
	Carbon	Hydrogen	Nitrogen
Foppen	54.9	5.8	6.3
Martin *et al.*	54.5-59.1	5.1	4.5-8.5
This work	41.6	5.9	5.6

Epirodins

The first chromatographic step (Column A) for isolating Epirodin A using column chromatography on silicic acid and eluting with 5 per cent methanol in chloroform gave the results shown in Tables 11 and 12 and Figures 9 and 10. The appearance of TLC of the crude extract and of Column A is shown in Figures 11 and 12, respectively. Of the approximately 2.5 g placed on the column over half the material came off in the first thirteen tubes collected. The material collected in tubes 5-13 left an oily residue which remained an oil no matter how long the material was left on the rotary evaporator.

As can be seen in Table 11 and Figure 9 these initial fractions contained little or no activity against B. megaterium spores. Thin layer chromatography of these fractions on prepared silica gel G plates (E. Merck and Eastman) gave a spot that had the same R_f as β-carotene (R_f = .88) when they were co-chromatographed in hexane-acetone solution (88:12). β-Carotene has been shown to be produced by E. nigrum also by Foppen.[3] A better separation of these earlier pigments is achieved if a 1 per cent solution of methanol in chloroform is used rather than a 5 per cent solution. Chromatography with the 5 per cent solution eluted a considerable amount of reddish brown material along with the suspected carotenoid pigments.

Table 11

Activity of fractions from Column A against B. megaterium.

Fraction	Avg. net zone of inhibition (cm)*				
	Conc. (mg/ml)				
	0.50	0.25	0.20	0.10	0.05
1-4					
5-7	0.30 0.25	neg.			
8-13	neg.				
14-31	0.70 0.60	0.20 0.20			
32-60	0.35 0.30	neg.			
61-80	1.00 1.05	0.95 0.95			
81-120	1.50 1.45	1.45 1.40	1.15 1.15	0.90 0.90	0.65 0.60
121-140	1.10 1.15	0.95 0.95		0.75 0.70	0.35 0.35
Remainder	neg.				

*Total diameter of zone minus 0.7 disc diameter. Two separate determinations run.

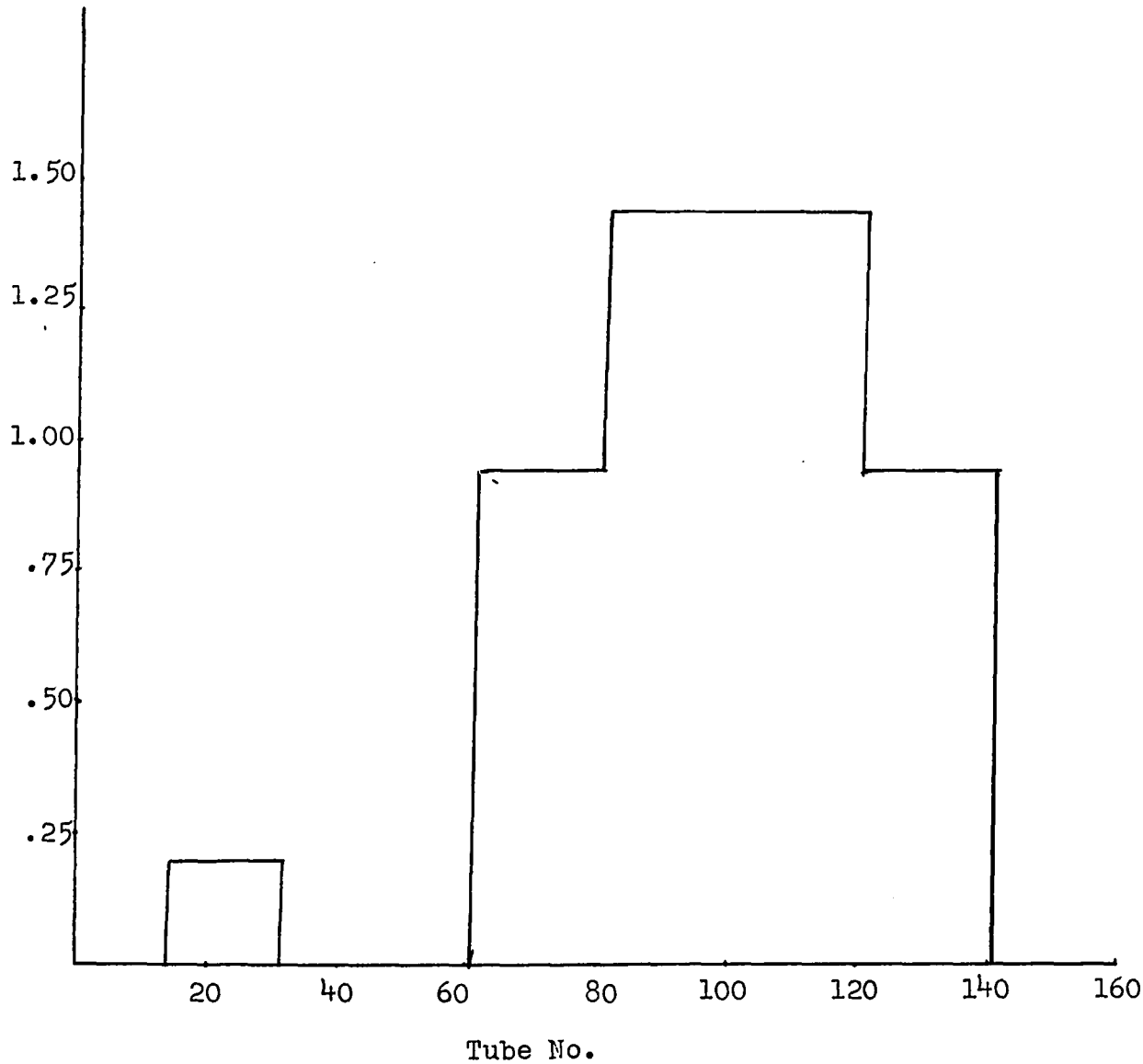


Figure 9

Activity of fractions from Column A, zones obtained at 0.25 mg/ml concentration.

Table 12

Weight of fractions from Column A.

Tube No.	Weight of Fractions
1-4	nil
5-7	1.195 g
8-13	0.292 g
14-31	0.113 g
32-60	0.131 g
61-80	0.124 g
81-120	0.096 g
121-140	0.029 g
Remainder	0.252 g

2.5 g approximately of crude extract were placed on a silicic acid column (5 X 58 cm) and 1300 drops per tube collected.

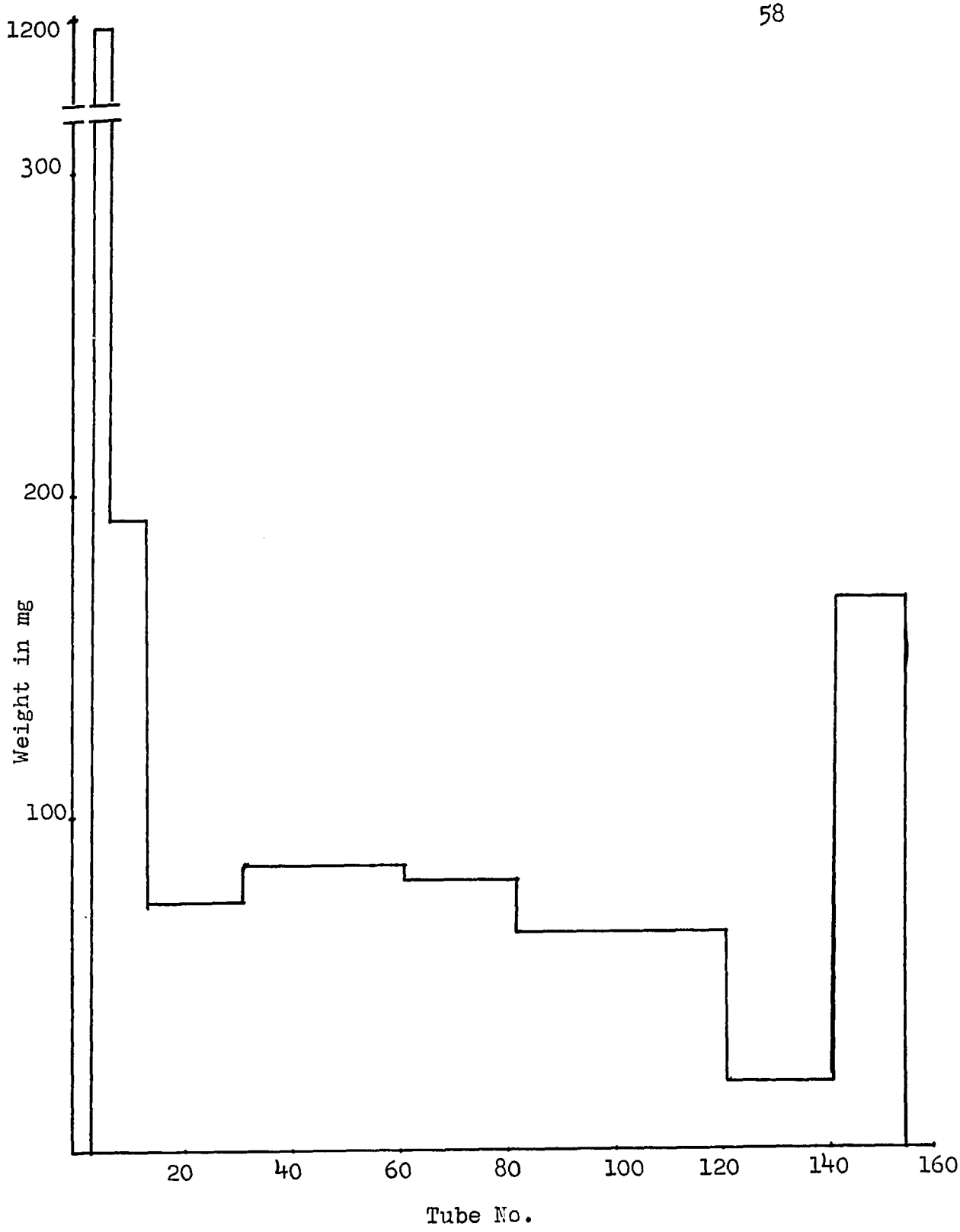
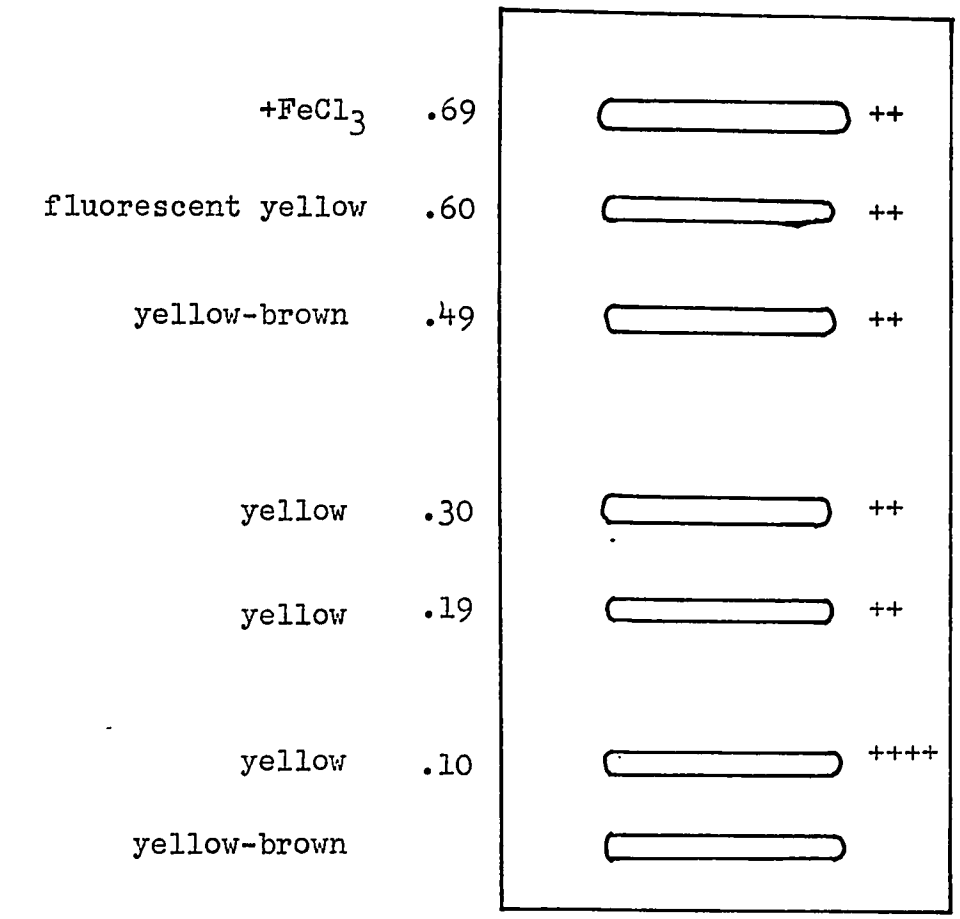


Figure 10

Weight of fractions from Column A.



+ Relative amounts

Figure 11

TLC pattern of crude 1st extract on Eastman plates in acetone-methanol (1:1) solvent system.

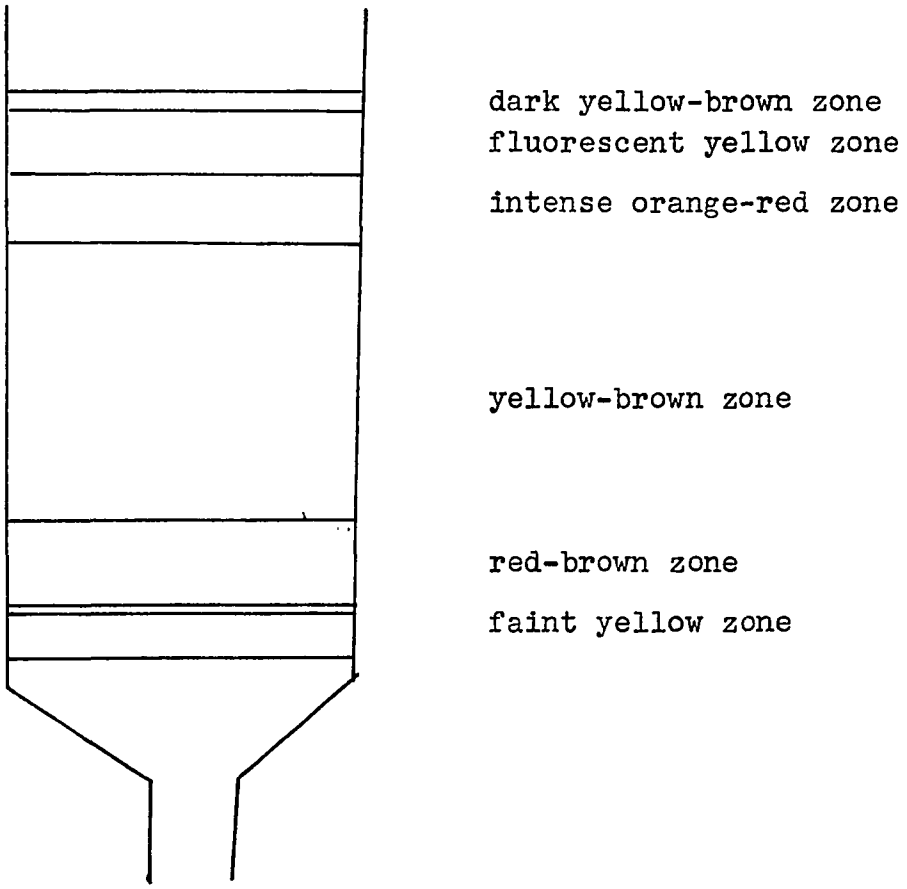


Figure 12

Appearance of Column A during chromatography.

The activity of fractions 14-60 was marginal. When these fractions were run on silica gel plates (Eastman) in acetone-methanol (1:1) solvent system a considerable amount of brown streaking along the path of migration was observed. Since the activity of these fractions was marginal, further investigations of these fractions were curtailed.

In examining the activity distribution in Table 11 and Figure 9, it can be seen that significant activity started to come off the column around tube 60. Since all the fractions were colored, there was difficulty at first in determining by sight when the active fractions had started coming off the column. With experience, however, the active fractions could be readily recognized by the intense color of the eluate. The outlet of the column becomes stained yellow as the material is being eluted. The beginning of the active fraction can also be determined by the absorbance at 429 nm. The color intensity and the absorbance at 429 nm were used to mark the beginning of the active fraction. The collecting of the active fraction was started when a concentration of 60 ug/ml or less caused the absorbance at 429 nm to be 0.80 or greater. In the case of the most intensely colored material in fractions 81-120, 10 ug/ml was sufficient to give an absorbance of 0.80 or higher.

Alternatively a biological assay was also used to determine where the active fraction began. By testing

the early fractions at a concentration of 0.25 mg/ml against B. megaterium spores one could tell where the active fraction started. Good agreement between the two methods was obtained. The variance was usually about 3-4 tubes. The bioassay was considered the more accurate but it suffered from a 14-16 hour time lag before results could be obtained.

Column A gave a very rough separation in the sense that the active components were not separated in pure form, but in terms of separation from the bulk of inactive material the column was very effective. Over 85 per cent of the weight of the applied material was separated from the active material by using this silicic acid column. This separation was completed within a 24 hour period after the column had been properly packed.

Column B was used to rechromatograph the active material from Column A. Column B was a column using silica gel H as support and acetone-methanol (1:1) as solvent. The appearance and results of this column are shown in Figure 13. Fractions were taken on the basis of fluorescent bands that were seen with a long wave (365 nm) ultraviolet lamp. Fraction 1-F included the first fluorescent band along with a yellow band associated with it. Fraction 2-F included the second fluorescent band, the yellow band associated with this fluorescent band, and the yellow material eluted by 500 ml of the acetone-methanol solvent used to elute this

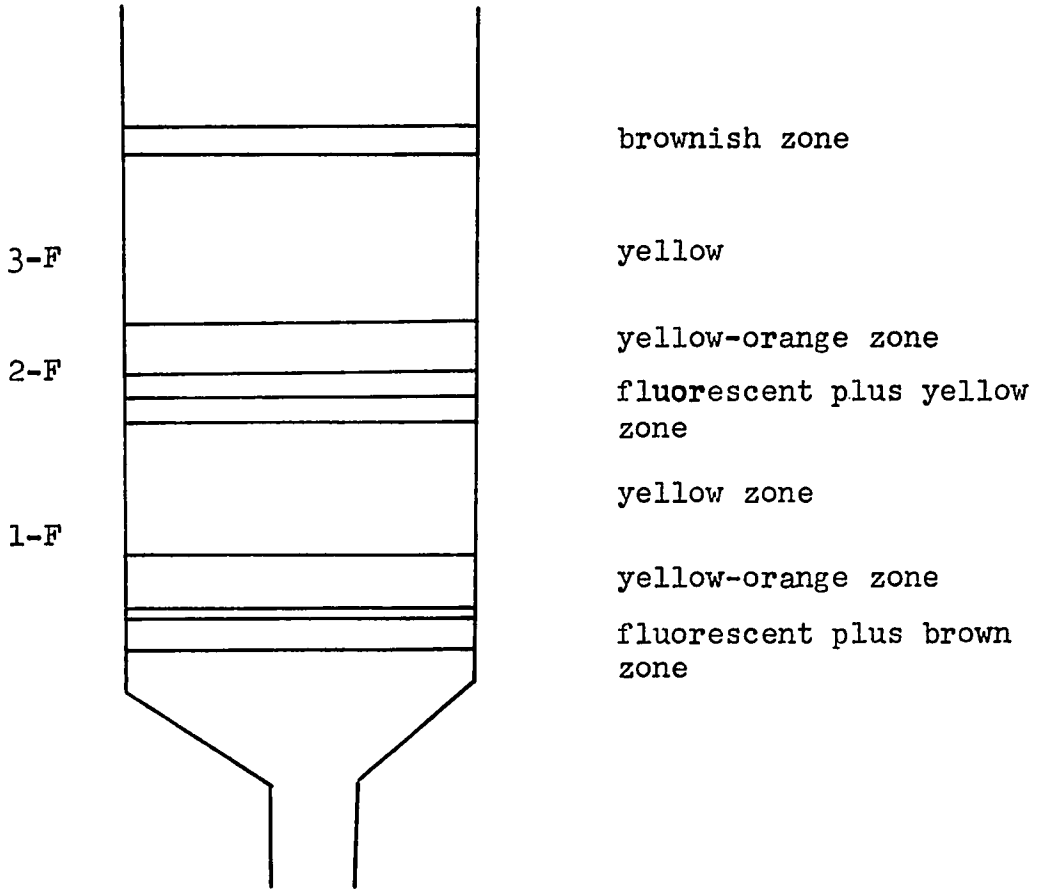


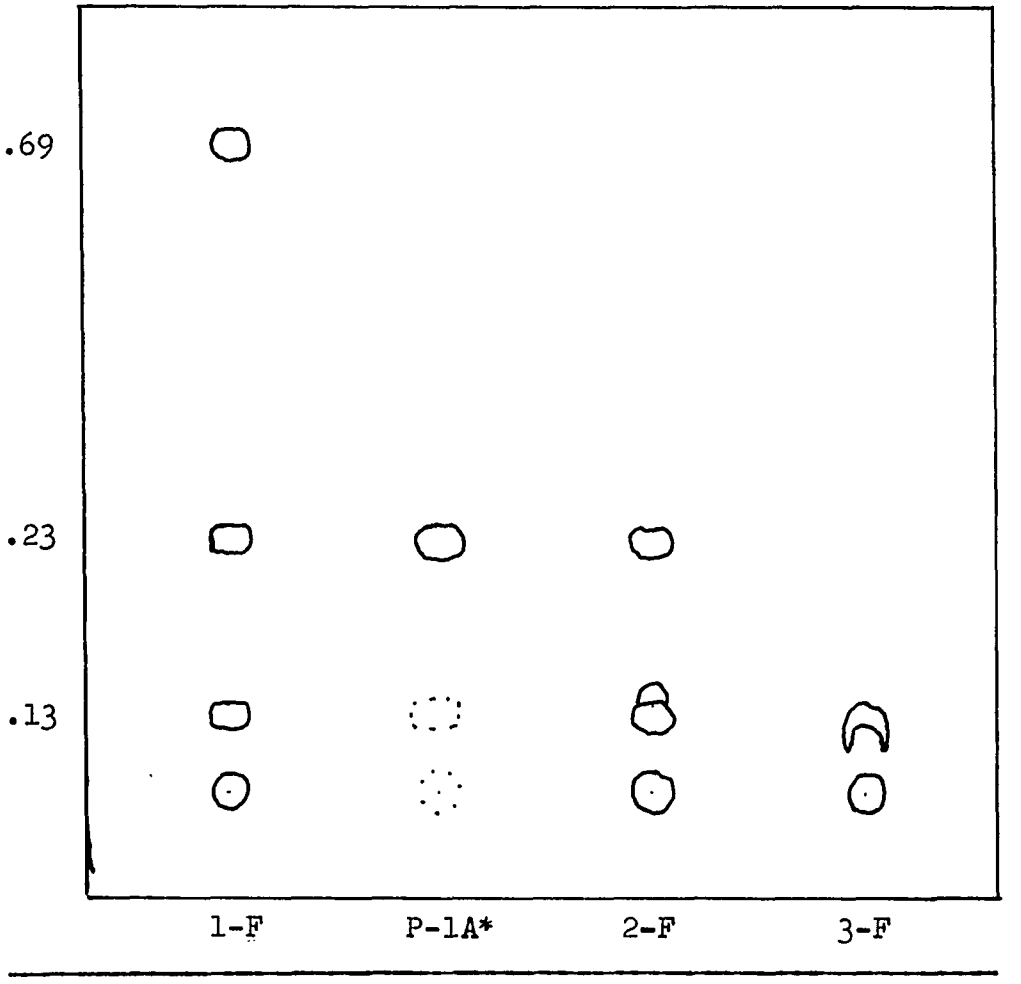
Figure 13

Appearance of Column B during chromatography.

fraction. The remaining yellow material was eluted with 400 ml of methanol and labeled fraction 3-F.

Thin layer chromatography of the fractions off Column B on prepared plates are illustrated in Figures 14 and 15. These two plates were run simultaneously in the same tank (paper lined) with acetone-methanol (1:1) as solvent. Effects normally seen with different absorbents or with different solvent systems were observed to occur here when using different brand TLC plates. As can be seen from the figures the separation obtained on Column B was not sufficient to purify the active components. Fraction 1-F contained mostly the materials that had a R_f of .23 on prepared Eastman TLC plates. However, significant amounts of the .13 R_f materials were also present. In fraction 2-F more of the .13 R_f materials along with a component that did not move from the origin. A very small amount of the material with the R_f of .23 was present in this fraction also. Fraction 3-F seemed to be mostly the .13 R_f materials along with the component that did not move from the origin. A very small amount of the materials with the R_f of .23 was just barely visible on the plate in fraction 3-F.

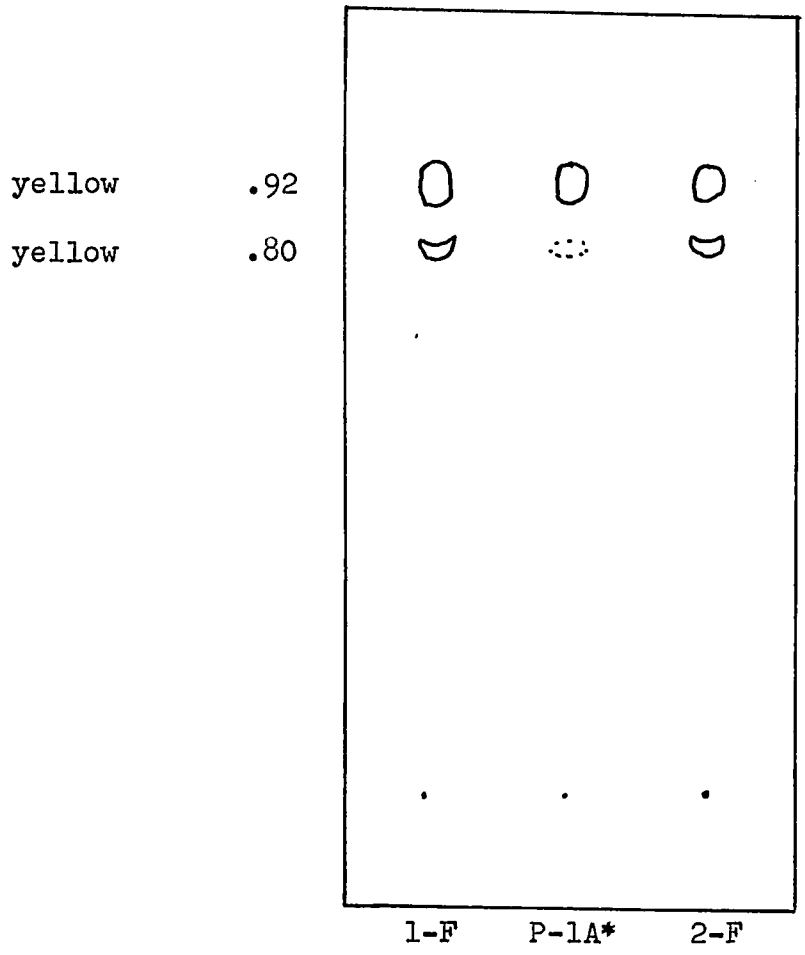
From the results shown in Figures 14 and 15 it can be seen that Column B did not yet give components that were pure enough to be suitable for spectral work or for structural determinations. Some separation was achieved, however. Several batches were run through Column B to



*Pigment obtained by subjecting another culture extract to the same purification steps as Epirodin A.

Figure 14

TLC pattern of fractions 1-F, 2-F, 3-F and pigment P-1A on Eastman prepared plates in acetone-methanol (1:1) solvent system.



*See Figure 14

Figure 15

TLC pattern of fractions 1-F and 2-F, and pigment P-1A on Brinkman prepared plates in acetone-methanol (1:1) solvent system.

accumulate enough material for the next step in the purification (Column C).

The 1-F material was subjected to chromatography on Column C. This column used silica gel G (E. Merck) as solid support and 95 per cent ethanol as eluant. A representation of the elution pattern off this column is shown in Figure 16. Thin layer chromatography was used on the fractions from Column C in order to determine their chromatographic purity. The migration patterns on Eastman TLC plates of these fractions are shown in Figure 17. All the fractions contained the .23 R_f material but the .37 R_f material decreased in the later fractions such that it did not seem to be present after tube 5. The .37 R_f material is of interest because of some pigments isolated earlier that had R_f values in this range. A more detailed explanation of the interest in the .37 R_f pigment will be included in the discussion of the molecular weight of Epirodin A.

The fractions collected from Column C were labeled 1-G, 2-G, 3-G and 4-G. The 1-G fraction consisted of some brownish material and a fluorescent yellow band. The 2-G fraction consisted of a yellow orange band. The 3-G fraction consisted of yellow material up to another yellow band that had a slight amount of fluorescence associated with it. The yellow band and the fluorescence associated with it along with a yellow orange band that closely followed comprised the 4-G fraction. The 4-G fraction will be referred to as the Epirodin B_{mix}.

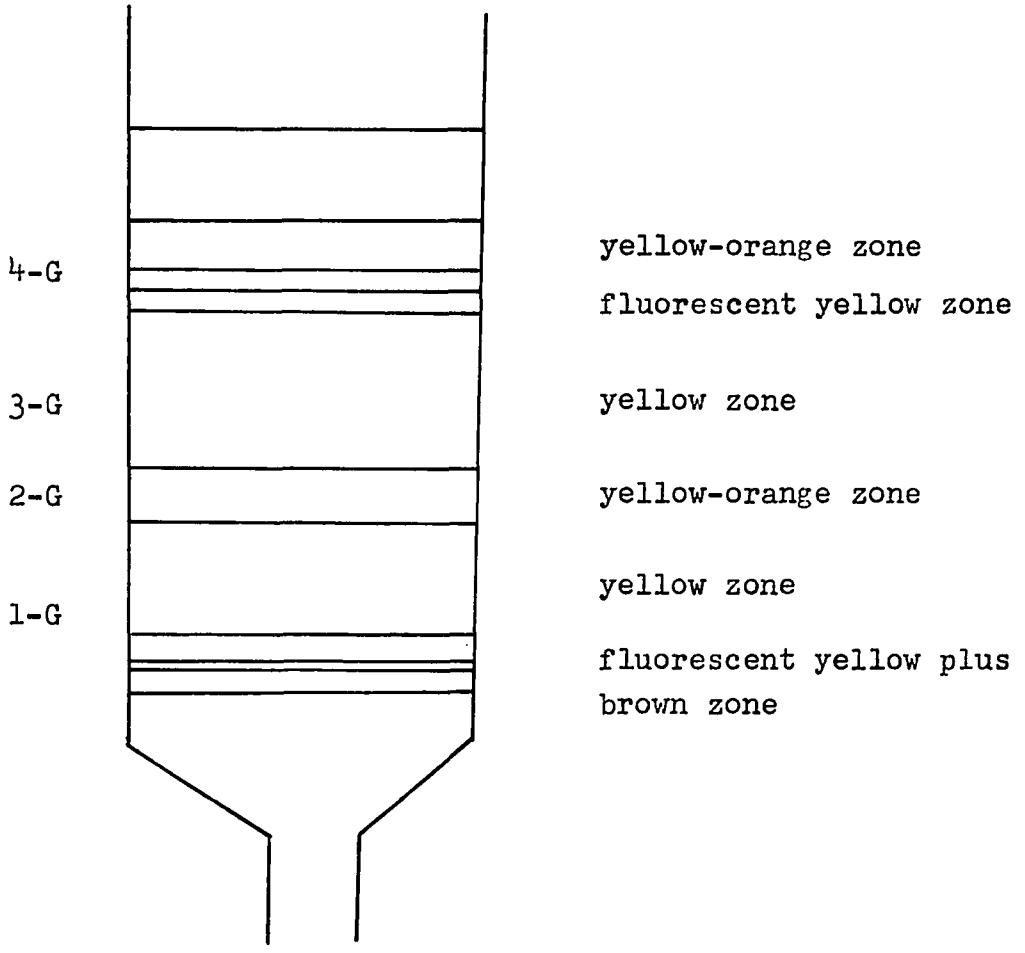
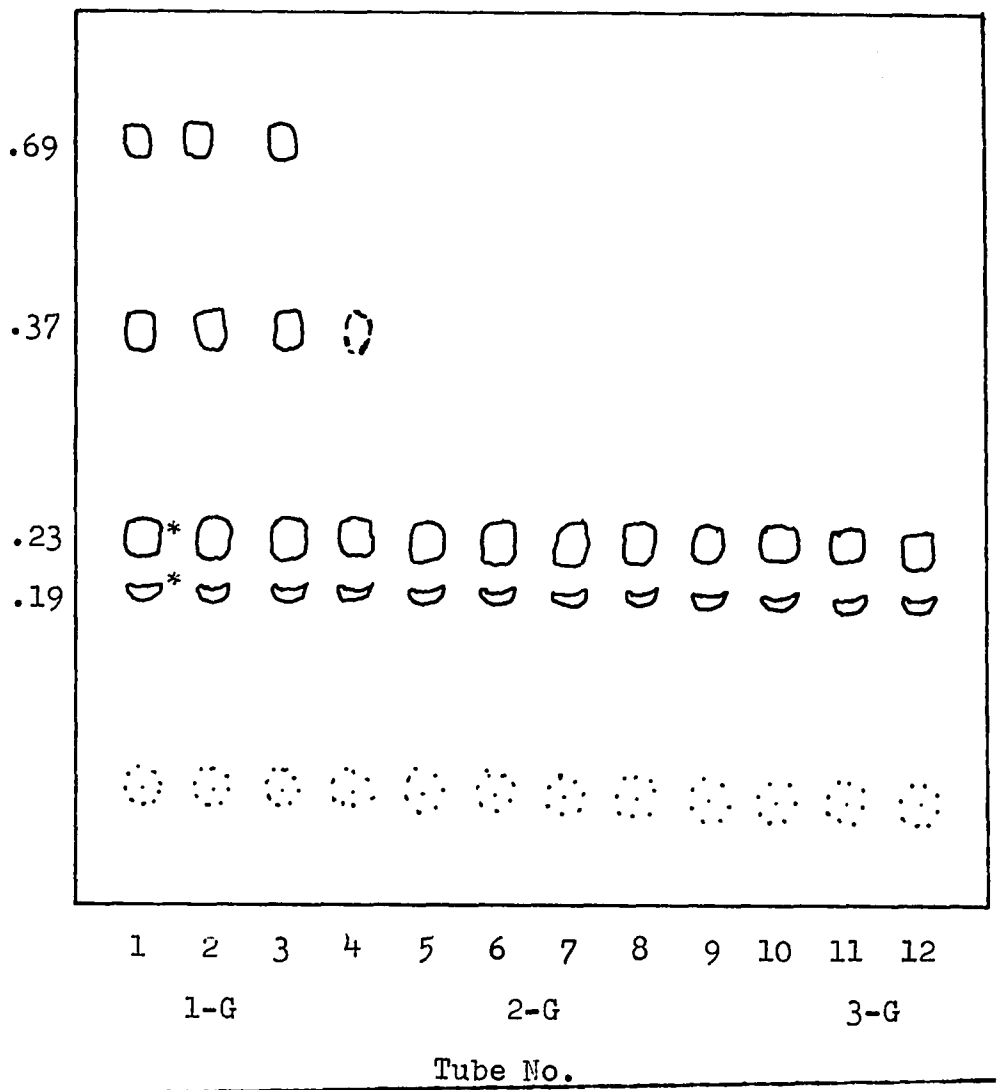


Figure 16

Appearance of Column C of 1-F material during chromatography.



*Biologically active against B. megaterium spores.

Figure 17

The TLC pattern of fractions from Column C using column 5 on Eastman prepared plates using acetone-methanol (1:1) solvent system.

The 2-F fraction from Column B was also subjected to chromatography on Column C. The elution pattern is shown in Figure 18. Essentially the same type of pattern obtained for the 1-F fraction was also obtained for the 2-F fraction. There did not seem to be any of the brown material associated with fraction 1-G (from the column of the 1-F fraction) present in the column of the 2-F fraction. There was only a small amount of fluorescence and the yellow orange band of the 2-G fraction (from the column of the 1-F fraction) was less in the 2-F fraction. The 4-G fraction was considerably more abundant in the 2-F fraction than the 1-F fraction.

The 2-G material from Column C of the 1-F fraction was rechromatographed on Column D. The elution pattern is shown in Figure 19. This column used silica gel H as support and acetone-methanol (1:1) as eluant. The yellow orange band was collected. This band was shown to contain two components by TLC on Eastman prepared plates. The material was chromatographed on Eastman TLC plates and the .23 R_f material taken. This material will be referred to as Epirodin A.

The visible and ultraviolet spectrum of Epirodin A is shown in Figure 20. The absorption maximum occurs at 429 nm. Shoulders occur between 445-455 nm and 234-245 nm. The visible and ultraviolet spectrum of Epirodin B_{mix} is shown in Figure 21. The absorption maximum also occurs at 429 nm with shoulders between 445-455 nm and 234-245 nm.

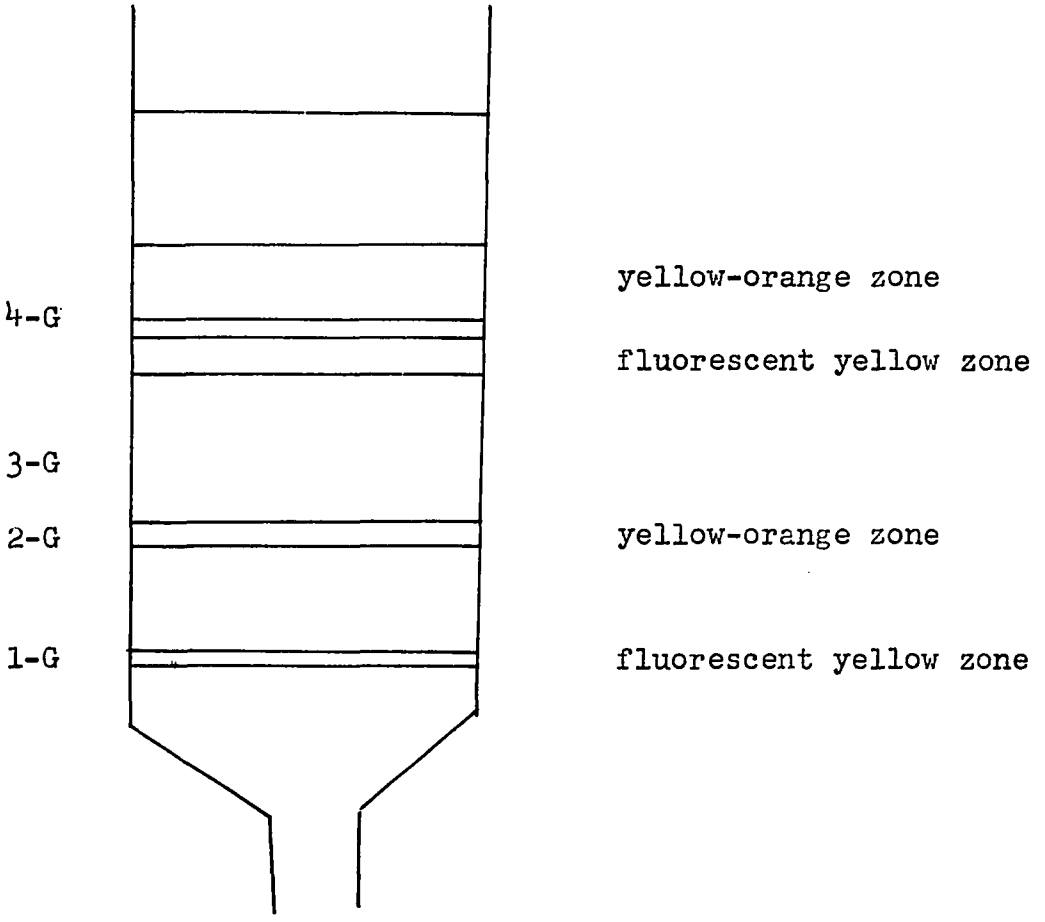


Figure 18

Appearance of Column C of 2-F material during chromatography.

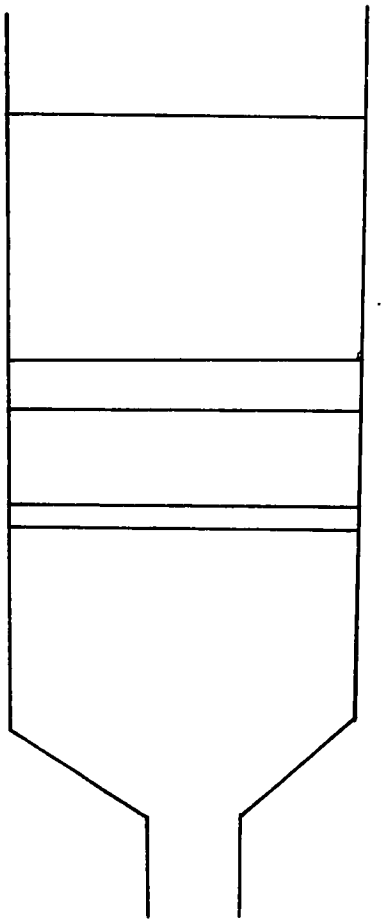


Figure 19

Appearance of Column D during chromatography.

Figure 20

Visible and ultraviolet spectrum of Epirodin A

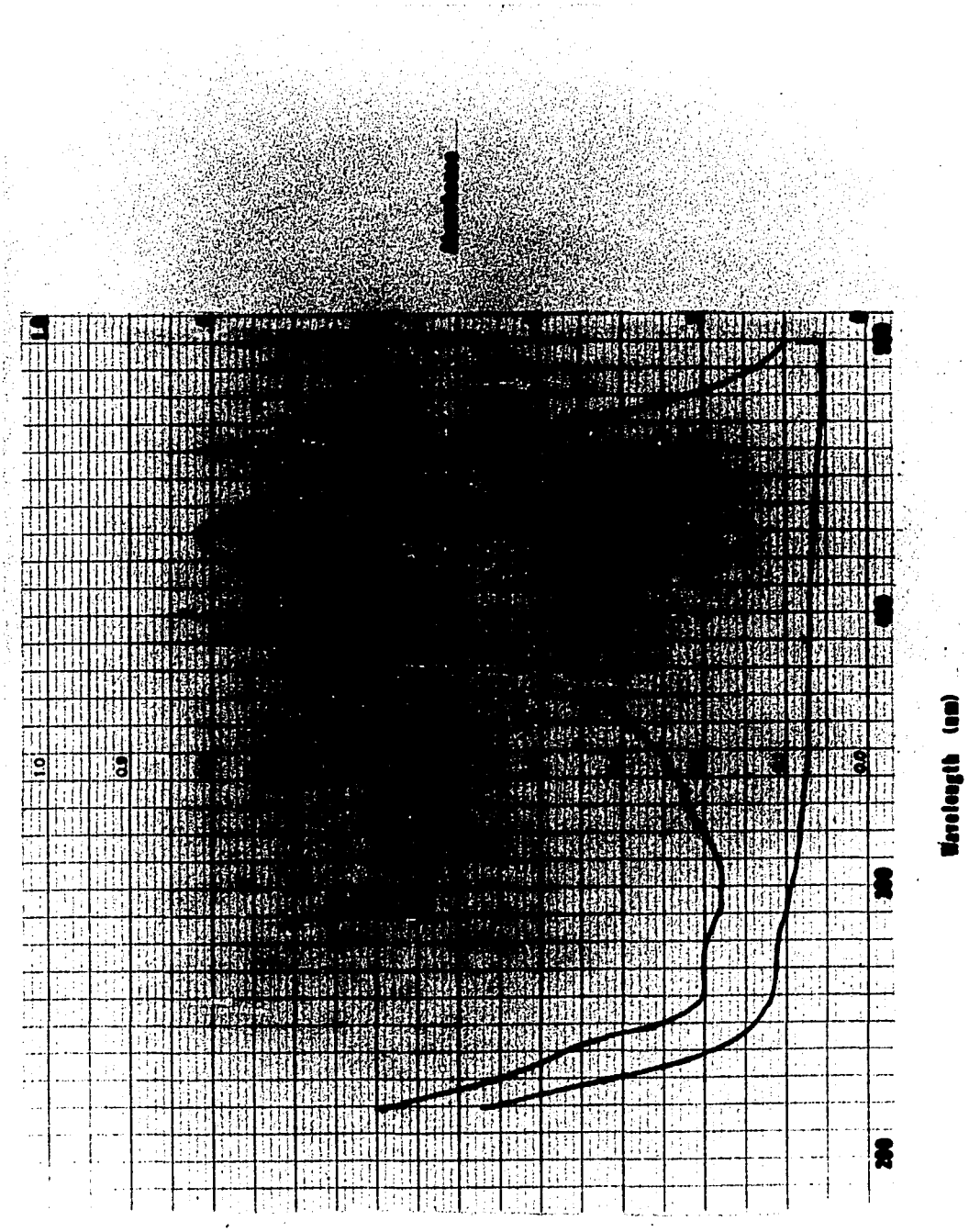
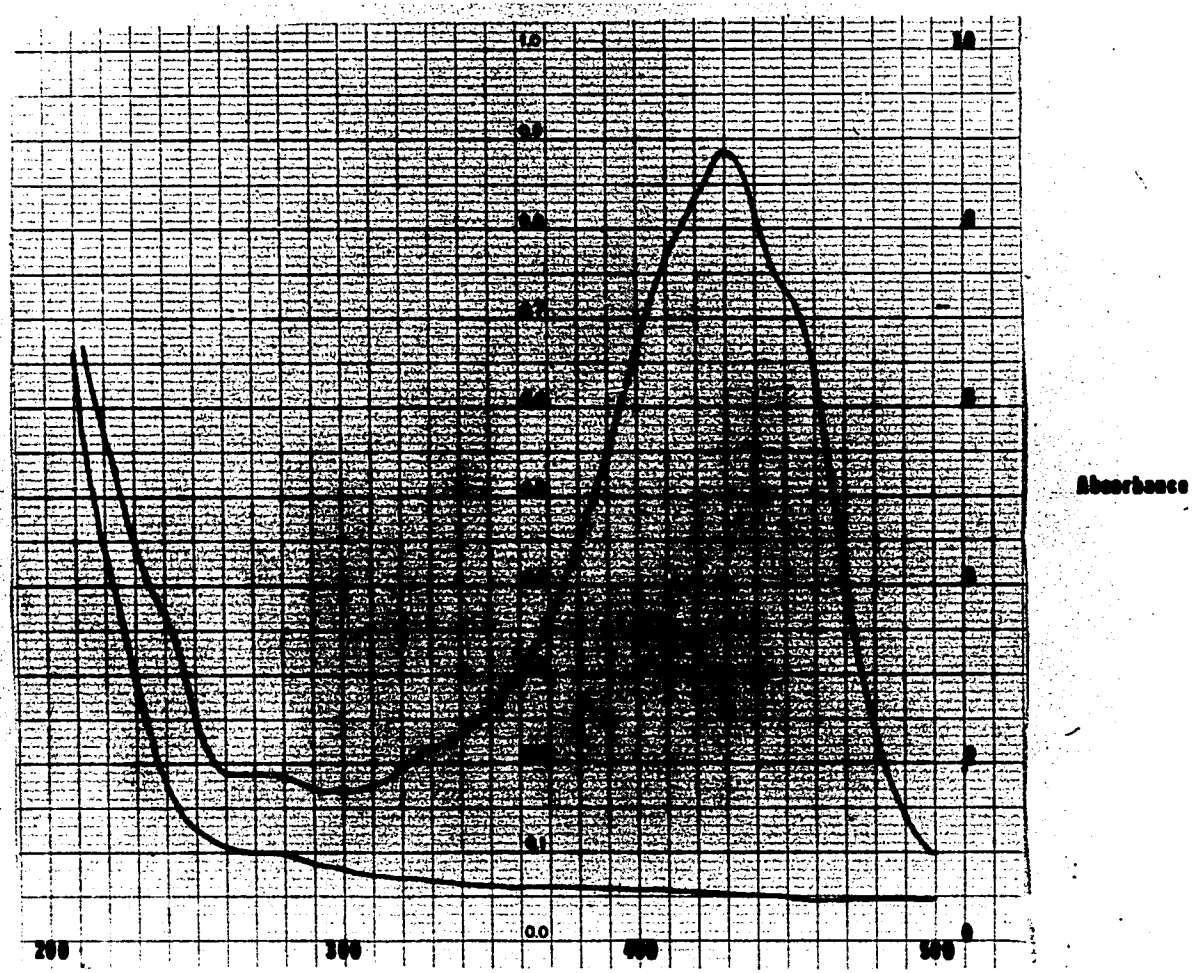

1.0
0.0
Wavelength (nm)

Figure 21

Visible and ultraviolet spectrum of Epirodin B $_{mix}$.


1.0
0.0
Absorbance
Wavelength (nm)

As in the case of Epirodin A no other absorption peaks are apparent in the visible and ultraviolet regions of the spectrum. Examination of the spectra of Epirodin A and Epirodin B $_{mix}$ reveals that these two pigments have essentially the same chromophore. This data suggests that the two pigments are related chemically. The nature of the chromophore cannot be determined by observation of the recorded spectra. However, since the spectra of Epirodin A and Epirodin B $_{mix}$ seem to be almost identical, substitutions on the chromophore of both Epirodin A and Epirodin B $_{mix}$ probably occur at the same positions. From the data obtained in the ultraviolet and visible spectra, it cannot be determined if the functional groups or atoms that are substituted in these positions are the same.

The fact that Epirodin A and Epirodin B $_{mix}$ have absorption maxima in the region above 350 nm suggests that there might be a good degree of unsaturation present in these molecules. The fact that Epirodin A and Epirodin B $_{mix}$ are not transparent rules out the possibility that these pigments are aliphatic or alicyclic hydrocarbons, amines, nitriles, alcohols, ethers, carboxylic acids, or alkyl chloride or fluoride. The fact that these compounds are colored means generally that they contain not less than four, and more probably at least five conjugated chromophores or auxochromes. The only common exceptions to this rule are the α dialdehydes and diketones, and monomeric nitroso compounds.[17,18]

The latter type compounds are colored because of the presence of the functional groups mentioned. There are other compounds that possess these functional groups but also have -C=C- systems in conjugation with these groups. This results in absorption at longer wavelengths and changes in color. The Epirodin A and Epirodin B $_{mix}$ pigments do seem to be different from those compounds that are exceptions to the rule by the long wavelength at which they absorb. Most of these compounds that are exceptions have maxima below 400 nm. The latter type compounds in which these functional groups are in conjugation may be similar to the Epirodins. The chromophore of the Epirodin A and Epirodin B $_{mix}$ pigments might also be explained by a conjugated -C=C- system with an auxochrome present which by itself does not possess any color.

The extinction coefficients of a 1 per cent solution of Epirodin A and a 1 per cent solution of Epirodin B $_{mix}$ are shown in Table 13. There is only a slight difference between the extinction coefficients of the pigments. This slight difference in the two pigments might be the result of slightly different substitutions at positions in the Epirodin B $_{mix}$ molecule that only weakly effect the chromophore. The effect seems to be one of increasing the intensity of the absorption maximum.

The stability of Epirodin A in a basic solution as measured by the change in absorption at 429 nm is shown

Table 13

Extinction coefficients of Epirodin A and Epirodin B $_{mix}$.

Pigment	Extinction Coefficients*		
Epirodin A	log ε	2.119	(429 nm)
Epirodin B $_{mix}$	log ε	2.121	(429 nm)

* 1 percent solutions in ethanol.

in Figure 22 and Table 14. In the study of the stability of the pigments in acid and base some interesting results were obtained. In the presence of base at pH 12 the absorbance declined very slowly at 25° C after the initial decrease in absorbance shown in Table 14. However, when the temperature was raised to 110° C at the same pH, Epirodin A was considerably more unstable as shown in Figure 23 and Table 15. In the experiment using the 110° C treatment, the yellow color of the sample disappeared completely with only a faint tan color being present at the end of the timed period. In the case of the 25° C treated sample of Epirodin A the color was observed to persist for several weeks more, but the intensity decreased. This data suggests that the chromophore is reasonably stable in a basic solution at low temperatures but unstable at higher temperatures. The color at the end of the timed period in the 110° C experiment did not return to yellow when the pH was lowered to 5.8. This indicates that the chromophore is irreversible destroyed by the treatment at pH 12 and 110° C.

In acidic solution pH 2 there was an immediate change in color of Epirodin A from a yellow to a tan color. The absorbance was reduced over 50 per cent by lowering the pH (Figure 24 and Table 16). A C=C conjugated system alone would not be expected to behave in the manner exibited by Epirodin A in such mildly acidic conditions. Usually in the case of the conjugated C=C alone there

Table 14

Decrease in absorbance of Epirodin A in distilled water at pH 12 with time at 25° C.

Time (hours)	Absorbance
0.1	.682
0.0	.641
1.5	.605
2.5	.598
4.5	.598
8.5	.567
22.5	.540
29.5	.498
32.5	.498
46.5	.498
56.5	.485
372.0	.302

(10 ug/ml solution of Epirodin A at pH 12).

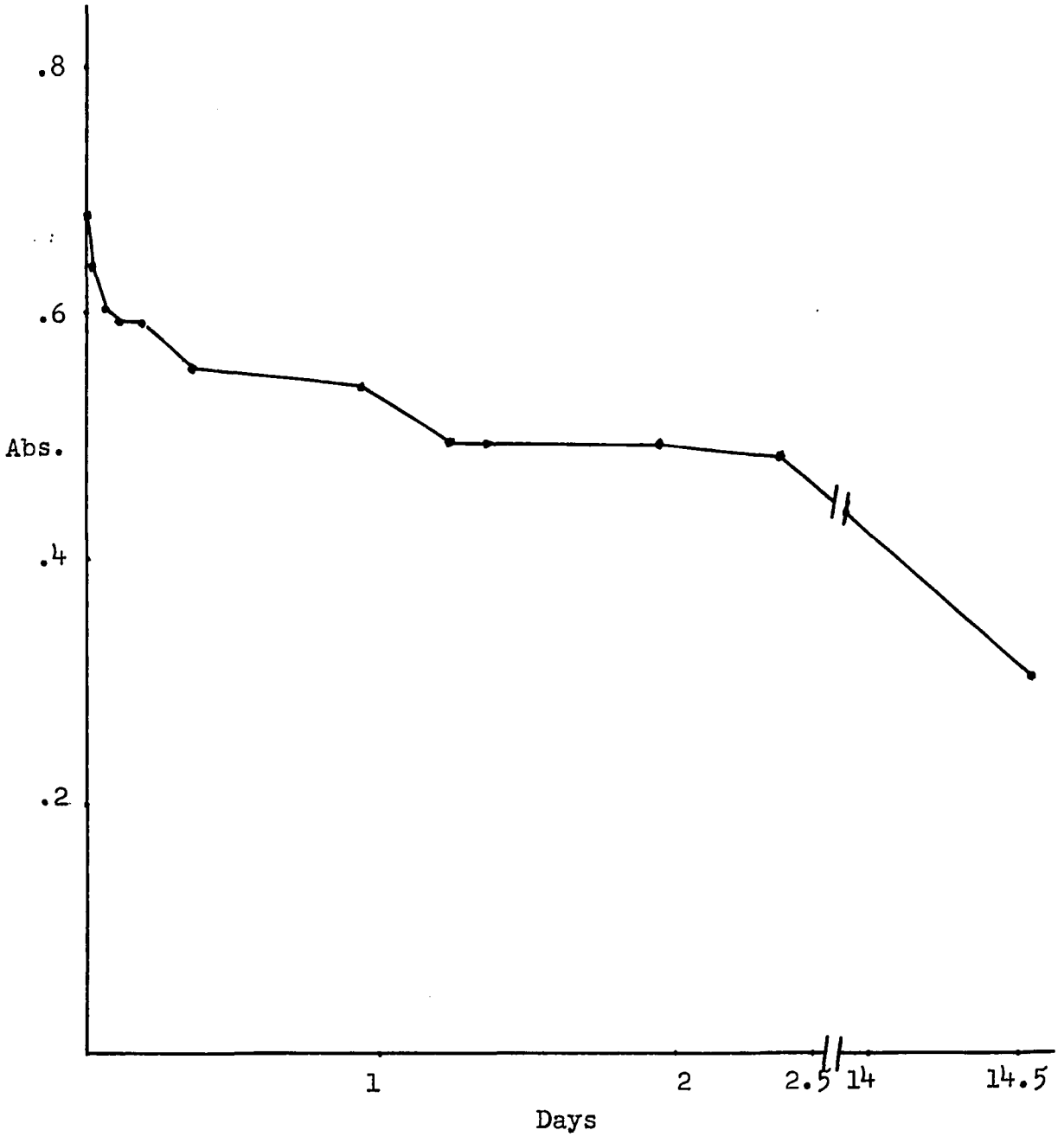


Figure 22

Stability of Epirodin A in basic solution at 25° C.

Table 15

Decrease in absorbance of Epirodin A in distilled water at pH 12 with time at 110 C.

Time (hours)	Absorbance
0.0	.541
5.0	.308
8.0	.248
22.0	.111
32.0	.098

(10 ug/ml solution of Epirodin A incubated at 25° C for 22.5 hours before placing in 110° C oven).

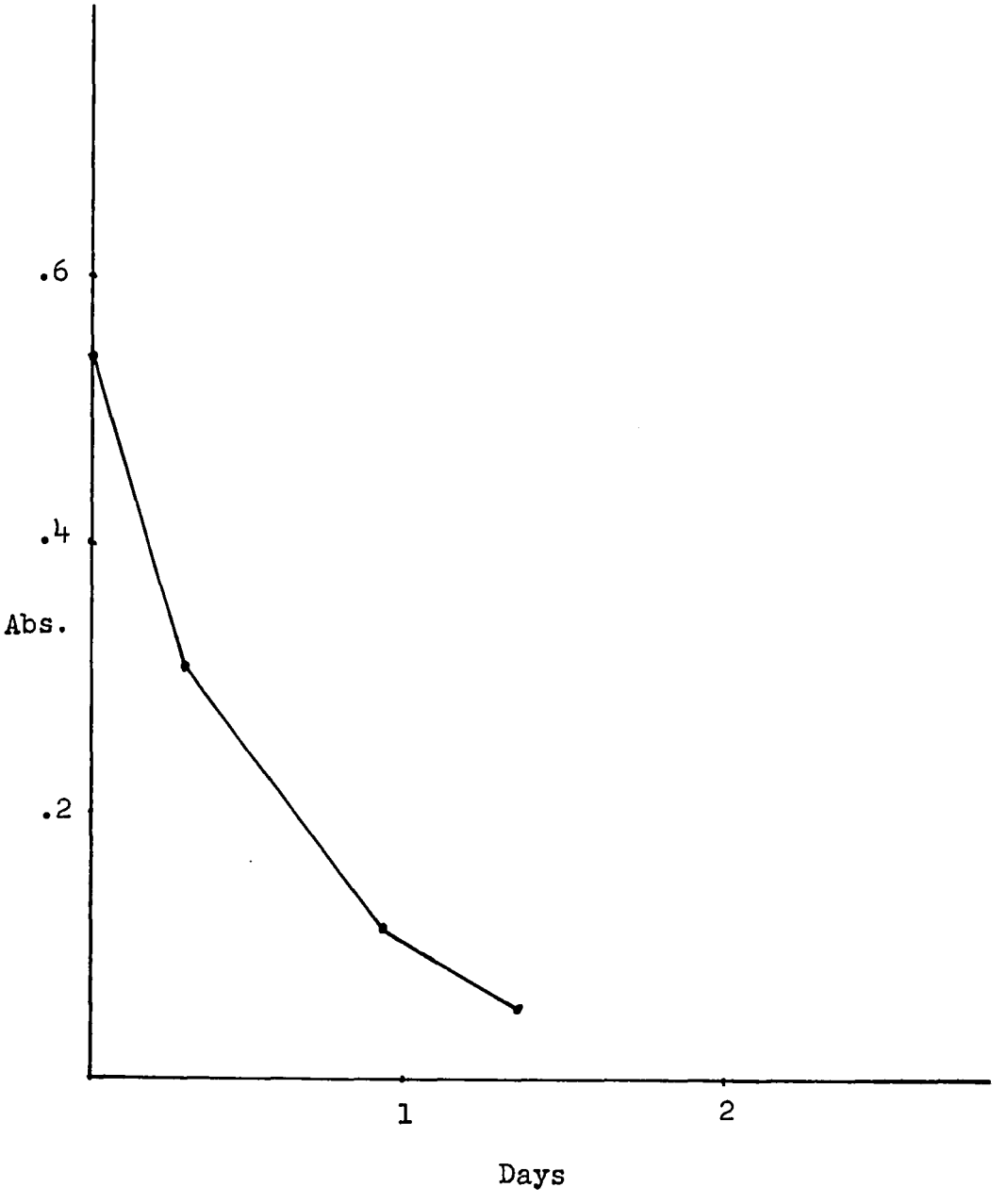


Figure 23

Stability of Epirodin A in basic solution at 110° C.

Table 16

Decrease in absorbance of Epirodin A in distilled water at pH 2 with time at 25° C.

Time (hours)	Absorbance
0.1	.682
0.0	.243
1.5	.206
2.5	.184
4.5	.173
8.5	.129
22.5	.091
29.5	.073
32.5	.072
46.5	.056
56.5	.047
372.0	.024

(10 ug/ml solution of Epirodin A at pH 2).

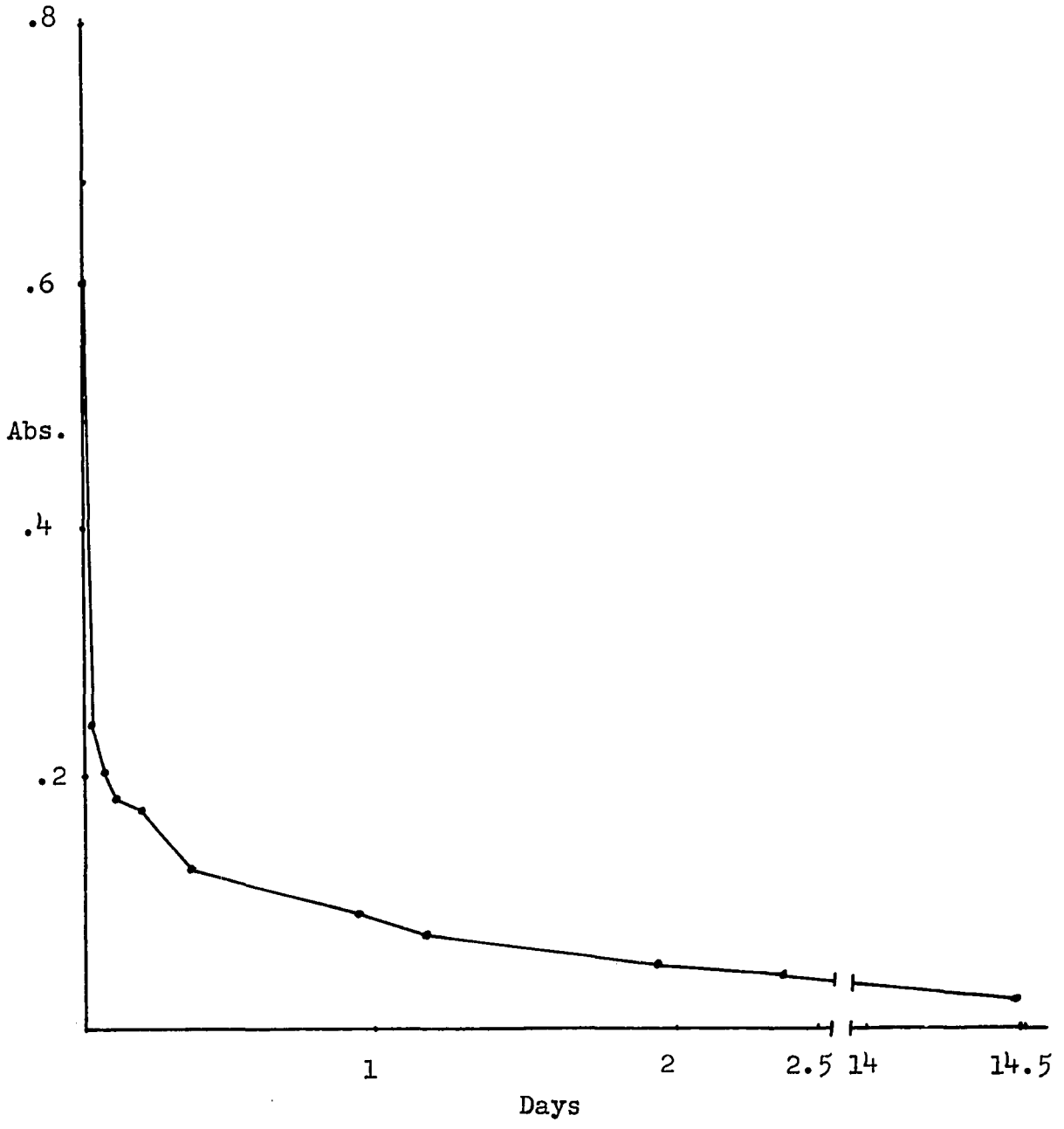


Figure 24

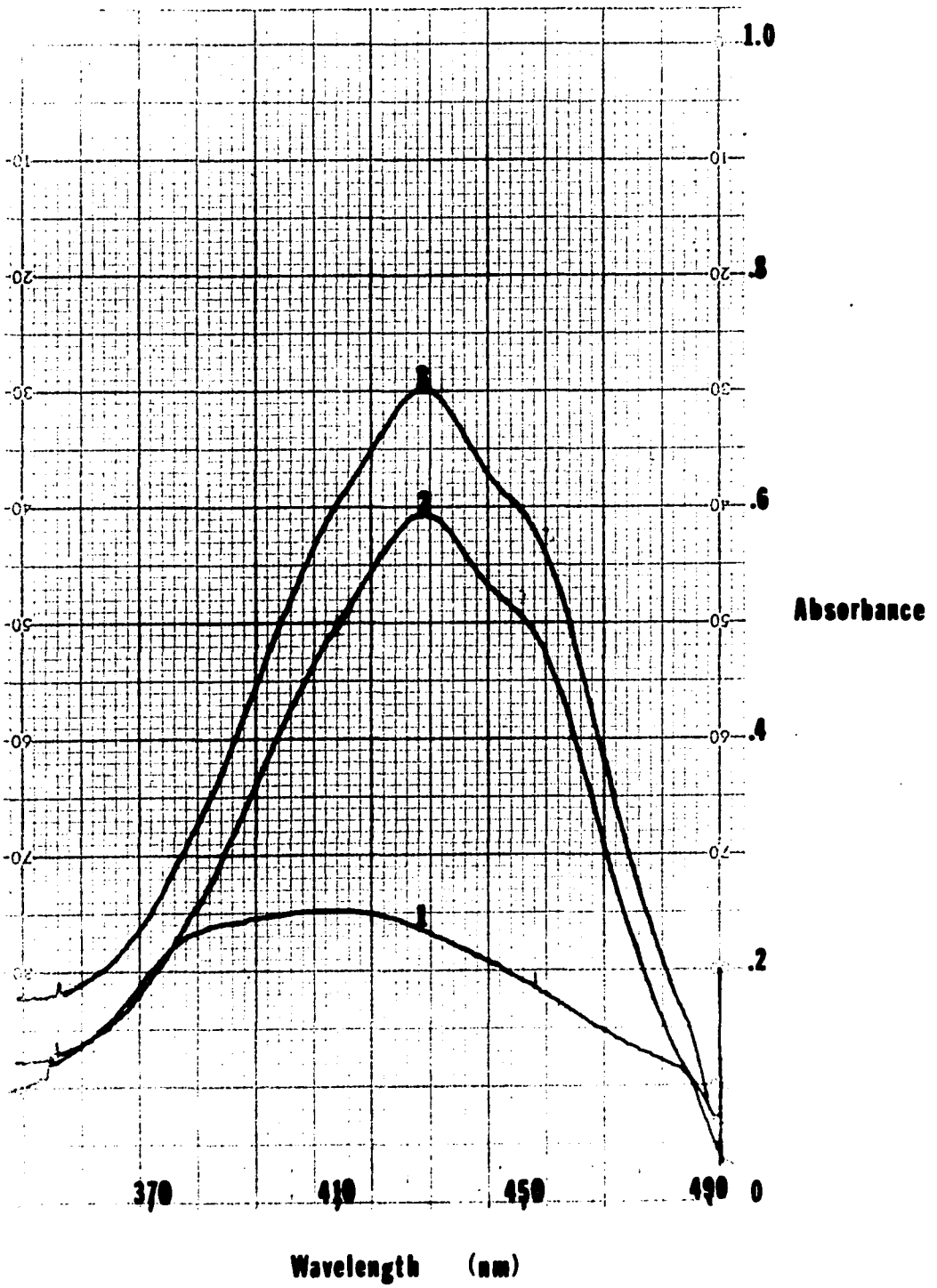
Stability of Epirodin A in acidic solution at 25° C.

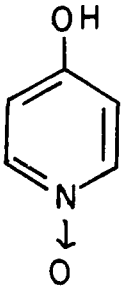
might be a shift in the absorption maximum but not such a drastic lowering of the absorbance at that maximum.

The recorded spectra of Epirodin A in acidic and basic solutions, and distilled water are shown in Figure 25. As can be seen in the figure there is very little difference in the spectra of the sample in distilled water and the sample in basic solution. There is, however, considerable difference in the spectrum of the pH 2 sample which suggests that the chromophore has an auxochrome present that is altered when in acidic solution. There seem to be two maxima in the spectrum of the acidic solution occurring at 429 nm and 401 nm. Usually when an acidic or basic center is present and is either a part of the chromophore or directly attached to it, occurrence of acid-base reactions can be expected to change the appearance of the recorded spectrum because of the different species present in these solutions. The different species present in the acidic, neutral and basic solutions will usually absorb differently. This seems to be the case in Epirodin A with the spectrum being considerably different under acidic conditions. There does not seem to be any drastic difference between the basic and neutral solutions, however. An example of pH effects is illustrated by 4-hydroxypyridine-1-oxide (Figure 26). In acid it absorbs at approximately 240 nm, in neutral solutions around 260 nm and in base around 272 nm. A similar type transition

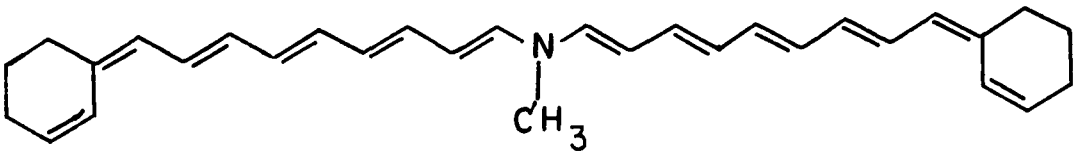
Figure 25

Recorded visible spectrum of Epirodin A in acidic solution (1), basic solution (2) and distilled water (3).


1.0
.8
.6
.4
.2
0
Absorbance
370
410
450
490
Wavelength (nm)



4-Hydroxypyridine-1-oxide



Indicator Yellow

Figure 26

Structures of 4-hydroxypyridine-1-oxide and indicator yellow.

occurs in indicator yellow which is also shown in Figure 26. In acid this compound absorbs at 440 nm and in base at 365 nm.

From the results of the study with the visible spectra of Epirodin A in the ethanolic, acidic, neutral and basic solutions, it was concluded that the pigment might possess some type of chromophore involving possibly a -C=C- system in conjugation. The study also suggested the possibility of an acid labile auxochrome being present, either being part of or directly attached to the -C=C- chromophore. The ultraviolet and visible spectra suggests the existence of some type of -C=C- chromophore which had been suspected because of the color of Epirodin A.

The similarity between Epirodin A and Epirodin B $_{mix}$ has already been discussed in the recorded visible and ultraviolet spectral data reported above. Epirodin B $_{mix}$ also seems to behave similarly to Epirodin A in both acidic and basic solutions. Epirodin B $_{mix}$ changed from a yellow to a tan color on acidifying to pH 2 and on raising the pH to 12 the color remained yellow but there was the slight decrease in absorbance at 429 nm. Although the Epirodin B $_{mix}$ pigments were not examined over a long period in either acid or base, the initial reactions were the same in those solutions (acidic, neutral, and basic) as in the solutions of Epirodin A. These results also seem to support the fact that these

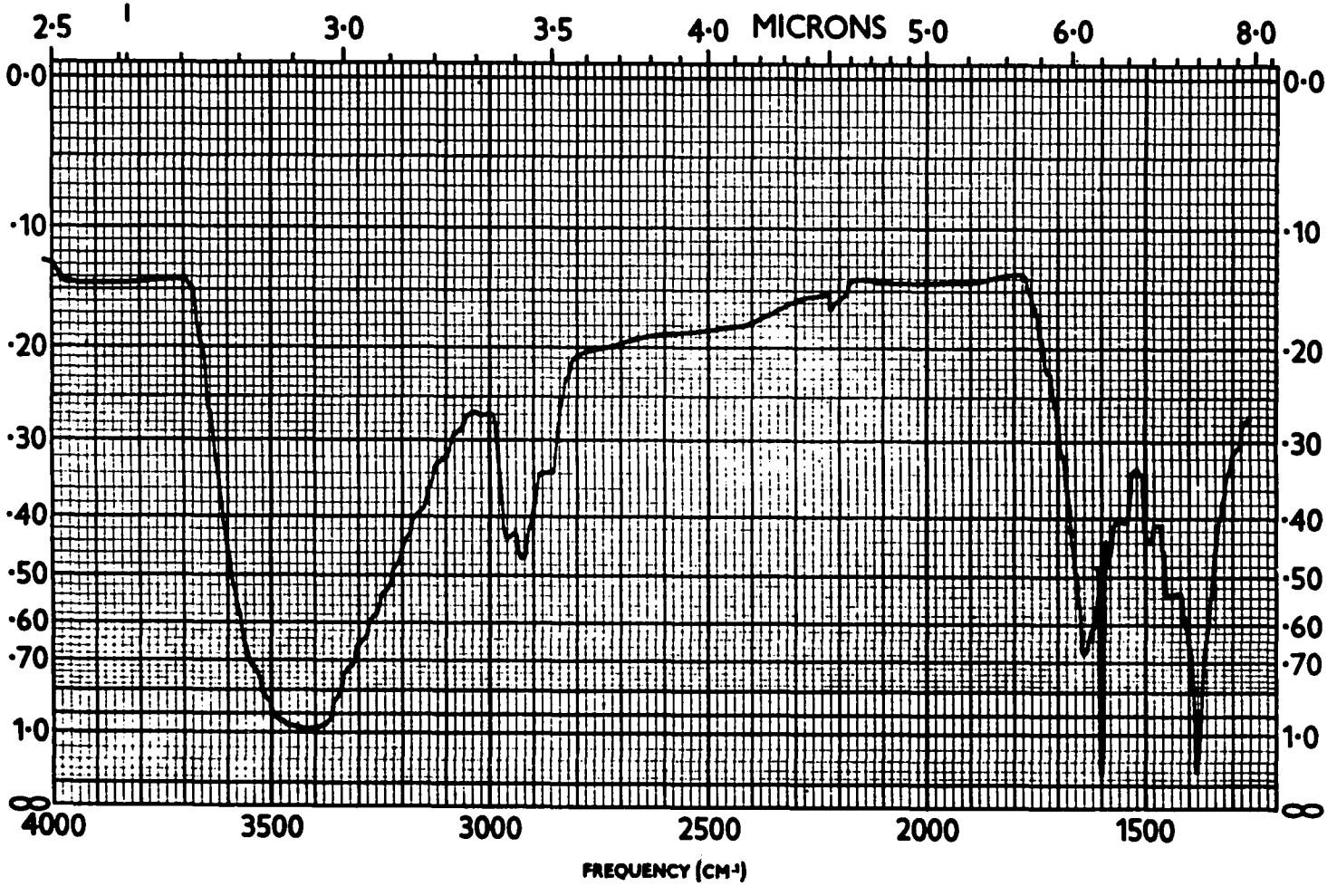
two pigments have basically the same chromophore and that the substitution of functional groups on the chromophore system responsible for the visible spectra was similar in both Epirodin A and Epirodin B $_{mix}$.

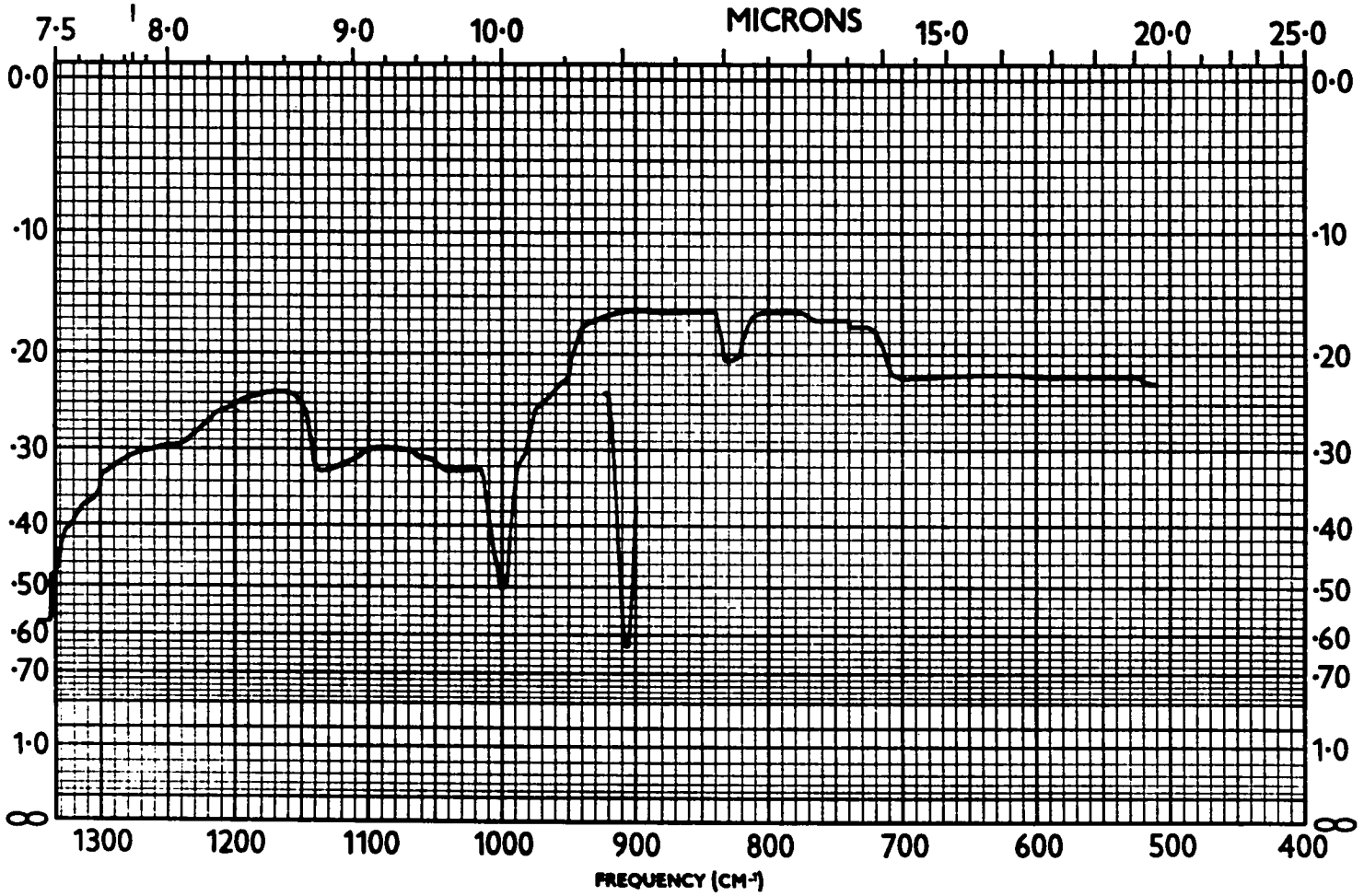
The infrared spectrum of Epirodin A is shown in Figures 27 and 28. The infrared spectrum of Epirodin B $_{mix}$ is shown in Figures 29 and 30, and Table 17 shows a comparison between the wavelength of the absorption bands in the spectrum of Epirodin A and the wavelengths of the absorptions in the spectrum of Epirodin B $_{mix}$. The infrared spectra of Epirodin A and Epirodin B $_{mix}$ are similar. The spectrum of Epirodin A shows a broad absorption band between 3390-3500 cm^{-1} with the peak occurring at 3420 cm^{-1}. In this region the O-H and N-H stretching vibrations occur. Epirodin B $_{mix}$ also shows an absorption peak in this region occurring between 3490-3220 cm^{-1} with the peak at 3360 cm^{-1}. In the infrared spectrum of Epirodin B $_{mix}$ the absorption in the O-H and N-H stretching region is much broader than the absorption peak in the corresponding region of the Epirodin A spectrum. There seems to be an additional absorption band present in this region of the Epirodin B $_{mix}$ spectrum.

The very weak absorption blip around 3010 cm^{-1} in the spectrum of Epirodin A seems to indicate the presence of aromatic protons being present in the pigment. The absorption around 3010 cm^{-1} in the spectrum of Epirodin

Figures 27 and 28

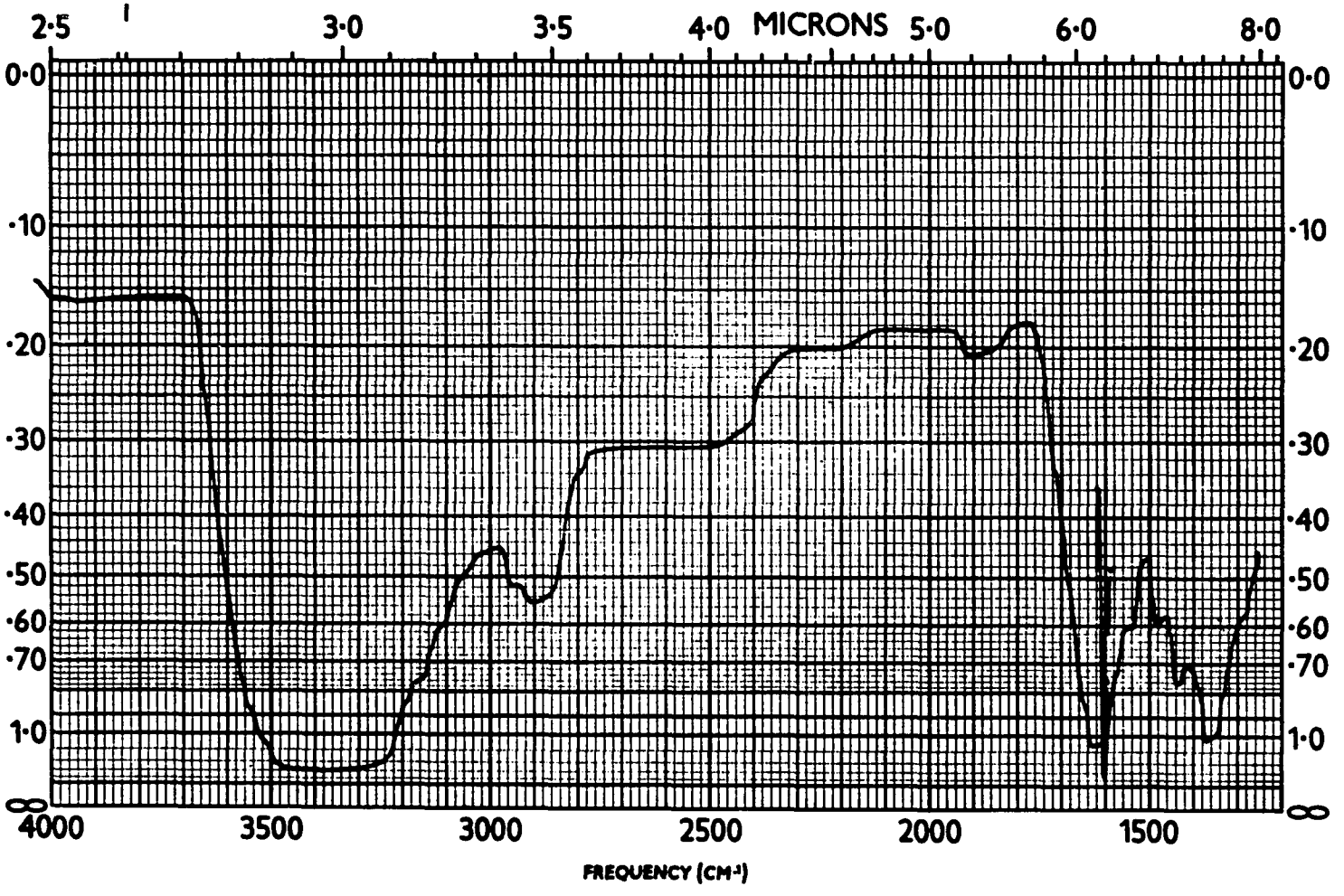
Infrared spectrum of Epirodin A.


2·5
3·0
3·5
4·0
MICRONS
5·0
6·0
8·0
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
∞
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)


MICRONS
7·5
8·0
9·0
10·0
15·0
20·0
25·0
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
∞
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

Figures 29 and 30

Infrared spectrum of Epirodin B mix.


2·5
3·0
3·5
4·0
MICRONS
5·0
6·0
8·0
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
∞
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)

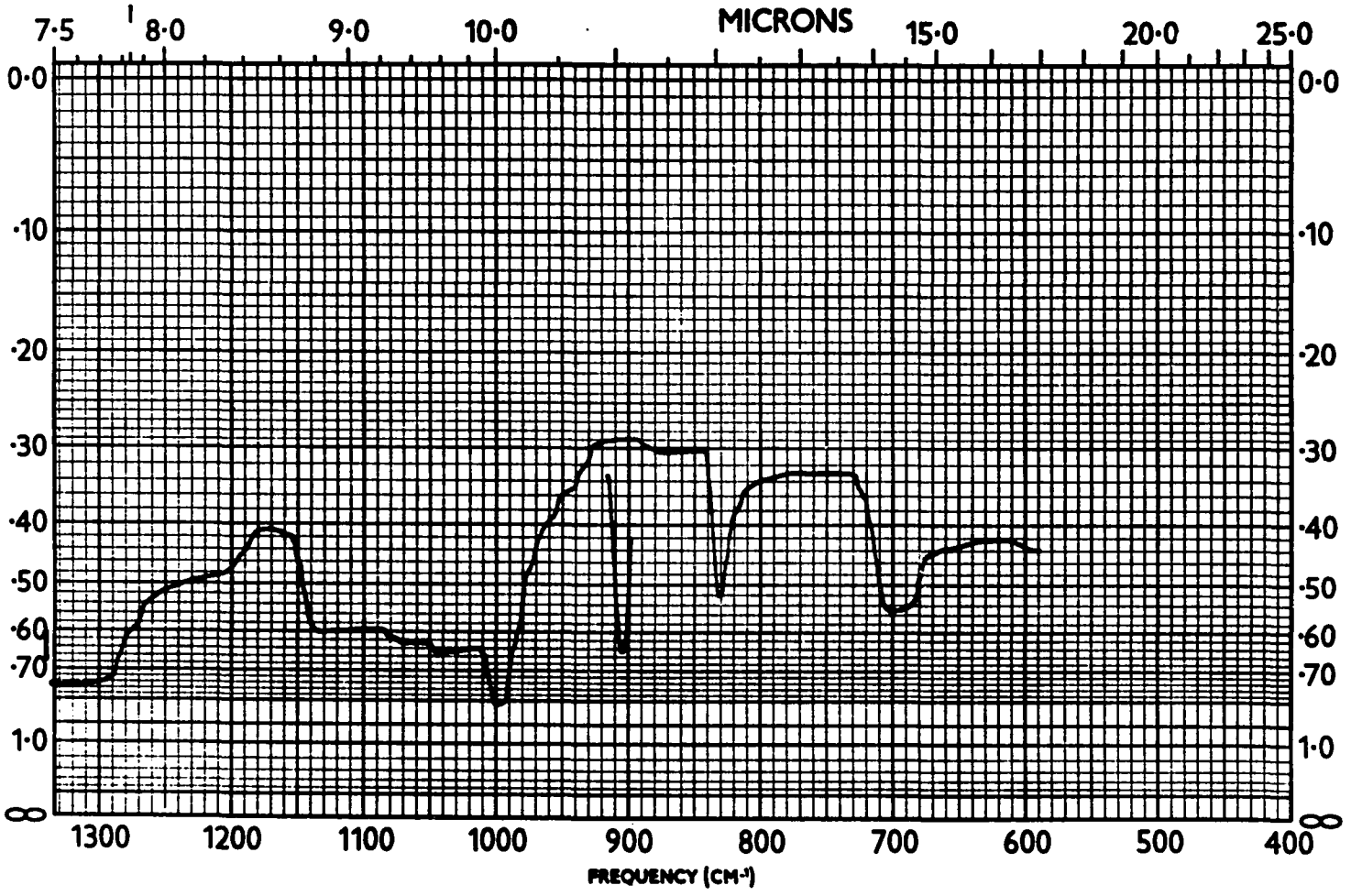

MICRONS
7·5
8·0
9·0
10·0
15·0
20·0
25·0
0·0
·10
·20
·30
·40
·50
·60
·70
1·0
∞
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

Table 17

Absorption wavelengths in the infrared spectra of Epirodin A and Epirodin B mix.

Epirodin A (wavelength in cm^{-1})	Epirodin B mix (wavelength in cm^{-1})
3420	3360
2960	2950
2923	2900
2870	
	1900
1640	1620
1540	1540
1490	1490
1440	1440
1390	1370
	1330-1300
1135	1140-1080
1080-1020	1080-1020
999	999
830	830
	700

B $_{mix}$ does not have the blip present in the spectrum of Epirodin A.

In the spectrum of Epirodin A the aliphatic protons were resolved in two distinct peaks at 2960 cm^{-1} and 2923 cm^{-1}, and a shoulder peak at 2870 cm^{-1}. In the spectrum of Epirodin B $_{mix}$ a shoulder peak occurs at 2950 cm^{-1} and broad peak at 2900 cm^{-1} which extends over to 2880 cm^{-1}.

A weak absorption band occurs at 1900 cm^{-1} in the spectrum of Epirodin B $_{mix}$. This region is another region where weak absorption bands due to aromatic protons show up. The spectrum of Epirodin A does not seem to have this weak band present. The absorption peak in this region is the first indication in the infrared spectrum that an aromatic system is present in Epirodin B $_{mix}$. The possible presence of an aromatic system in Epirodin A was indicated by the blip at 3010 cm^{-1}. The aromatic center lends support to the idea that some type of conjugated system is present in the pigments.

The infrared band at 1640 cm^{-1} in the spectrum of Epirodin A is in the region where absorptions due to conjugated C=O stretching and N-H bending vibrations occur. The intensity of the absorption band at 1640 cm^{-1} is moderate. If the band is the result of a carbonyl stretching then the band is not as intense as the usual carbonyl absorption. A carbonyl stretching band at

1640 cm^{-1} would require a conjugated carbonyl as one possibility for the lowering of the absorption below 1700 cm^{-1}. Another possibility for the lower frequency is that the carbonyl might be that of an amide. The carbonyls of amides absorb below 1700 cm^{-1} but most do not absorb below 1650 cm^{-1}.

The spectrum of Epirodin B $_{mix}$ also shows an absorption band in this region but it occurs at 1620 cm^{-1} with what seems to be a shoulder at 1640 cm^{-1}. The band at 1620 cm^{-1} is more intense in the Epirodin B $_{mix}$ spectrum than the 1640 cm^{-1} band in the spectrum of Epirodin A. The band at 1620 cm^{-1} could also be due to a conjugated carbonyl or the carbonyl of an amide. These bands could also be the result of N-H bending vibrations. Other possibilities are the absorptions in this region due to C=N and C=C vibrations.

Epirodin A and Epirodin B $_{mix}$ show absorption bands at 1540 cm^{-1}, 1490 cm^{-1} and 1440 cm^{-1}. These peaks at 1540 cm^{-1} and 1490 cm^{-1} might be the result of C=C vibrations also. The absorption bands due to N-H bending vibrations also extend into this region.

A sharp peak at 1390 cm^{-1} is present in the spectrum of Epirodin A might be the result of a C=C vibration or the C-H vibration of a methyl or methylene group. The spectrum of Epirodin B $_{mix}$ shows a broader band at 1370 cm^{-1}. These two absorption bands occur in what is known as the finger print region of the infrared spectrum.[19,20]

The region is especially useful in distinguishing between similar compounds. Even though similar compounds may show identical spectra in the region 4000-1430 cm^{-1}, there will always be discernable differences in the finger print region. There is enough difference in the 4000-1430 cm^{-1} region of the spectra of Epirodin A and Epirodin B $_{mix}$ to show that they are different compounds. The finger print region only reinforces the differences in the two pigments.

The spectrum of Epirodin A shows a weak absorption band at 1135 cm^{-1}. The absorption in the spectrum of Epirodin B $_{mix}$ is a little different, however. There does not seem to be a clear peak but rather a leveling off of the absorbance at around .60 in the spectrum of Epirodin B $_{mix}$.

The next clearly discernable absorption band occurs at 999 cm^{-1}. There seems to be a broad absorption band between 1080-1020 cm^{-1} particularly noticeable in the spectrum of Epirodin B $_{mix}$ and to a lesser extent in the spectrum of Epirodin A. The broad 1080-1020 cm^{-1} absorbance relative to the band absorbance at 999 cm^{-1} can be seen to be more intense in the spectrum of Epirodin B $_{mix}$ than in the spectrum of Epirodin A.

The finger print region contains many of the absorption bands caused by bending vibrations. It also contains the C-C, C-O, and C-N stretching vibrations. Thus this region of the spectrum usually complex, with many absorption bands and shoulders appearing.

A weak peak occurs in the spectrum of Epirodin A at 830 cm^{-1}. This peak also occurs in the spectrum of Epirodin B $_{mix}$ but seems to be a stronger absorption than the band in the spectrum of Epirodin A. There appears to be absorbance at 700 cm^{-1} in the Epirodin A spectrum, but a distinct peak does not seem to be present. These two bands occur in the region of the spectrum where absorptions due to the C-H out of plane bending vibrations of unsaturated systems occur. The non-aromatic unsaturated systems show intense peaks due to bending vibrations in the range of 1000-800 cm^{-1}. As can be seen in the spectra of Epirodin A and Epirodin B $_{mix}$ there seem to be two weak bands in this region but only one falling in the range mentioned. The aromatic out of plane bending vibrations usually give rise to peaks below 900 cm^{-1}. These two bands observed occur in the aromatic region. There are other bending and stretching vibrations which occur between 830-600 cm^{-1}, but if these two absorptions at 830 cm^{-1} and 700 cm^{-1} are the result of the aromatic protons then this would provide more evidence for a conjugated chromophore being present in Epirodin A and Epirodin B $_{mix}$

The elemental analysis data in Table 18 shows the relative percentages of carbon, hydrogen and nitrogen. From previous mass spectral work it was not certain whether the remaining percentage was oxygen or oxygen plus chlorine. Several high resolution mass spectra were obtained on some samples isolated earlier in the

Table 18

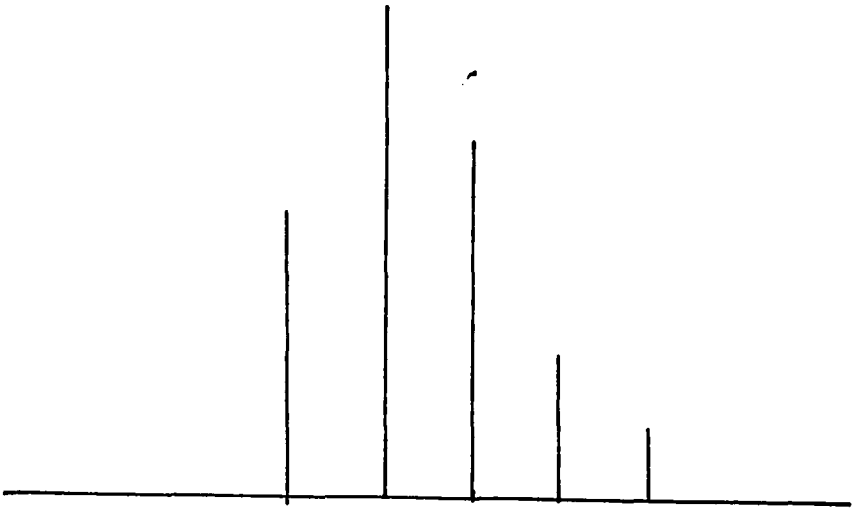
Elemental analysis data on Epirodin A and Epirodin B $_{mix}$.

Pigment	Percentage		
	Carbon	Hydrogen	Nitrogen
Epirodin A	40.40	4.85	1.62
Epirodin B $_{mix}$	44.13	5.49	0.75

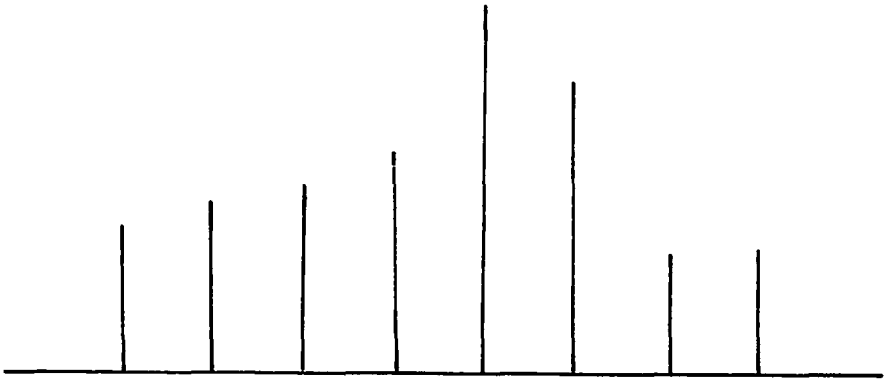
research when autoclaving and acid-base treatment were employed in the isolation of these pigments from E. nigrum. One pigment isolated indicated the presence of several halogen atoms being present. The isotope cluster observed seemed to be more than the cluster that results from 5 chlorine atoms (illustrated in Figure 31). Epirodin A did not give a precipitate with $AgNO_3$ which indicated that either a halogen was not present or that the halogen was bound to an aromatic system. Thus only the percentages of carbon, hydrogen and nitrogen were obtained.

The molecular weight of Epirodin A was determined roughly by thin layer gel filtration and found to be 750 ± 100 (Figure 32 and Table 19). An attempt to get the molecular weight by mass spectroscopy proved to be futile. Epirodin A was not volatile enough to go up into the inlet valve even when heated to 250° C. The pigment probably decomposed at the high temperature of 350° C at which temperature some low mass ions did show up on the spectrum.

Early in the research, as previously mentioned in the discussion of the elemental analysis data, mass spectra were obtained on several pigments. The lower molecular weight pigment had a molecular weight of 356 while the highest molecular weight pigment had a molecular weight of 602. Another mass spectrum taken



5 Chlorine Atoms[22]



Mass 602 Pigment

Figure 31

Isotope cluster in 602 molecular weight pigment

Table 19

Results of thin layer gel filtration on *Epirodin A*

Compound	$1/R_{cytochrome\ c}$	
Flavine mononucleotide	1.51	3.63 (trimer)
Vitamin B_{12}	1.74	
Epirodin A	2.66	

(1/R = 1/migration distance of each compound divided by 1/migration distance of the reference compound cytochrome c).

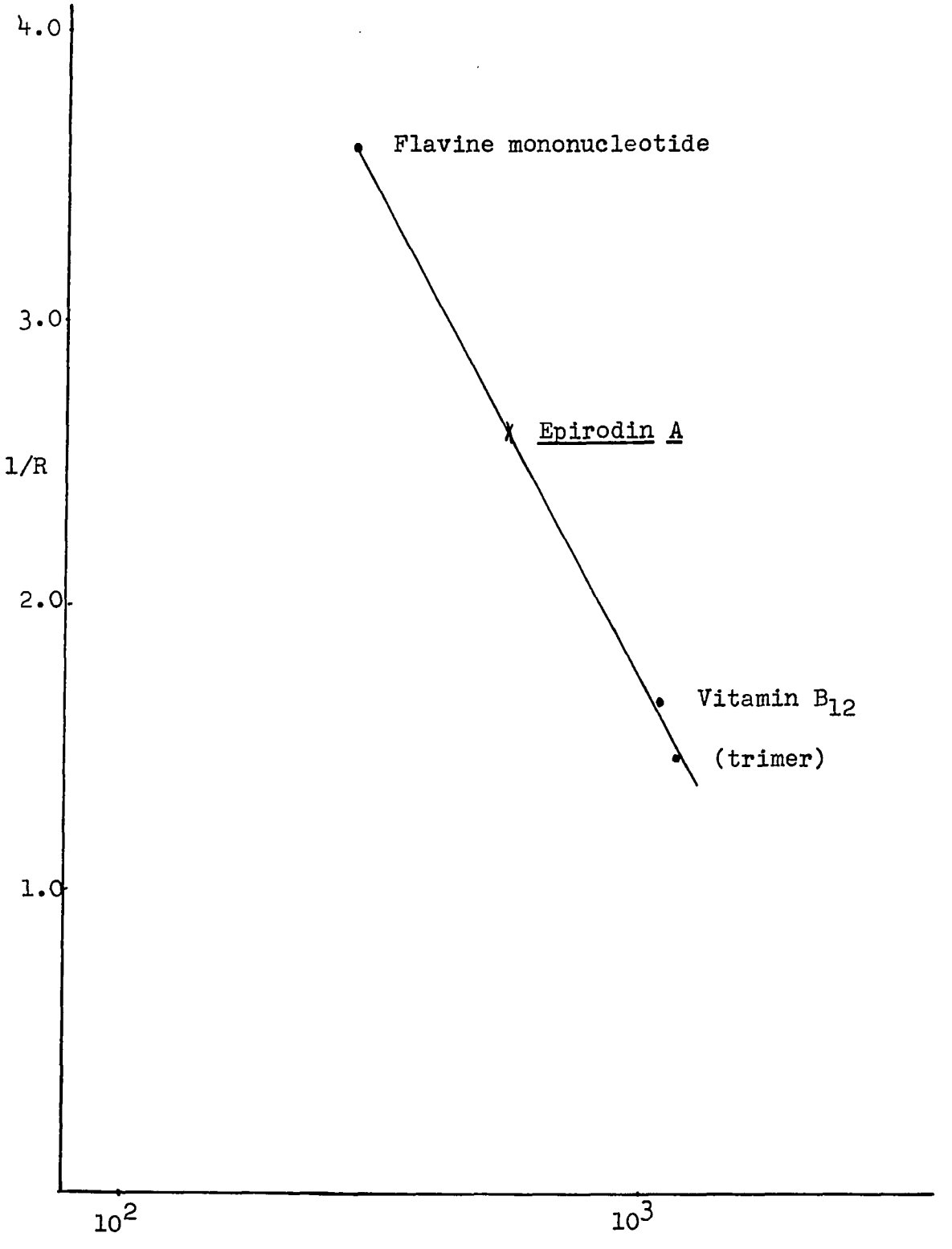


Figure 32

Molecular weight of Epirodin A by gel filtration.

prior to these two spectra had indicated the possible presence of two compounds, one with a molecular weight of 429 and the other of 385. These latter two pigments were the first pigments isolated and the R_f on Eastman TLC plates were .64 and .58 using the acetone-methanol (1:1) solvent system. The pigment with the molecular weight of 602 had an R_f of .15 and the 356 molecular weight pigment had an R_f of .59 in this solvent system using Eastman TLC plates. With the high resolution mass spectra molecular weights with three decimal point accuracy were obtained. The most probable elemental composition was obtained by consulting Beynon's Table of Mass Abundance.[21] The mass 429 pigment corresponded to the formula $C_{23}H_{17}N_4O_5$, the mass 385 pigment to a formula $C_{21}H_{13}N_4O_4$, and the mass 356 pigment corresponded to a formula of $C_{13}H_7NO_9Cl$. No formula was obtained for the mass 602 pigment. Another pigment submitted along with the mass 602 pigment failed to go up into the mass spectrometer at a temperature of 250° C as Epirodin A. It was suspected to be of higher molecular weight than the mass 602 pigment because of its migration pattern on Eastman TLC plates.

The fact that Epirodin A and Epirodin B_{mix} decomposed at 190-195° C when a melting point was attempted on these pigments probably explains why Epirodin A did not give a suitable mass spectrum above 200° C. Evidently at temperatures below 190° C, Epirodin A is not volatile enough to give a mass spectrum.

The molecular formulas that were obtained on the earlier pigments did not seem to be the formulas for the parent compounds. In the case of the formula $C_{13}H_7NO_9Cl$ there is an odd number of nitrogen atoms but the molecular weight is even. In the case of the $C_{23}H_{17}N_4O_5$ and the $C_{21}H_{13}N_4O_4$ there is an even number of nitrogen atoms but each pigment had an odd numbered molecular weight. If an odd number of nitrogens is present then the molecular ion should occur at an odd mass number and if an even number of nitrogens are present then the molecular ion should be at an even molecular weight.[22] The fact that the molecular ions of the pigments mentioned are exact opposites of what the nitrogen rule requires suggest that these ions and the formulas are probably not those of the parent compounds. A possible explanation at the time these spectra were taken was that these peaks could be p+1 or p-1 peaks of the parent ion. But now that the Epirodin A pigment has been isolated by much milder conditions, by elimination of the heat and the acid-base treatment used in the isolation of the earlier pigments, it seems that these formulas probably belong to break down products of either Epirodin A or Epirodin B $_{mix}$. Epirodin A has been shown by TLC to be different from the other pigments whose molecular formulas have been presented. From the migration pattern of these pigments on Eastman TLC plates, it seemed that Epirodin A was a larger molecule than the

three small pigments with molecular weights of 356, 385 and 429. This is also confirmed by the molecular weight reported in the thin layer gel filtration data. All the pigments reported here inhibited the growth of B. megaterium spores. Table 20 gives the activity of Epirodin A. The fact that all of these compounds are active would also suggest that they are related.

The most probable formula corresponding to the molecular weights did provide information on the extent of unsaturation in these compounds and possibly in the parent compounds. From the results it seems that these pigments are very unsaturated. The formula used in the calculation of rings plus double bonds is shown in Table 21. Using the formula, $C_{13}H_7NO_9Cl$ corresponds to a total of 10 rings plus double bonds, $C_{21}H_{13}N_4O_4$ corresponds to 17 rings plus double bonds, and $C_{23}H_{17}N_4O_5$ corresponds to 17 rings plus double bonds. The mass spectra thus confirms the presence of C=C systems being present in these pigments and possibly in the parent pigments.

Epirodin A can also be shown to be different from the earlier pigments by using other criteria. These pigments had absorption maxima in the visible region at 398-402 nm. The R_f on Eastman TLC plates were in the vicinity of .60 except for the 602 molecular weight pigment which had a R_f of .15 in the acetone-methanol (1:1) solvent system. The R_f of Epirodin A in this solvent system is .23 on Eastman plates. From the results

Table 20

Activity of Epirodin A against B. megaterium and B. subtilis.

Organism	conc. mg/ml		
	0.10	0.05	0.01
B. megaterium	0.82 (9)*	0.48 (6)	neg. (3)
B. subtilis	0.31 (6)	neg. (6)	neg. (9)

* Number of determinations.

Table 21

Formula for determining rings plus double bonds.

$I_y II_n III_z IV_x$	General elemental formula
	I = H, F, Cl, Br, I
	II = O, S
	III = N, P
	IV = C, Si, etc.

Total rings plus double bonds = $x - \frac{1}{2}y + \frac{1}{2}z + 1$

(for an even-electron ion, the true value will be followed by "$\frac{1}{2}$")

presented on the molecular weights of the pigments isolated earlier it can not be ascertained with certainty that they were derived from Epirodin A. There appeared to be a small amount of a yellow pigment that ran slightly above the spot of Epirodin A in the TLC of Column C material but there did not seem to be as much present as in the isolation of the earlier pigments which ran slightly higher on the TLC plate. Further work on this faint yellow material and the Epirodins will have to be done before the origin of the earlier pigments can be determined, however. It is worth noting that in acid solution (pH 2) there does seem to be material generated from Epirodin A that absorbs around 400 nm.

Comparison of the Humic Acid and Flavipin with Epirodin A

The humic acid can easily be differentiated from Epirodin A by the data presented in this thesis. The lack of any significant absorption in the visible and ultraviolet spectrum clearly differentiated the humic acid from Epirodin A. The spectrum of the humic acid showed only minor absorption in the region of 429 nm and what seems to be a shoulder between 270-285 nm. The fact that the humic acid is reddish brown in color is probably due to the minor absorption above 300 nm shown in the recorded spectrum of the humic acid (Figure 8). The molecular weight serves to accentuate the difference in the humic acid and Epirodin A. The fact that Epirodin A does not run with the void volume of a Sephadex G-15 plate limits the molecular weight to below 1500. The molecular weight was actually determined to be 750 ± 100 on a Sephadex G-15 plate, while the molecular weight of humic acid on G-50 plates was determined to be 4,350 ± 350. The infrared spectrum of the humic acid has a broad absorption in the N-H and O-H region as does the spectrum of Epirodin A although that of the latter is not as broad. The 1650 cm^{-1} absorption in the humic acid spectrum (Figures 6 and 7) is more intense than the 1640 cm^{-1} absorption in the spectrum of Epirodin A (Figures 27 and 28). The absorption at 1650 cm^{-1} in the case of the humic acid spectrum is well below 1700 cm^{-1} and suggests that

the carbonyls of the phenols and the amino acids are probably in conjugation with C=C systems or in amide linkage. The elemental analysis data reinforces the difference between the humic acid and Epirodin A, as does the biological activity data. Epirodin A has activity against B. megaterium spores but hardly any activity against Chlorella species while the humic acid has been shown to have no activity against either B. megaterium spores or Chlorella species.

From the results it seems certain that the Epirodin A pigment is different from the humic acid elaborated by E. nigrum. The visible and ultraviolet spectra along with the biological activity data confirm the difference most notably. The elemental analysis data, the molecular weight data, and the infrared spectrum of each pigment provide additional data to support the difference in these two pigments.

Flavipin can also be differentiated from Epirodin A by the data presented in this thesis. Flavipin is a pale yellow pigment while Epirodin A is an orange pigment. The recorded spectra of Epirodin A and flavipin confirm the difference observed in viewing the pigments. Epirodin A has a maximum at 429 nm while flavipin has a maximum at 347 nm. Flavipin decomposes around 228° C and Epirodin A around 192° C. The infrared spectra also show differences in the two pigments. Both have absorptions in the N-H and O-H region of the spectrum. In the spectrum of flavipin

two distinct peaks can be clearly seen between 1650 cm^{-1} and 1615 cm^{-1} while only one peak can be seen in the spectrum of Epirodin A. There are other differences in the spectra and these too serve to illustrate the differences in the two pigments. The difference in the two pigments is also revealed in the elemental analysis data and the biological activity data. Flavipin is considerably more active against Chlorella species than Epirodin A, while Epirodin A is considerably more active against B. megaterium (Table 20) than flavipin. The molecular weight data on flavipin shows that it has a molecular weight of 196 while that on Epirodin A shows that it has a molecular weight of 750 ± 100. All the data presented seems to confirm the difference in these two pigments produced by E. nigrum.

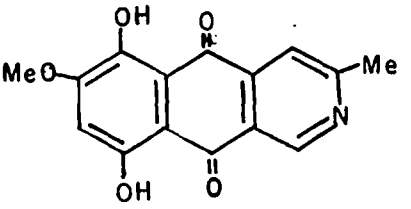
Fusarium Pigments

Having examined the previously described pigments of E. nigrum and finding that none of these resembled the pigment Epirodin A, it was decided that a survey of pigments produced by a related fungus might be helpful in elucidating the possible nature and structure of Epirodin A. The genus Fusarium and the genus Epicoccum are both classified in the same family (Tuberculariaceae), and Fusarium species also produce a number of pigments ranging in color from orange to red.

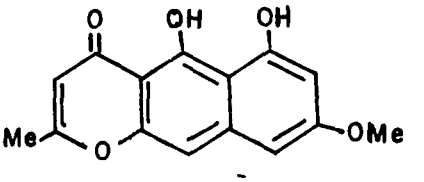
Fusarium graminearum a pathogen of wheat plants has been shown to produce different proportions of aurofusarin and rubrofusarin.[23] The structures of these compounds are shown in Figure 33.

Fusarium bostrycoides produces bostrycoidin, another of the quinone type pigments, the structure of which is shown in Figure 33.[24,25] It crystallizes as red or brown laths. Bostrycoidin is an aza-anthraquinone and takes its place alongside phomazarin in a rather select group of natural products. It is the first known naturally occurring 2-aza-anthraquinone. Bostrycoidin has been shown to have inhibitory action against tubercle bacillus strain H-37.

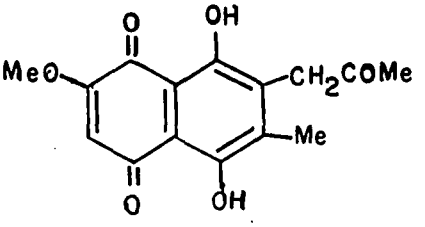
Fusarium javanicum culture solutions have been shown to inhibit the growth of Staphylococcus aureus at dilutions of 1:100 and to be very active against the acid fast



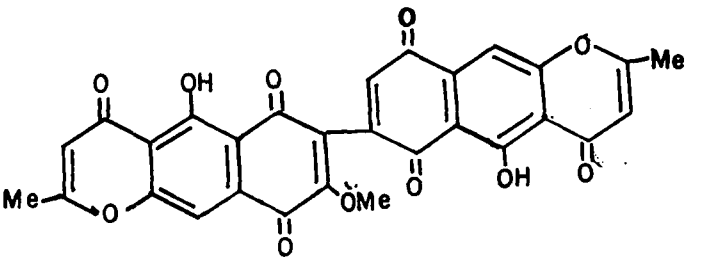
Bostrycoidin



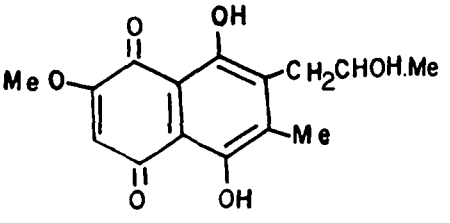
Rubrofusarin



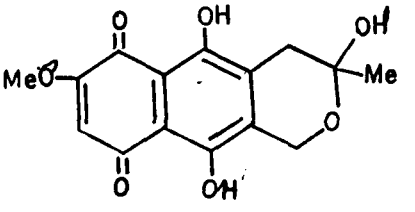
Javanicin



Aurofusarin



Solaniol



Fusarubin

Figure 33

Structures of Fusarium pigments.

Mycobacterium phlei.[26,27] The active principle has been isolated and given the name javanicin (Figure 33).

Fusarium solani has been shown to produce several pigments, which include bostrycoidin, fusarubin, javanicin, and a new metabolite solaniol.[38] The structure of solaniol is shown in Figure 33.

Most of the other pigments isolated from other Fusarium species are derivatives of the ones already mentioned. These compounds are of interest to the author because of their visible spectra and the chromophores which they possess. As can be seen in Table 22 none of these compounds seem to show the same absorption exibited by Epirodin A.

These quinone derivatives produced by the Fusarium species are also phenolic and therefore would be expected to give color reactions with $FeCl_3$ such as exibited by solaniol whose methanolic solutions give a green color when $FeCl_3$ is added.[28] Epirodin A does not react with the $FeCl_3$ reagent and thus the presence of phenolic groups in the pigment are ruled out.

One interesting comment might be made concerning the infrared absorption bands of the carbonyl groups of the quinones. They absorb around 1660 cm^{-1} in the case of non-intramolecular hydrogen bonded and at 1602 cm^{-1} in the case of the intramolecular hydrogen bonded quinones. The fact that the Epirodin A spectrum shows absorption below 1700 cm^{-1} in the C=O region suggests that a

Table 22

Visible maxima and molar absorptivity of several Fusarium pigments.

Pigment	Absorption Wavelength (maxima)
Solaniol	500 nm (log ε 3.97)
Javanicin	512 nm (log ε 3.86)
Fusarubin	505 nm (log ε 3.64)
Aurofusarin	381 nm (log ε 3.99)
Bostrycoidin	488 nm (log ε 4.00)
Rubrofusarin	343 nm (log ε 3.14)

carbonyl might be in conjugation with other double bonds as is the case in the quinone system. A spot test using 2,4 dinitrophenylhydrazine, however, failed to give any positive reaction both when used as a spray reagent on thin layer chromatography plates of Epirodin A and as a drop reagent with Epirodin A in solution. From these results it was concluded that the absorption present in the spectrum of Epirodin A was not due to a quinone type system nor an aldehyde or ketone.

From the recorded visible and ultraviolet spectra, the solubility properties and spot tests for functional groups, it seems unlikely that Epirodin A is related to the pigments produced by the Fusarium species. It seems that although the related Fusarium species are able to produce pigments ranging in color from orange to red, the basic structure of their pigments seems to be derived from a quinone type system, which does not seem to be the case with E. nigrum. Although the Fusarium pigments possess considerable antibacterial activity against Mycobacterium species, the author was not able to come across any reports where the mode of action of these compounds on sensitive organisms was established. It seems now certain that Epirodin A inhibits the DNA - dependent RNA polymerase of E. coli in vitro. Whether this is also the primary site of inhibition in vivo has not been determined.

RNA Polymerase Inhibitors

A survey of the literature did not reveal any data that showed the quinone pigments elaborated by the Fusarium species to have any antiviral or anticancer activity. Species belonging to the genus Streptomyces have been shown to produce compounds that show anticancer activity and these also can be classified as quinone derived compounds. These compounds besides showing anticancer activity possess strong antibacterial activity most notably against Gram positive organisms. Because these compounds possess an unusual macrocyclic ring system, they are referred to as ansamycins.

The naturally occurring rifamycins have been isolated from Streptomyces mediterranei and represent a class of ansamycins.[29] Derivatives of the parent compounds have been shown to be active against Mycobacterium tuberculosis and are being used clinically.[30,31] In general the derivatives on the rifamycins have been found to be more active than the parent compounds. The bacteria are not the only organisms affected by the rifamycins. The blue green alga Anacystis montana was completely blocked by rifamycin B, S and rifampicin (See Figure 34) while the growth of Chlorella pyrenoidosa (green alga) was unaffected.[32]

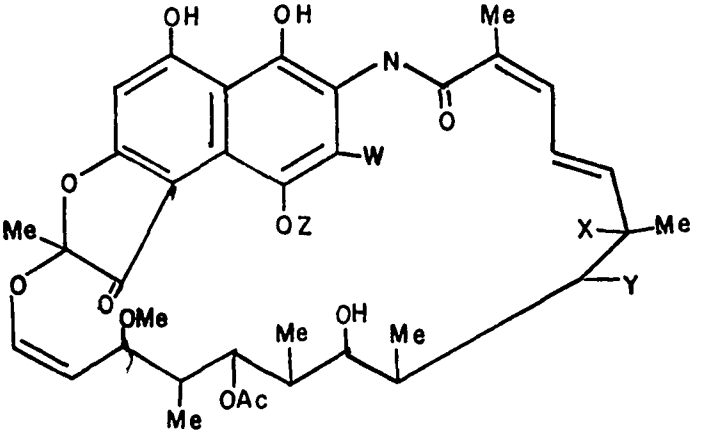
The ansamycins are also antivral agents. Rifampicin inhibits replication of vaccinia poxvirus.[33,34] Other derivatives have been shown to be even more potent than

Table 23

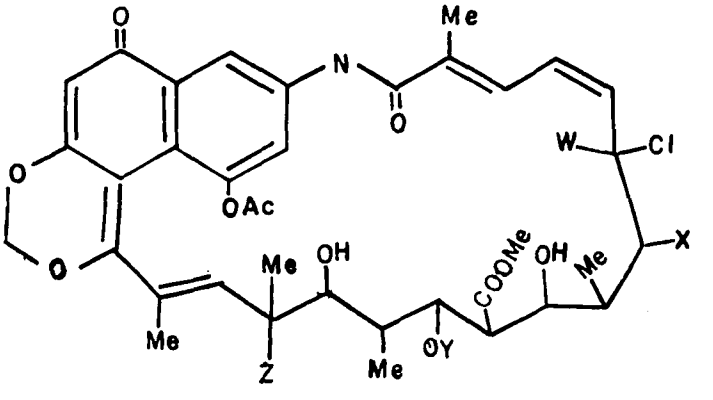
Substitutions on rifamycin and streptovaricin structures in order to obtain a particular rifamycin or streptovaricin.

Rifamycin	W	X	Y	Z
B	H	H	OH	CH_2COOH
Y	H	OH	O=	CH_2COOH
L	H	H	OH	$COCH_2OH$
SV	H	H	OH	H
Rifampicin	CH=N-N	$N-CH_3$	H	OH	H

Streptovaricin	W	X	Y	Z
A	OH	OH	Ac	OH
B	H	OH	Ac	OH
C	H	OH	H	OH
D	H	OH	H	H
E	H	O=	H	OH
G	OH	OH	H	OH



Rifamycins



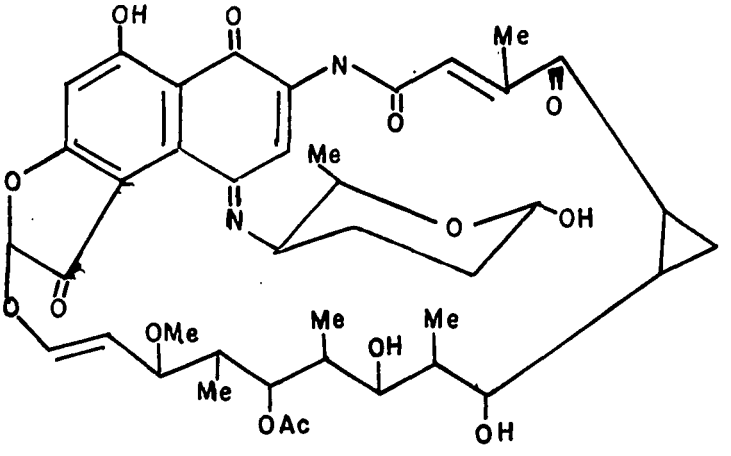
Streptovaricins

Figure 34

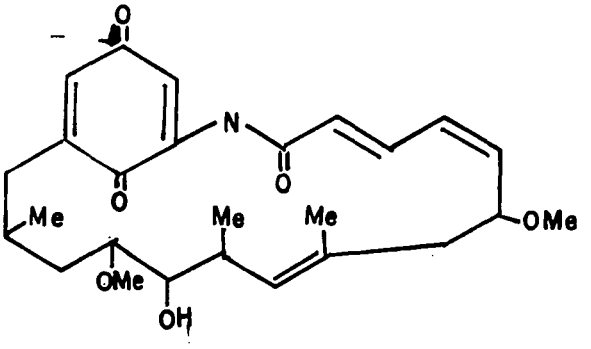
Structures of Rifamycins and Streptovaricins.

rifampicin in inhibiting vaccinia virus, herpes simplex and pseudorabies.[35] The mode of action seems to be different from that ascribed to the antibacterial activity. The mode of action seems to be the inhibition of virus particle formation by the prevention of the conversion of one polypeptide to another.[36,37] The other ansamycins also show antiviral activity but the degree of activity varies.[9,38] The streptovaricins from Streptomyces spectabilis,[39] the tolypomycins from Streptomyces tolypophorus,[40] and geldanamycin from Streptomyces hydroscopicus var. geldanus var. nova[41] possess antibiotic and antiviral activity. Geldanamycin is an interesting variant from the other ansamycins in that it has its principal activity against protozoa rather than bacteria.[41]

Probably the most dramatic activity of the ansamycins is their potential antitumor activity. A number of derivatives of formylrifamycins SV (Figure 34) have been shown to inhibit the RNA-dependent DNA polymerase activity of several RNA tumor viruses.[42] In this system rifampicin was without effect. The streptovaricin complex has been shown to be more active against RNA-dependent DNA polymerase activity of a murine leukemia virus than the purified streptovaricin A or C (Figure 34).[12] It is also reported to be more active than rifamycin SV which in turn is more active than rifampicin. The general structures of the ansamycins are shown in Figures 34 and 35.



Tolypomycin



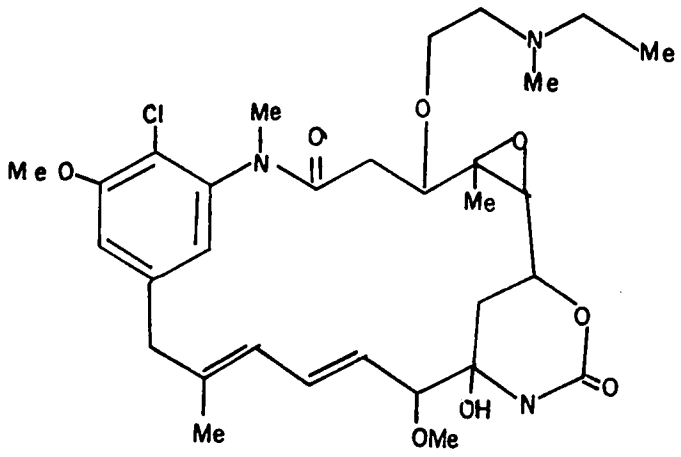
Geldanamycin

Figure 35

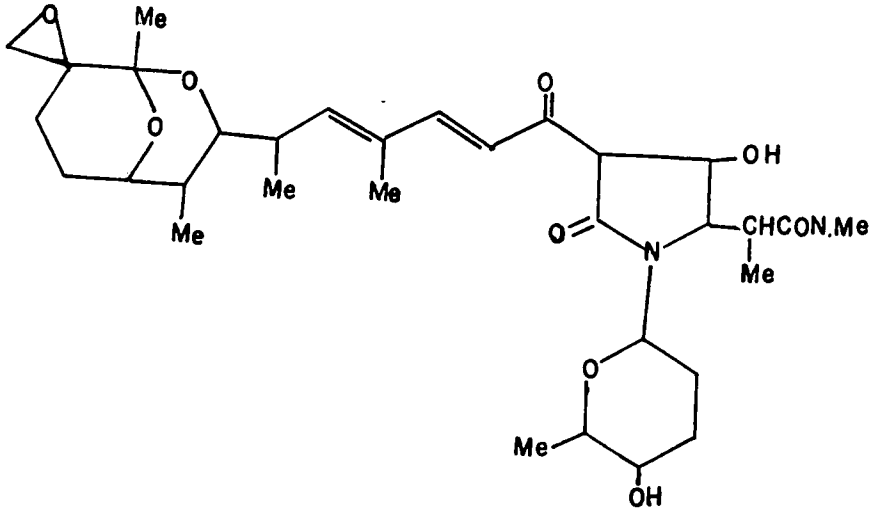
Structures of Geldanamycin and Tolypomycin.

The activity of the ansamycins against bacteria has been shown to be directed toward the DNA-directed RNA polymerase.[9,43,44] In the synthesis of RNA in bacteria there are four steps that can be distinguished: DNA Binding in which the enzyme binds to the DNA template; Chain Initiation in which there is a ternary complex between DNA, RNA polymerase and the nucleoside triphosphate which forms the 5'-terminus of the RNA chain; Chain Elongation in which nucleoside triphosphates are added sequentially to the 3' end of the growing RNA chain; and finally Chain Termination in which the complex dissociates releasing the completed sequence.[45] It has been shown that the initiation step is the one that is inhibited by the rifamycins.[46,47] If the rifamycins are added after chain initiation has begun they have no effect on that round of RNA synthesis, but further synthesis is inhibited. From the data available it seems that the antibiotics inhibit chain initiation by binding to the bacterial RNA polymerase.[10]

More recently an ansamycin derived from a small East African shrub Maytenus ovatus has been shown to have high activity against animal tumors.[48,49] The other naturally occurring antitumor compounds are active at levels of mg/kg while maytansine (Figure 36) is active at levels of ug/kg. Because of the very small quantity of this compound present in the plant it is not certain that the plant actually produces it. The other ansamycins



Maytansine



Streptolydigin

Figure 36

Structures of Maytansine and Streptolydigin.

are derived from micro-organisms, and it is possible that maytansine is also of microbial origin. Maytansine is the first ansamycin shown to contain the carbinolamine epoxide and aryl halide function and appears to be the first member of the series of show significant *in vivo* tumor inhibitory activity. Maytansine has not been shown yet to inhibit RNA polymerase, however.

Streptolydigin (Figure 36) produced by *Streptomyces lydicus*[50,51,52] is not related to the ansamycins in that it is neither a benzoquinone nor napthoquinone derivative. In fact it does not have any aromaticity. It does possess inhibitory activity against bacterial DNA-dependent RNA polymerase, but the mechanism of action seems to be quite different from that reported for the ansamycins. The ansamycins, exemplified by the data reported for the rifamycins, do not inhibit chain elongation once the initiation of RNA synthesis has begun. It has been shown that streptolydigin inhibits not only chain initiation but also chain elongation. It has also been shown that streptolydigin stabilizes the enzyme template complex and that this stabilization may distort the product site, thereby altering the 3'hydroxyl orientation that is critical for the α phosphate of the incoming trinucleotide. Since translocation of the polymerase along the template requires transient dissociation of the enzyme from template, it is proposed that streptolydigin also inhibits this translocation.[53]

Epirodin A seems to behave like streptolydigin in activity against E. coli DNA-dependent RNA polymerase. The pigment inhibits initiation when added at the beginning when the other components (enzyme, template, etc.) are added. It also inhibits chain elongation when added after initiation has already taken place. From these results it seems that Epirodin A behaves like streptolydigin biologically but differs from it spectrally. It seems unlikely that Epirodin A or the other pigments elaborated by the fungus that have similar activity are related to streptolydigin chemically. In the case of the ansamycins their spectral data is similar to the data reported for the quinone pigments produced by the Fusarium species. The biological properties and spectral properties serve to differentiate Epirodin A from the ansamycin type antibiotics.

SUMMARY

The *Epicoccum nigrum* strain used in the research reported in this thesis has been shown to produce both the humic acid and flavipin pigments that had been isolated earlier by other workers. The humic acid was found to have no inhibitory activity against *B. megaterium* and *Chlorella* species. The pigment flavipin was shown to have only very minimal activity against *B. megaterium*, but was shown to have considerable activity against *Chlorella* species. The lack of any significant activity against the bacterium was in keeping with literature data. Flavipin's activity against *Chlorella* species had not been previously reported.

E. nigrum was also shown to produce several other pigments that had biological activity. The pigments were named *Epirodins*. One pigment, *Epirodin A*, was isolated and shown to be chromatographically pure. A mixture of pigments labeled *Epirodin B* $_{mix}$ was also isolated and used to compare with *Epirodin A*. Both *Epirodin A* and *Epirodin B* $_{mix}$ were shown to have inhibitory activity against *B. megaterium*. *Epirodin A* and *Epirodin B* $_{mix}$ had only weak activity against *Chlorella* species. The spectral data, elemental analysis data and visible appearance of the pigments suggest that the pigments are unsaturated compounds. The data presented in this thesis serves to differentiate the *Epirodin* pigments from

the humic acid and flavipin pigments. The biological activity data serves to illustrate this difference in the pigments of E. nigrum most noticeably. The infrared, visible and ultraviolet spectra, elemental analysis and melting point data all lend support to the fact that these pigments are different. The data shows that Epirodin A represents a new class of pigments elaborated by E. nigrum.

The Epirodin A pigment was also compared with pigments from Fusarium species. These Fusarium pigments have been shown to be basically quinone derivatives. Chemical and spectral data tends to differentiate these pigments of Fusarium species from Epirodin A. Most of the Fusarium pigments are phenols and would be expected to react with $FeCl_3$. The Fusarium pigments would also be expected to react with 2,4 dinitrophenylhydrazine reagent. Epirodin A has been shown not to react with either of these reagents.

Epirodin A was also shown to inhibit the DNA-dependent RNA polymerase of E. coli. The pigment was shown to inhibit RNA synthesis by binding to the RNA polymerase directly and not to DNA. Only the ansamycins antibiotics which consist of the rifamycins, streptovaricins, tolypomycins, and geldanamycin, and streptolydigin are known to inhibit the RNA polymerase. The ansamycins have been shown to inhibit the initiation of RNA synthesis while streptolydigin inhibits both chain initiation and

chain elongation. The Epirodin A pigment was shown to inhibit chain initiation and chain elongation as does streptolydigin. Although Epirodin A has the same activity as streptolydigin, the visible and ultraviolet spectral data clearly differentiate streptolydigin from Epirodin A. Epirodin A was shown to be different from the ansamycins by chemical and spectral means.

Epirodin A is a new inhibitor of DNA-dependent RNA polymerase. It does not seem to be related to any of the inhibitors previously reported. Also, Epirodin A and probably Epirodin B_{mix} are the first inhibitors of RNA polymerase to be isolated from the true Fungi Imperfecti. The other inhibitors have been isolated from the Actinomycetes (mold-like bacteria).

The infrared, visible and ultraviolet data along with the elemental analysis data on Epirodin A are included in this thesis. There is considerable structural work that has yet to be done on these pigments. The Epirodin B_{mix} pigments also provide another avenue for further research. The verification that the DNA-dependent RNA polymerase as the main site of activity *in vivo* has yet to be done. Epirodin B_{mix} will probably be easier to identify once the structure of Epirodin A is known. The fact that the structures are still unknown and the possibility that Epirodin A and Epirodin B_{mix} might be potential antiviral agents and might possess some anticancer activity should stimulate further research on these pigments.

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APPENDIX

Method for Determining RNA Polymerase Inhibitory Activity*

The assay reaction mixture consisted of the following:

0.1 M Tris buffer pH 7.9

5.0 mM $MgCl_2$

0.4 mM ATP, GTP and CTP

0.04 mM ^3H UTP (sp. act. of 17 uc/mM)

0.2 mM Dithiothreitol

60 ug/ml of Calf Thymus DNA (except where indicated)

27.5 ug/ml of E. coli RNA polymerase (except where indicated)

In a final volume of 0.2 ml

Twenty five ul aliquots were removed at timed intervals and spotted onto Whatman No. 40 ashless 2.4 cm filter paper disks. The filter paper disks were dried for 15 minutes on the desk top, soaked in cold 10 per cent TCA, twice with 95 per cent ethanol, once with absolute ethanol, and twice with anhydrous ether. The disks were then dried and counted in a Nuclear Chicago scintillation counter using a toluene-ominiflour counting cocktail.

* This work was done by D. Nuss (Biochem. Dept., UNH, Durham, N.H) on a sample of Epirodin A at the suggestion of the author.

RESULTS AND DISCUSSION

In the first experiment when Epirodin A was incubated with the in vitro system, inhibition of RNA synthesis occurred. Fifty per cent inhibition of RNA synthesis occurred at a concentration of 1×10^{-4} M (based on a molecular weight of 950). In the next two experiments the site of inhibition was revealed. In the second experiment the RNA polymerase concentration was held constant while the DNA concentration was increased (other reagents as cited above). In the third experiment the DNA concentration was held constant and the RNA polymerase concentration was increased. Only in the third experiment did RNA synthesis occur. It was concluded from these preliminary experiments that Epirodin A was an inhibitor of RNA polymerase.