AN ABSTRACT OF THE THESIS OF

Vale Ann (Yount) Scheve for the Master of Science Degree

in Biology presented on May 17, 1985

Title: The Effects of Dimethyl Sulfoxide on Component Fractions of Aspergilline

Abstract approved: Richard P. Keeling

The effect of varying concentrations of dimethyl sulfoxide (DMSO) on component fractions of the brownish-black pigment, aspergilline, of Aspergillus carbonarius was investigated. The fungus was grown in the presence of varying concentrations of the pigment, inhibitor DMSO. Pigment components were separated by column chromatography. Spectrophotometric analysis established the presence of three component fractions within the pigment. An elution profile revealed that the fractions consisted of a large first peak with increasingly smaller second and third peaks. DMSO inhibited production of all fractions of the pigment. Over 30 per cent of the original first fraction was retained while only 5 per cent or less of fraction two and fraction three were retained. The role of these components of aspergilline is not known. It may be that the second and third fractions are precursors to the pigment in the larger first peak. An investigation was also conducted to establish a procedure which would allow separation of the components of aspergilline utilizing the simpler method of zone electrophoresis. A variety of procedures were tried in which support type, buffer pH and pigment pH were varied. Separation was poor and not satisfactory for analysis.

THE EFFECTS OF DIMETHYL SULFOXIDE ON COMPONENT FRACTIONS OF ASPERGILLINE

A Thesis
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of the Requirements for the Degree
Master of Science

by
Vale Ann (Yount) Scheve
May 17, 1985

Richard P. Keeling
Approved for Major Department

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Approved for Graduate Council

ACKNOWLEDGEMENT

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INTRODUCTION

Fungi produce a wide variety of pigments which may fall into several broad categories which include: pteridines, melanins, quinones, carotenes and carotenoids. The function of these pigments is quite diverse and ranges from serving as an energy reserve to that of providing a protective function. In some fungi quinones have been shown to function in their oxidation-reduction systems (Wolf, 1973). Zhdanova and Pokhodenko (1973) suggested that melanin may serve a role in the protection of fungus from desiccation.

Fungi in the Aspergillus group are known to produce yellow (Reid, 1950; Zajic, 1962), red (Assante, 1981), and brown (Barbetta et al, 1967) pigments. Linossier (1891) was the first to discuss the brown pigment, aspergilline. He found the pigment associated with conidia of fungi in the Aspergillus niger group. In a recent paper, Ray and Eakin (1975) refer to the pigment as aspergillin. Barbetta et al (1967) suggested that aspergilline was composed of a perylene quinone nucleus which upon hydrolysis releases aspartic acid, glutamic acid, phenylalanine and thirteen other amino acids in smaller amounts. Ray and Eakin (1975) found that aspergilline comprises approximately 25 per cent of the dry weight of the spores with maximum pigment synthesis occurring at 80 to 140 hours of incubation. Curtis (1970) established that aspergilline serves to protect the fungus from the effects of ultra-violet light. Curtis grew Aspergillus carbonarius in the presence of the pigment inhibitors dimethyl sulfoxide (DMSO), dimethyl sulfone, and dimethyl sulfite. The loss of pigmentation that ensued was not accompanied by a corresponding loss in conidial germination. There was, however, a sharp decrease in the resistance to UV light. Lysenko and Lyakh (1978) found that when

aspergilline was extracted from conidia of Aspergillus niger and added to suspensions of unpigmented microorganisms the degree of protection from UV light increased significantly. According to Carley et al (1967) pigmentation does not appear to be directly related to the life cycle of A. niger. They established that neither the growth rate nor sporulation was affected by DMSO until concentrations greatly exceeded that necessary to inhibit pigment formatiion. Their work indicated that the S=O radical was responsible for inhibition of pigment formation. Earlier work by Keeling (1984) showed conidia grown in the presence of DMSO adhered more tightly to the conidiophores of A. niger than did conidia in which the pigment was uninhibited.

DMSO was first developed in 1866 by Alexander Saylzeff. A by-product of the paper industry, DMSO is an unusual solvent. Schiller (1967) explored its usefulness as a cryoprotective agent. Clinically it has been used to treat rheumatoid arthritis (Matsumoto, 1967). DMSO has also been used to treat mental patients. Ramirez and Luza (1967) injected 42 severely disturbed psychiatric patients with DMSO and found it to have antipsychotic and anti-anxiety properties. DMSO has the unique ability to substitute for water and alter the configuration of certain enzyme proteins (Rammler and Zaffaroni, 1967). The alteration appears to be reversible leaving no permanent effect on the affected enzyme.

Carley et al (1967) observed a "bleaching" effect of DMSO on A. niger. Maximum color inhibition occurred at 4000 ppm DMSO. There was approximately 25 per cent reduction in the size of the conidial head at concentrations of 10,000 ppm DMSO. Although a slight growth reduction was noticed at this concentration, no decrease in spore viability was

observed. Teel (1965) determined that "spines" usually associated with the conidia of A. niger were not produced when the fungus was grown in the presence of DMSO. The effect of DMSO on fungal pigment and spines was reversible. When DMSO inhibited spores were seeded on media free of DMSO the natural brown-black pigment and spines reappeared in the resulting colonies. It is possible that DMSO acts to interrupt lipid synthesis within the fungus (Sauter, 1969). This inhibition occurs only in conidia and no correlation has yet been drawn between "bleaching", loss of "spines", and lipid synthesis.

The pigment fraction or fractions of aspergilline inhibited by the presence of DMSO in culture media have not been well established. Ray and Eakin (1975) in their work with A. niger described a high molecular weight native pigment present in fungal spores. Spores grown in the presence of 2,4-dithiopyrimidine (DTP), exhibited green and brown pigment fractions. Those spores grown in the presence of DMSO had a single brown pigment fraction. However, in 1967 the work of Barbetta et al established the presence of three separate fractions in untreated pigment from A. niger.

Although Curtis (1970) used DMSO to study the role of pigment in the protection of A. carbonarius from UV light, the majority of studies utilized A. niger. Barbetta et al (1967) used gel column chromatography to separate the fractions of aspergilline. Techniques of this type can be expensive and require time to perform. If zone electrophoresis could be successfully adapted to the separation of native pigment fractions it would be possible to analyze pigment components with a much smaller investment in time and money.

This study was undertaken to establish the fraction, or fractions

of the pigment, aspergilline, obtained from Aspergillus carbonarius which are affected by treatment of the fungus with DMSO. In addition, a number of trials was made in an effort to develop a procedure which would allow the separation of the components of aspergilline utilizing the simpler method of zone electrophoresis.

MATERIALS AND METHODS

Experimental Stock

A culture of Aspergillus carbonarius (from NRRL #67) was obtained from the mycology collection maintained at Emporia State University. Subcultures were made as needed.

Sporulation Media

The organism was grown on agar plates of two media types. Modified Moyer-Wells Media was prepared according to the directions of Raper and Fennell (1973). Coors premium beer was added to the medium. Potato Dextrose Agar (Gibco Diagnostics--#M39400) was also used. It was prepared according to directions.

Transfer Medium

To facilitate transfer of conidia to sporulation media, a transfer medium was prepared by adding one drop of Tween 80, poloxyethylene (20) sorbitan monooleate, (Fisher Scientific Co.--T-164) to 500 ml distilled water with a wooden applicator stick.

Inoculation of Media and the Formation of Spores

Two Modified Moyer-Wells Media agar slants were washed with the transfer medium. A sterile loop was used to transfer spores to the agar plates. A single inoculation approximately 18 millimeters in diameter was made in the center of each plate. Plates were incubated upside down at 30 C. Those cultures cultivated on Modified Moyer-Wells Media were incubated 32 days while those cultivated on Potato Dextrose Agar were incubated 18 days.

Harvest of Conidia

Collection of conidia was accomplished by inverting the bottom portion of each petri dish containing the fungus over a single petri dish

lid, one at a time. The bottom of each dish was tapped lightly to achieve release of the conidia. They were then pooled and transferred to an Erlenmeyer flask.

Extraction of Pigment

One hundred milliliters of 1 N NaOH were added to the harvested conidia. This mixture was heated for 20 minutes in a boiling water bath. It was then autoclaved for ten minutes at five psi. The mixture was refrigerated until needed. This suspension was centrifuged at 5000 rpm for 15 minutes in a SerVall fixed angle centrifuge (type SS4). The supernatant containing the pigment was reserved. The pellet was discarded. This procedure was repeated one additional time. The pigment solution was then titrated with 5N HCl until precipitation occurred at pH 3.4. Titration was continued to pH 3.1. The pigment was washed twice in acidified distilled water (4 drops concentrated HCl per liter of distilled water). The container was covered lightly and the pigment was dried at room temperature for eight days. It was then transferred to a vacuum desiccator containing calcium chloride for further drying and storage until needed.

DMSO Treated Pigment

Pigment from culture which had been treated with DMSO (ACS) was obtained from Dr. Richard P. Keeling, Emporia State University. It was prepared according to the previously outlined procedure with DMSO added aseptically to the sterile media. The medium was modified by adding DMSO in increments of 10 mM from 10 mM to 100 mM.

Gel Chromatography

Delivery System

A gravity delivery system was constructed from an I.V. bottle

connected to the gel column by plastic tubing. The flow rate was adjusted by changing the bottle height.

Columns and Support

Gel columns (Bio Rad Labs, Richmond, CA -- #737-2240) of the size one centimeter by 20 centimeters were used. The gel support was composed of Sephadex G-50 superfine beads (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Beads were soaked overnight in an excess of 0.01 N NaOH. Columns were filled and allowed to settle overnight. The columns were equilibrated for a minimum of twelve hours at a flow rate of less than 4.5 ml/hour of 0.01 N NaOH.

Pigment Preparation

Dried pigment was dissolved in 1 N NaOH at a concentration of three milligrams of pigment per one milliter of NaOH. The sample was heated in a boiling water bath for 20 minutes to dissolve the pigment. The pigment solution was stored at room temperature in tightly capped tubes until needed.

Procedure

A routine sample size of 0.5 ml was analyzed. The flow rate was adjusted to 0.075 ml/min. Samples were collected at five minute intervals. An additional 3.0 ml of eluant was added to each of the collection tubes to facilitate analysis in the Bausch and Lomb Spectronic 20 at a wavelength of 420 nm.

Analysis of Data

Elution profiles were obtained by plotting absorbance against tube number for each concentration of DMSO. Peak height for each fraction was determined from the elution profiles. Peak height for each of the three fractions was plotted independently against concentration of DMSO.

Peak weight was obtained by cutting out and weighing individual peaks to the nearest milligram on a Mettler, type H-15, analytical balance. Peak weight was plotted against concentration of DMSO for each of the three fractions independently. An arbitrary base line of 0.03 Absorbance was used in peak weight comparisons. No arbitrary base line was used in peak height comparisons. A simple linear regression was performed on graphs of fraction one in which peak weight as well as peak height were plotted against concentration of DMSO independently. Both peak weight and peak height plots for fractions two and three were determined to be exponential functions of the concentration of DMSO.

Electrophoresis

A variety of techniques were tried in an effort to perfect a procedure of electrophoresis which could be applied to the analysis of aspergilline. It would be impractical to list all of the variations employed in this work. Therefore, procedures and techniques shall be limited to a general discussion with specific examples given.

Equipment

A 9.5 x 12.5 x 2 inch Gelman electrophoresis chamber (Gelman -- #51101) was used for all electrophoretic procedures. Direct current was supplied by a Gelman (115v/50-60Hz) power supply unit (#38201).

Buffers

A barbital buffer was prepared by mixing sodium barbitol (Fisher Scientific Co.) which had been diluted in distilled water to the desired molarity (i.e. 0.025, 0.05, and 0.075 M) with barbituric acid (Sigma) which had been diluted in distilled water to the same molarity. One component was titrated into the other to achieve the desired pH. A sodium glycinate buffer was prepared according to the directions of E. C. Corporation (Philadelphia, PA 19104), Technical Bulletin #134

and titrated to pH 10 with 1 N NaOH.

Migration Time

Electrophoresis was continued from ten minutes to one hour. The mode time was 20 minutes.

pH

The pH of the buffer system, pigment and support were all varied. Trials were made at pH levels of 4.5, 5.5, 6.5, 8.8 and 10.0.

Voltage

The majority of trials were conducted at 300 volts or 500 volts.

Preparation of Pigment for Analysis

Electrophoretic studies were performed with the pigment at a concentration of 20 milligrams of pigment per milliliter of 1 N NaOH. Samples were heated in a boiling water bath for 20 minutes to totally dissolve the pigment granules. Dissolved pigment was stored at room temperature in tightly capped tubes until needed.

pH of Pigment for Electrophoresis

Pigment pH was adjusted empirically. One part of the appropriate buffer and one part of 5 N HCl were added to 20 parts solubilized pigment in that order. The pH of the buffer used established the pH of the pigment.

Sample Application

Several different methods of sample application were utilized. Application onto solid supports such as cellulose polyacetate strips or thin layer chromatography strips was done with 10 ul micro-applicators (Cordis Laboratories, Miami, FL 33137 - #711-110). Sample application onto gel supports was done with a pasteur pipette into a trench mold or into a razor cut. Trench molds were prepared by embedding

an entomology pin cut to the approximate length of 1.5 cm in the liquid gel which was then allowed to solidify. Pins were removed with a magnet immediately prior to sample application.

Supports

Sepraphore III, Cellulose Polyacetate Electrophoresis Strips (Gelman Sciences, Inc., Ann Arbor, MI 48106 - #62038) were soaked in the desired buffer overnight. Immediately prior to an experiment the strips were removed from the buffer, quickly blotted, and the sample applied. Electrophoresis was initiated as quickly as possible after application of the sample to prevent diffusion of the pigment.

Agarose, type III gel support, was prepared by mixing the desired amount of Agarose (Sigma Chemical Co., St. Louis, MO 63178) (i.e., 150 mg, 250 mg, or 300 mg) with 50 ml of the desired buffer. The material was boiled until dissolved and pipetted onto glass microscope slides (3" x 1"). The slide containing the support was placed on a glass plate in order to bridge both buffer chambers. Wicks were attached to either end of the support and extended into the buffer wells for the opposing electrodes. Electrophoresis commenced immediately after application of sample.

Agarose-Sephadex gel supports were also utilized. A buffer-Sephadex solution was prepared by mixing four grams of Sephadex with 20 ml of buffer and allowing this mixture to stand for 24 hours at room temperature. Three hundred milligrams of Agarose were boiled with 16.7 milliliters of the desired buffer until dissolved. Thirty-three and three-tenths milliliters of the buffer-Sephadex solution were added to the agarose solution. While still warm the materials were pipetted onto 3" x 1" microscope slides. The support contained approximately

two per cent Sephadex (w/v).

A polyacrylamide gel support employing Cyanogum-41 (E. C. Corporation, Philadelphia, PA 19104, Technical Bulletin #128) was prepared according to directions and with the addition of the desired buffer at the selected pH. Polymerization was catalyzed chemically using TMED (N,N,N',N' tetramethylethylene diamine - Sigma) with ammonium persulfate as the initiator used to generate oxygen free radicals. The material was pipetted onto 3" x 1" microscope slides and placed in a vacuum desiccator to remove oxygen.

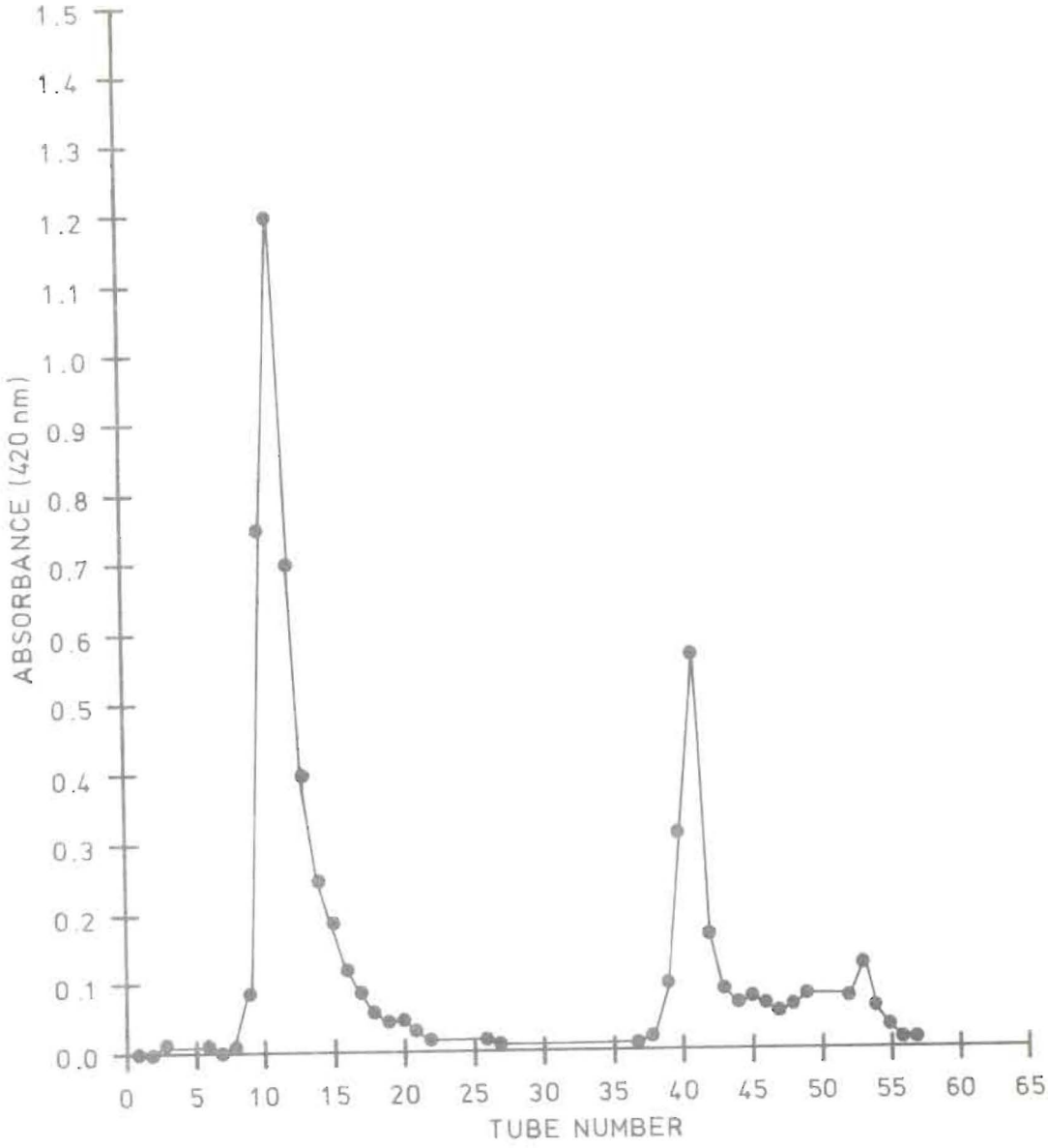
The thin layer chromatography support consisted of precoated thin layer chromatography plastic sheets, 20 cm x 20 cm (E. M. Reagents, Darmstadt, Germany - #5502) which were cut to 2.5 cm x 7.5 cm strips. The strips were placed on a glass plate. Wicks were used to bridge electrodes. Prior to electrophoresis strips were soaked in buffer for several hours.

RESULTS AND DISCUSSION

Gel filtration has found routine use in the separation of protein, nucleic acid, polysaccharide and lipid molecules since its introduction in 1959. Separation is accomplished mechanically by molecular sieving. It is a very "gentle" approach which minimizes the effect of electrostatic and adsorptive factors. Barbetta et al (1967) used gel chromatography to establish the presence of three component fractions of aspergilline. Ray and Eakin (1975), however, found only one component present in the native pigment. In this study gel column chromatography was performed as previously outlined in the materials and methods section of this work. Chromatographic separation of control pigment yielded three components which could be distinguished at 420 nm on the Bausch and Lomb Spectronic 20. The first fraction was eluted after approximately 50 minutes of flow time. While in the column, the band appeared broad and diffuse, but on collection and analysis it was found to have the most prominent peak. The second fraction was compact. There was very little "wall" effect. It was eluted after three hours. The third fraction was collected at approximately four hours of elution. Visually this band was compact but not as dark as the second, nor as dense. The "wall" effect was minimal. Visual observations of collected fractions of aspergilline indicated the first fraction would be expected to have the largest peak, followed by fraction two and, finally, fraction three. Spectrophotometric data confirmed this observation.

Control pigment extracted from conidia grown in the absence of DMSO was visibly darker than that grown in concentrations of DMSO of 50 mM or greater. Elution profiles (Fig. 1 through Fig. 11) also show a definite decrease in absorbance at 50 mM concentration. A graph of

Figure 1. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline.


ABSORBANCE (420 nm)
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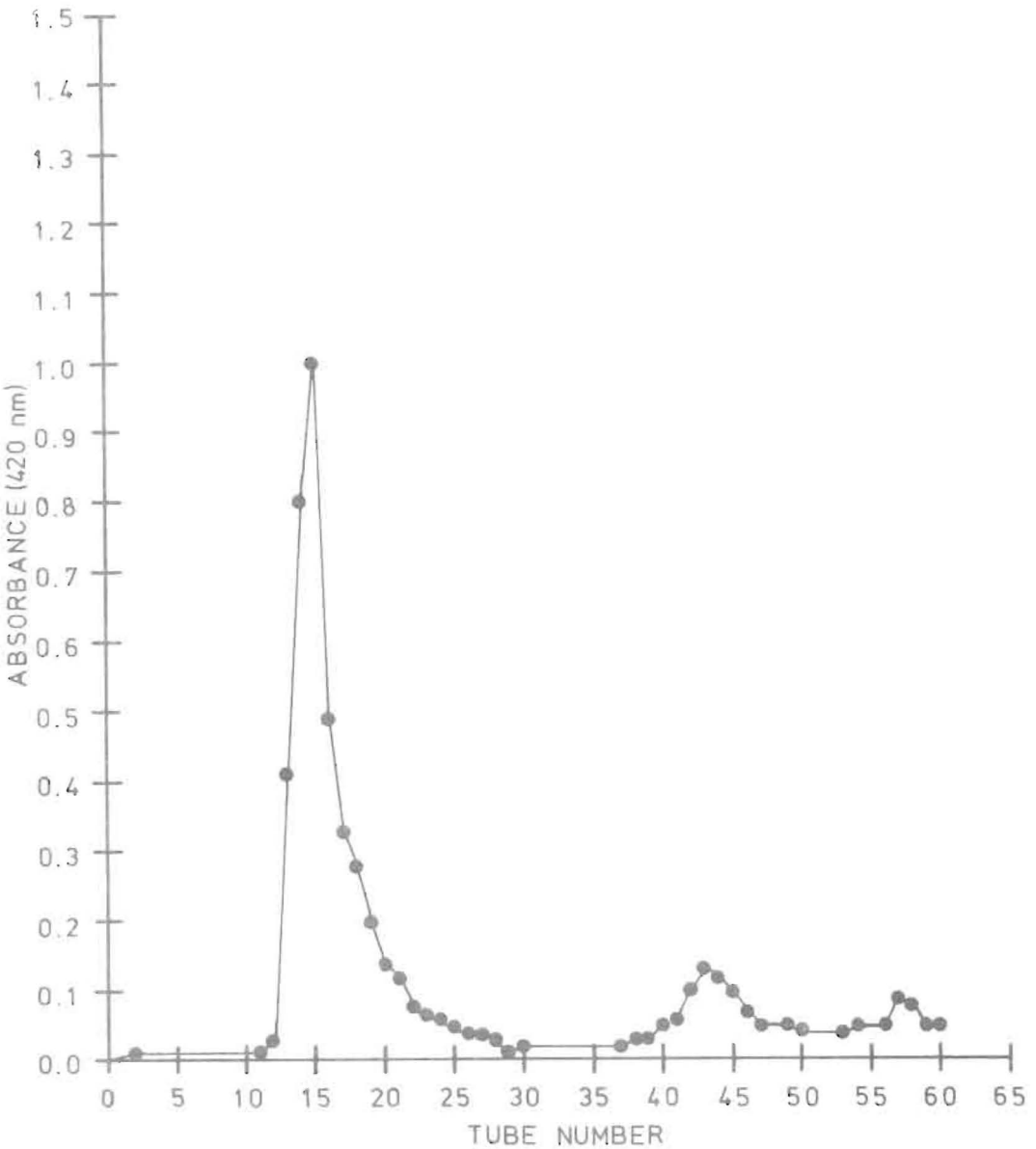
TUBE NUMBER

Figure 2. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 10 mM concentration.


ABSORBANCE (420 nm)
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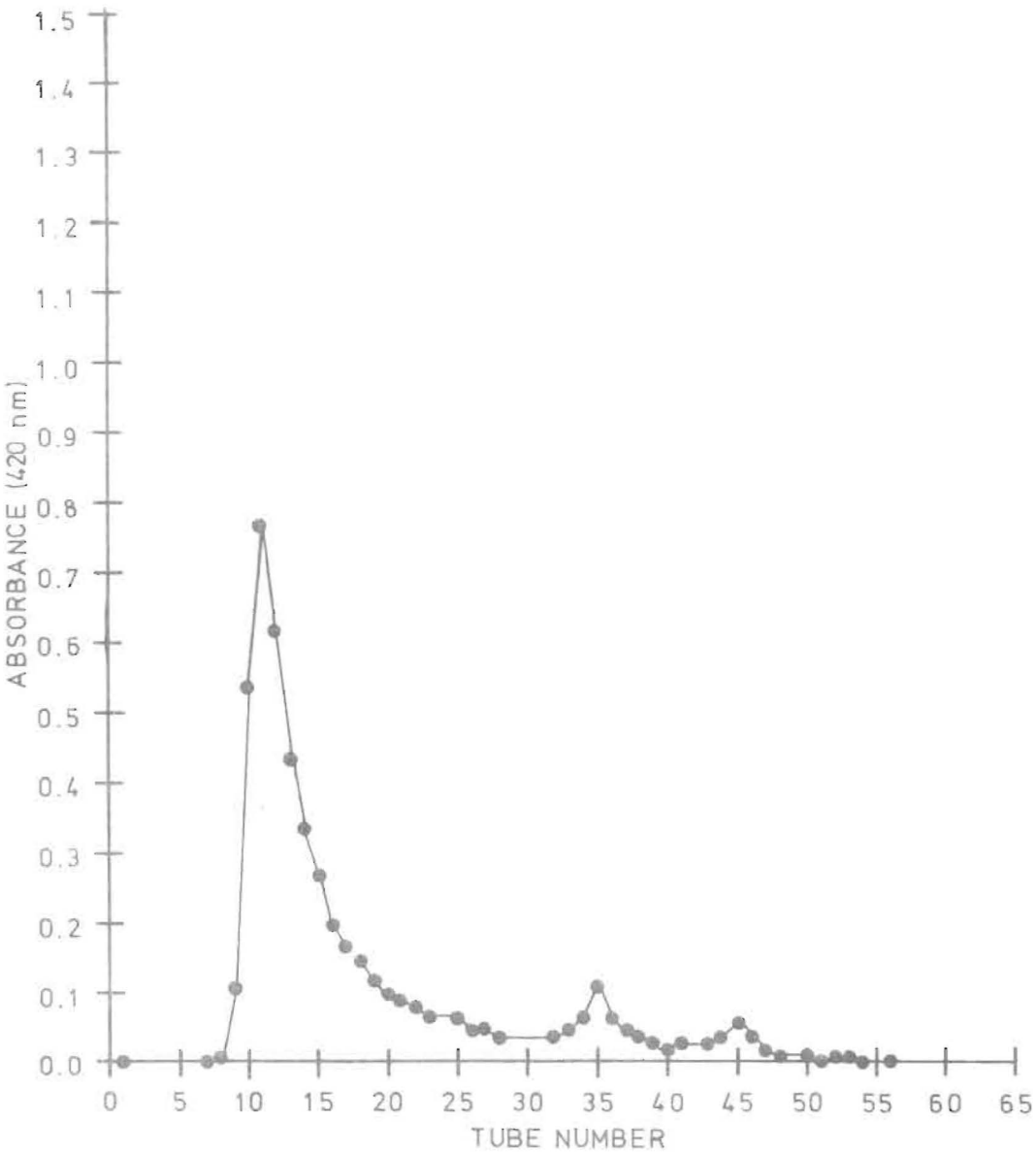
TUBE NUMBER

Figure 3. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 20 mM concentration.


ABSORBANCE (420 nm)
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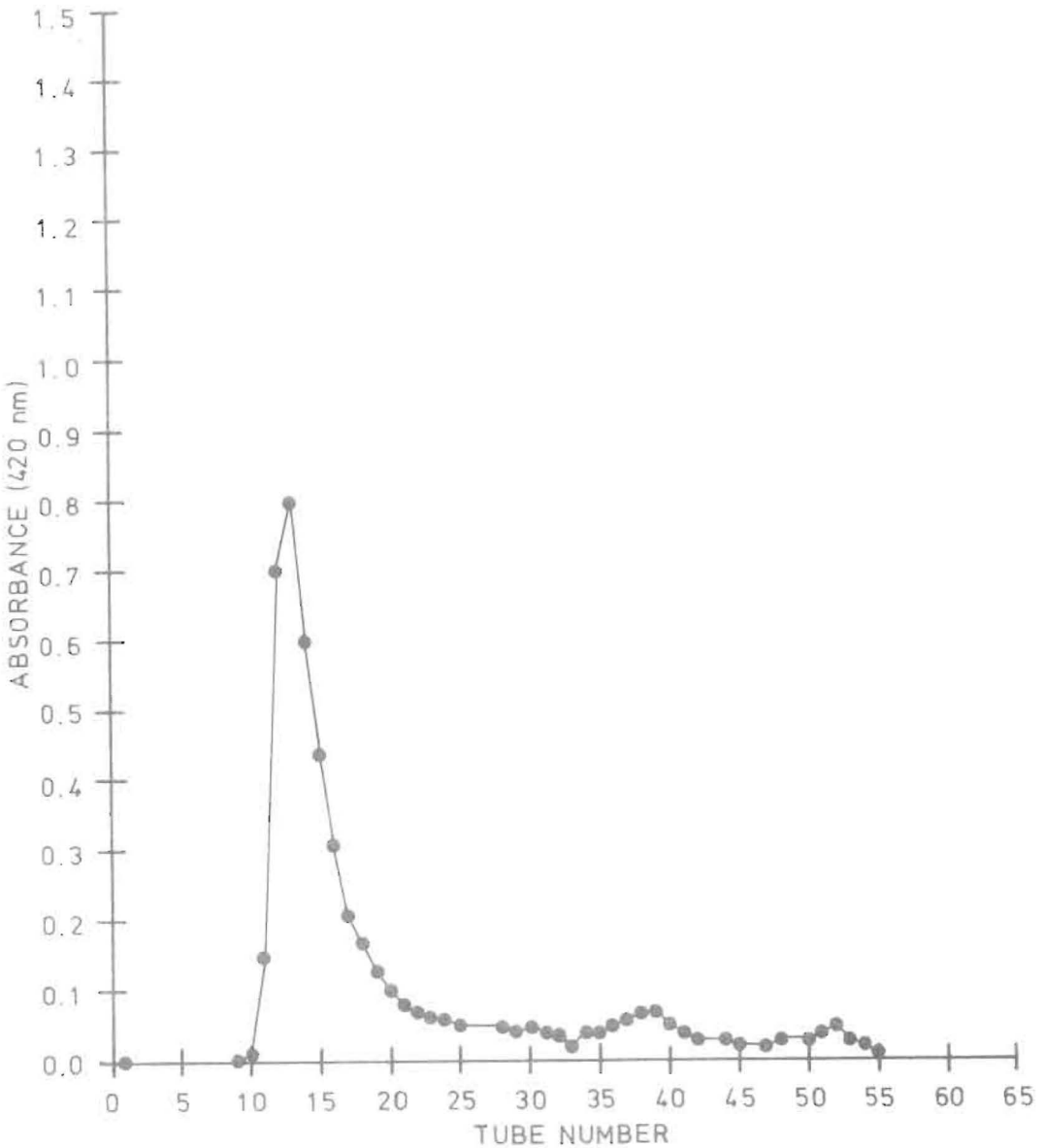
TUBE NUMBER

Figure 4. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 30 mM concentration.


ABSORBANCE (420 nm)
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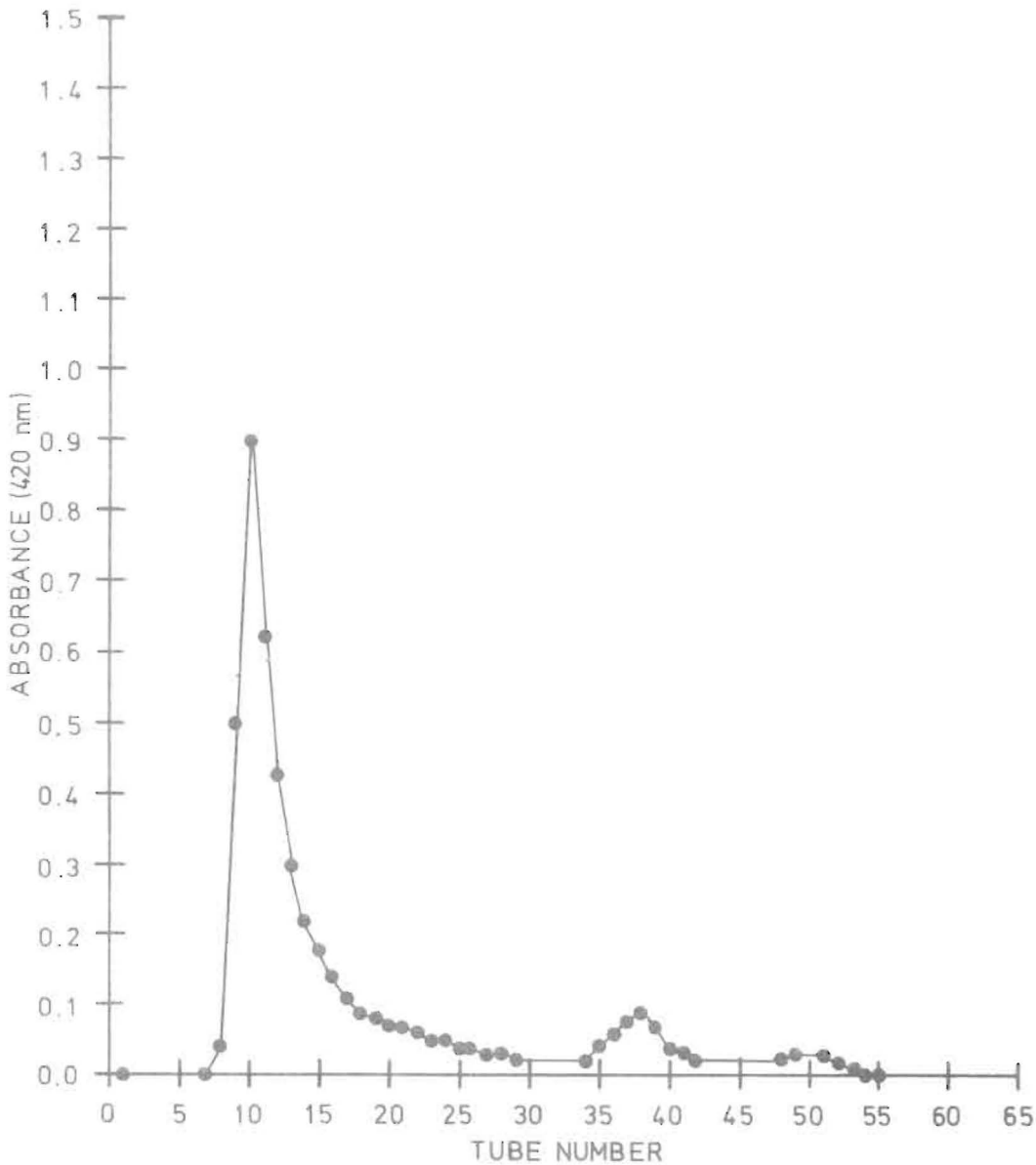
TUBE NUMBER

Figure 5. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 40 mM concentration.


ABSORBANCE (420 nm)
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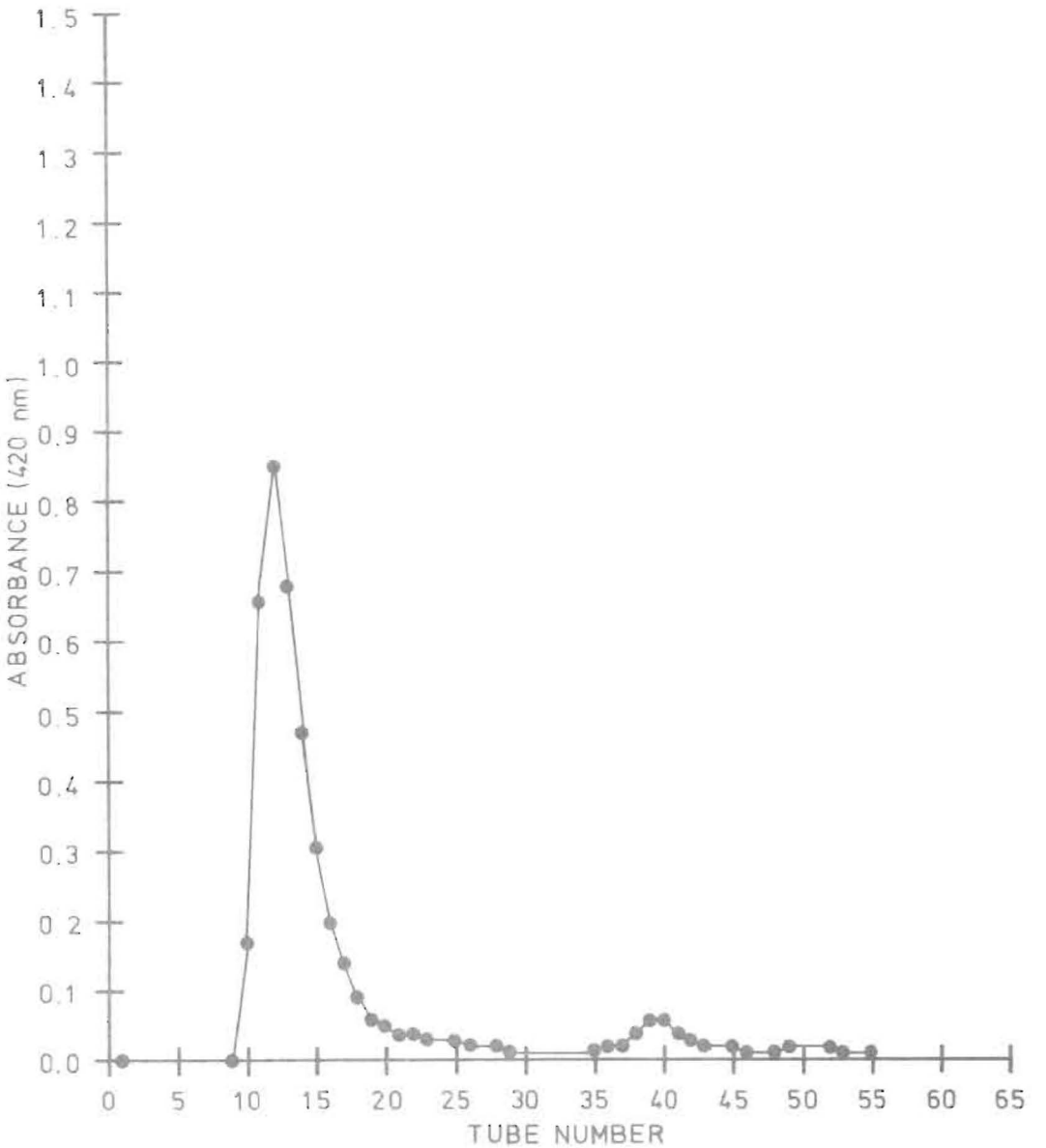
TUBE NUMBER

Figure 6. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 50 mM concentration.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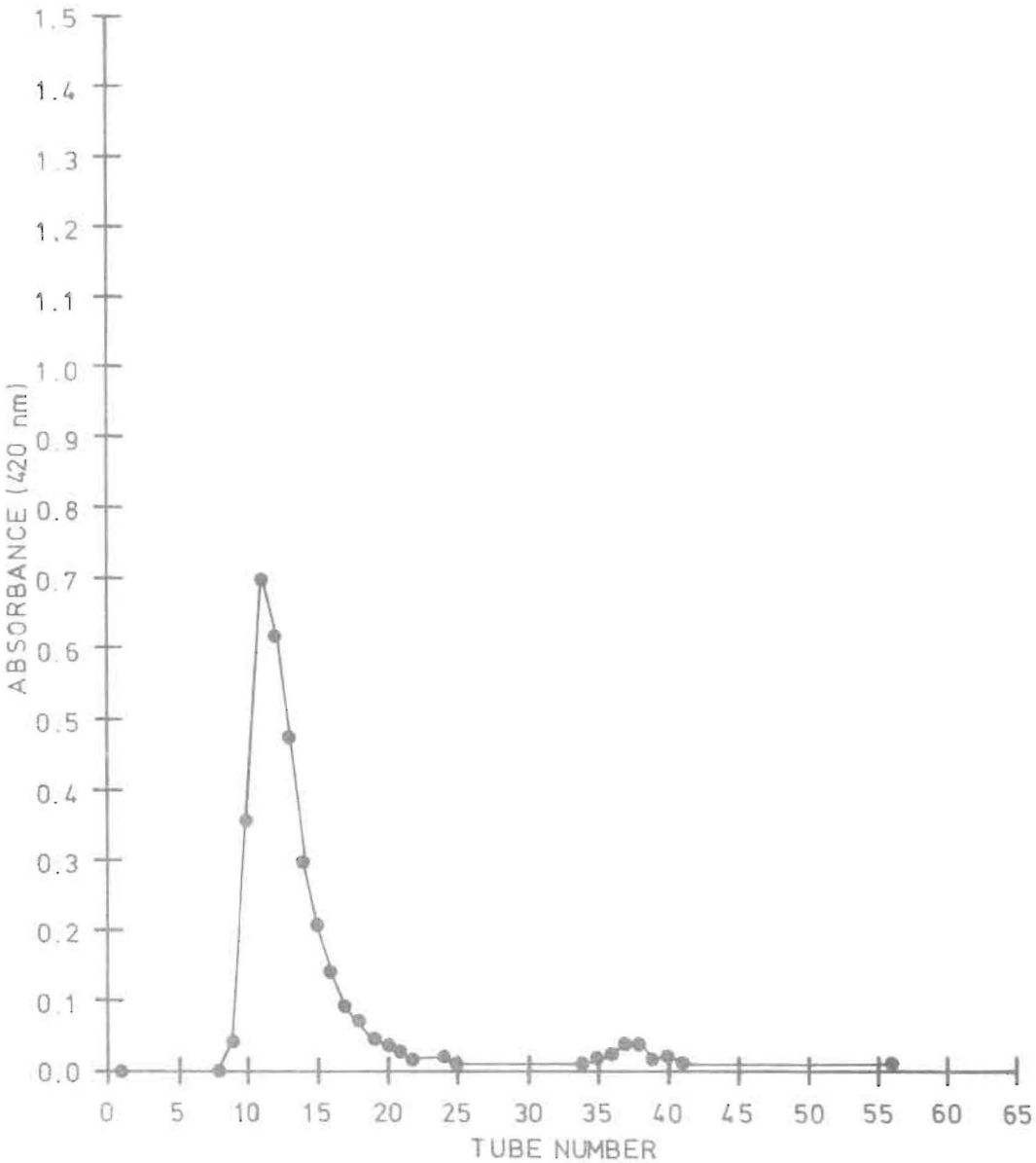
TUBE NUMBER

Figure 7. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 60 mM concentration.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ABSORBANCE (420 nm)
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Figure 8. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 70 mM concentration.

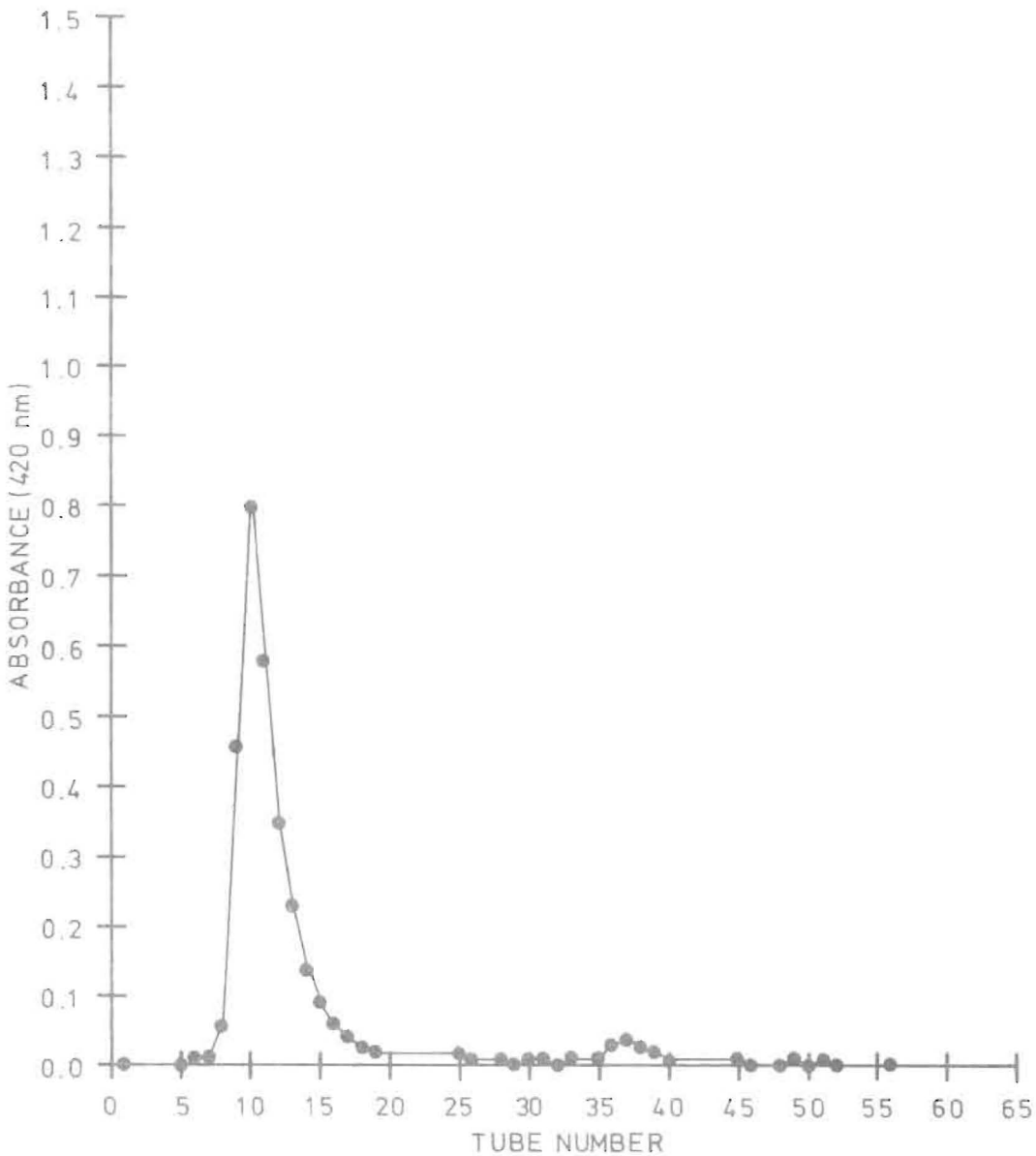
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Figure 9. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 80 mM concentration.

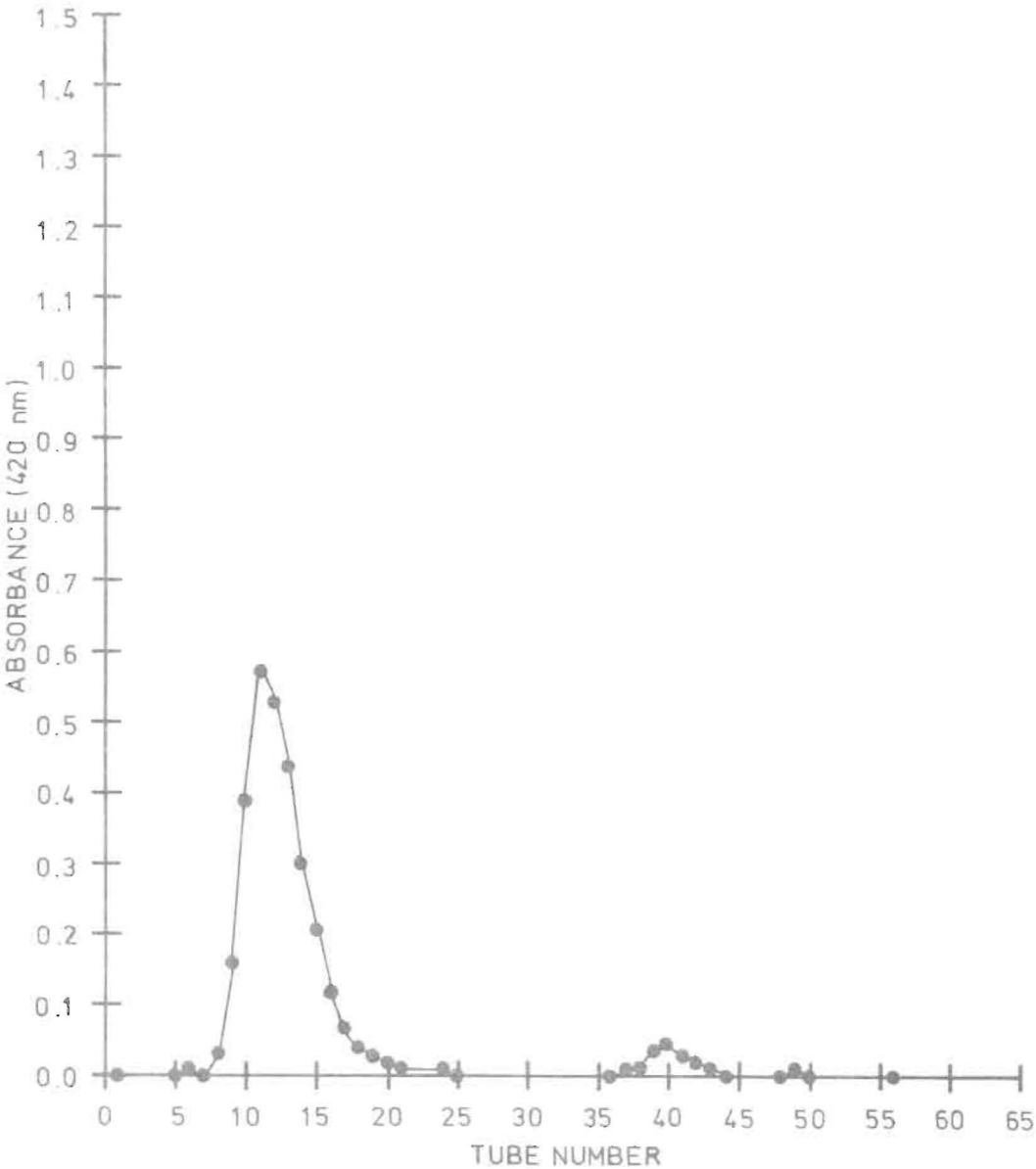
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Figure 10. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 90 mM concentration.

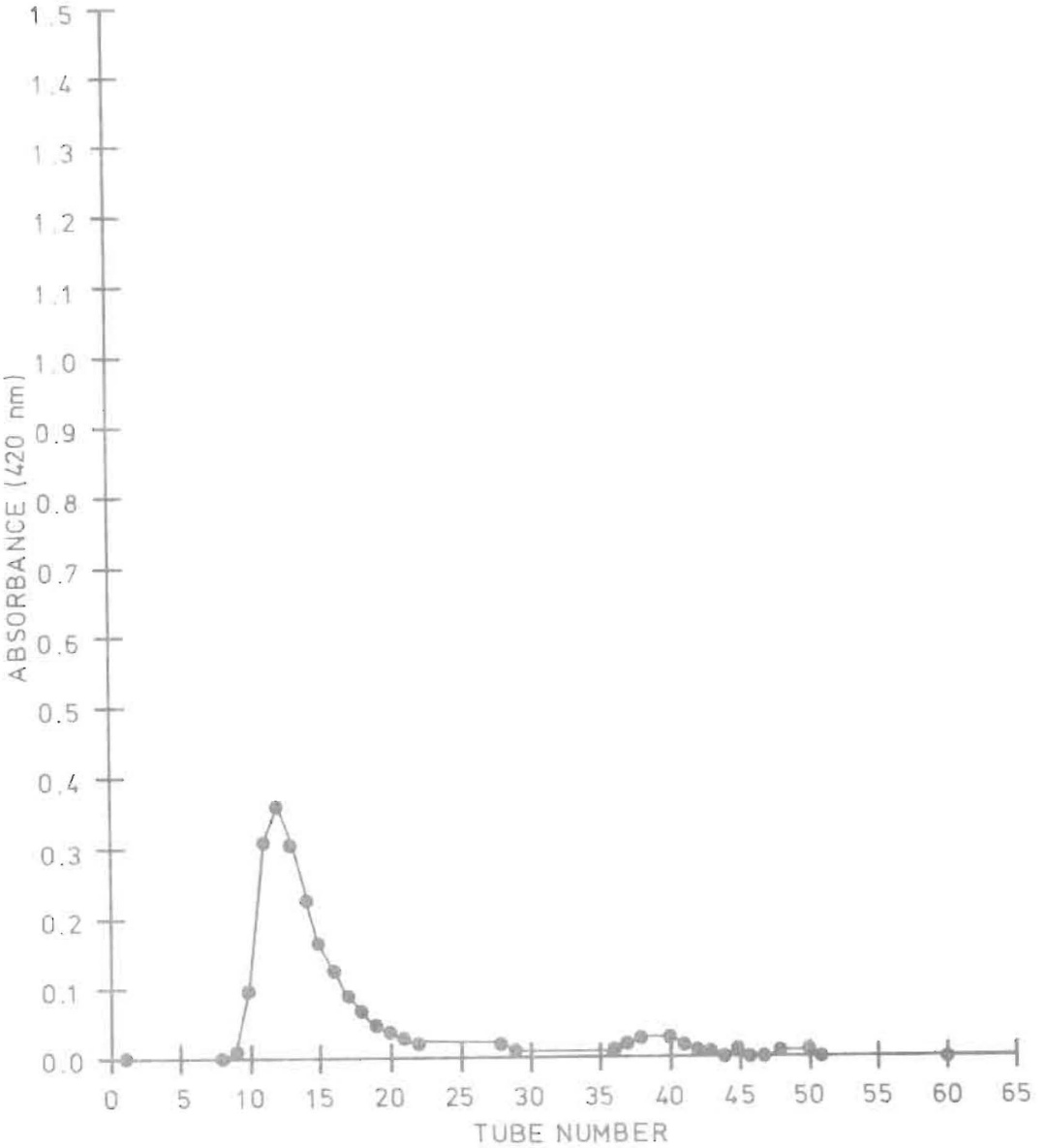
ABSORBANCE (420 nm)
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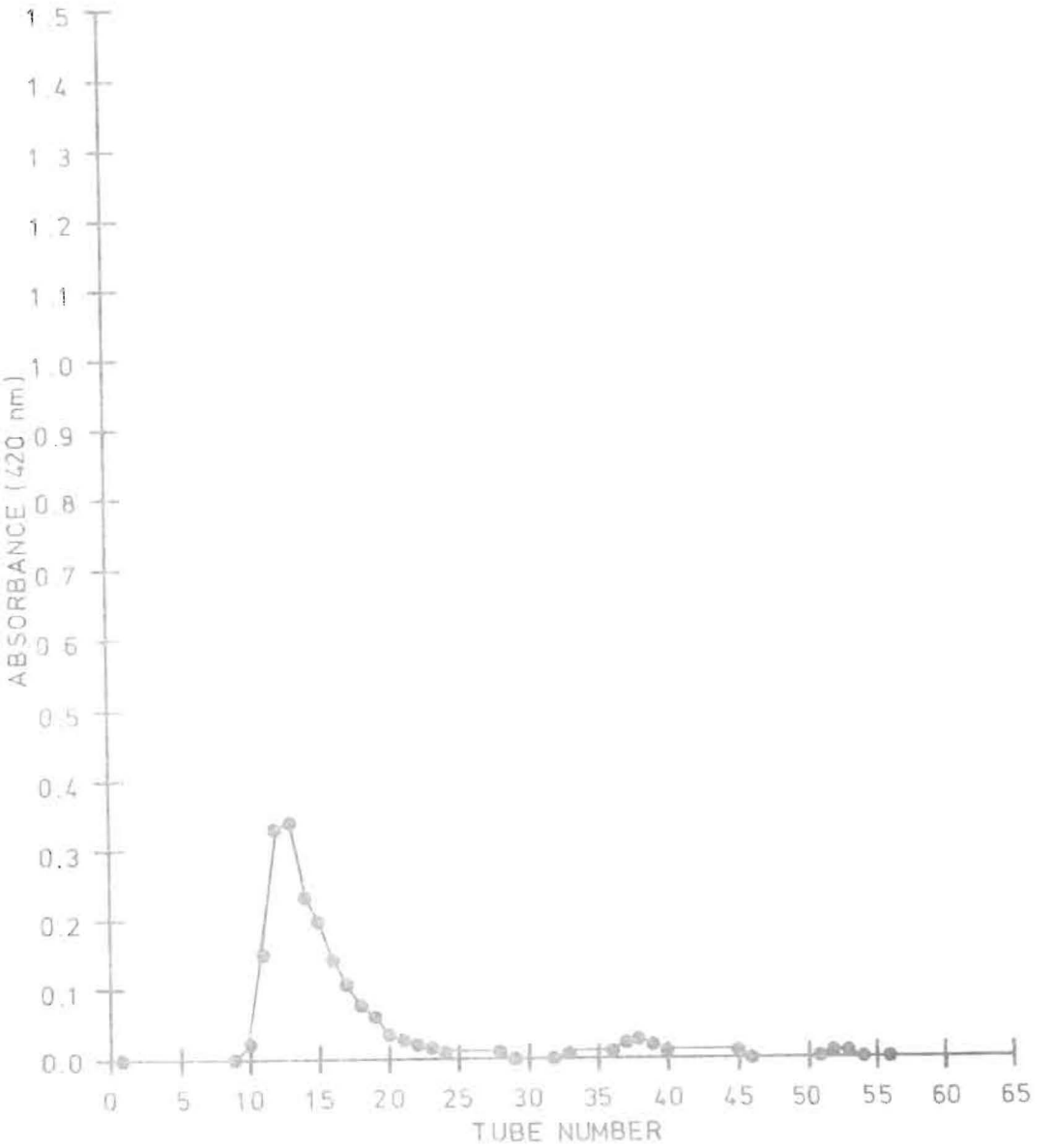
Figure 11. Light absorbance at a wavelength of 420 nm by three fractions of aspergilline inhibited with DMSO at the 100 mM concentration.


ABSORBANCE (420 nm)
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TUBE NUMBER

fraction one in which peak weight was plotted against concentration of DMSO showed less change in the per cent decrease from the control than did either fraction two or fraction three (Fig. 12, Fig. 13 and Fig. 14). Fractions number one, two and three revealed a 62.4 per cent, 100 per cent, and 100 per cent decrease in weight, respectively. Graphs of peak height as compared to concentration of DMSO showed a 72 per cent, 94 per cent and 95 per cent decrease, respectively (Fig. 15, Fig. 16 and Fig. 17). Both peak height and peak weight comparisons reflected similar results.

If magnitude of decrease is measured, the first fraction is decreased in magnitude by about twice that of the second fraction and almost four times that of the third fraction. However, over 30 per cent of the original first fraction is retained while only 5 per cent or less of fraction two and fraction three are present at the 100 mM DMSO concentration. The essential elimination of fractions two and three would indicate the greatest total effect of DMSO occurs in these fractions. The elution profile of the pigment of Aspergillus carbonarius reflected the work done by Barbetta et al (1967) in which three component fractions were found in the pigment of Aspergillus niger. The pattern of peak distribution is very similar in both cases. However, the presence of three fractions was not corroborated by the work of Ray and Eakin (1975). In their work only one fraction was found in the control pigment and only one fraction was found in the pigment inhibited by DMSO. This may be accounted for by procedural differences. In their study, DMSO was used at a single concentration of 4 mg/ml (50 mM) while this investigation used molarity ranging from 10 mM to 100 mM DMSO. In addition, the flow rate in their study was 0.25 ml/min while this research utilized a flow rate of 0.075 ml/min. Their stationary phase was composed by G-25 Sephadex while that of this

Figure 12. The relationship between peak weight and concentration of DMSO for the first fraction of aspergilline.

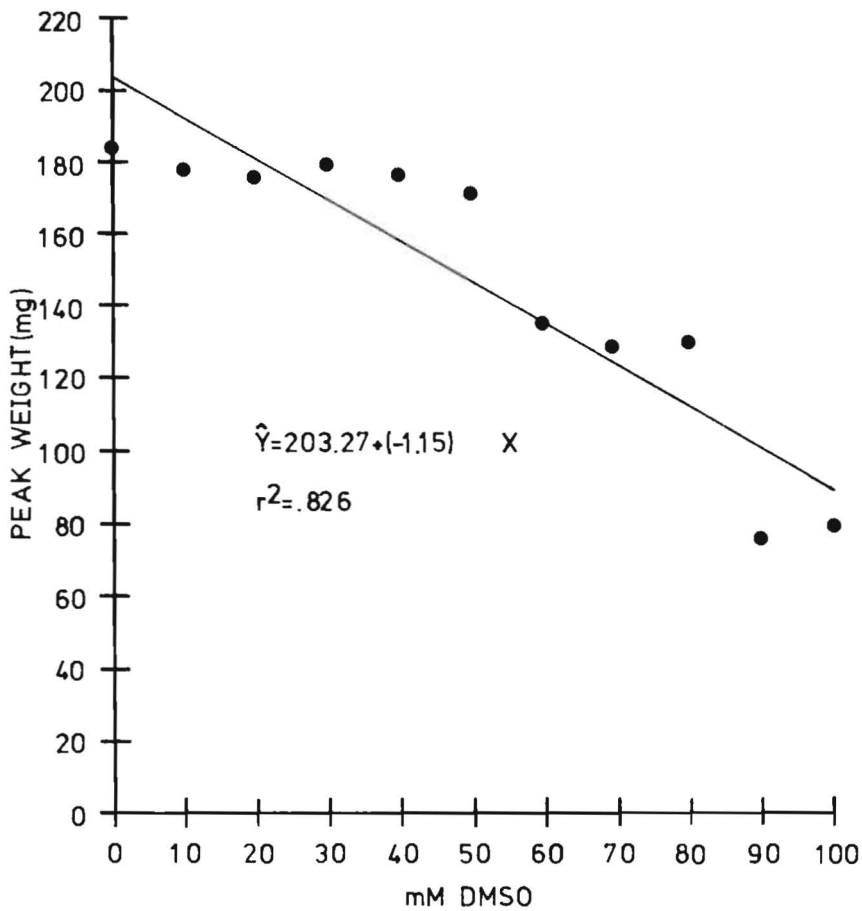

220
200
180
160
140
120
100
80
60
40
20
0
PEAK WEIGHT (mg)
$\hat{Y}$=203.27+(-1.15) X
r^2=.826
0
10
20
30
40
50
60
70
80
90
100
mM DMSO

Figure 13. The relationship betwen peak weight and concentration of DMSO for the second fraction of aspergilline.

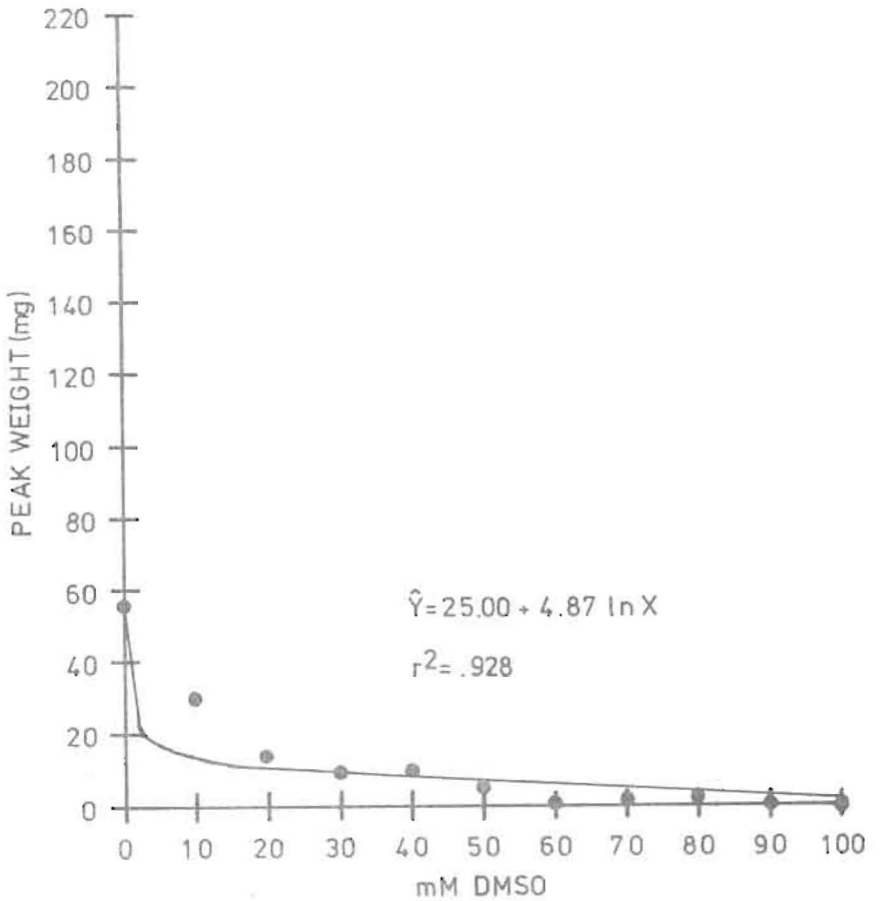

220
200
180
160
140
120
100
80
60
40
20
0
PEAK WEIGHT (mg)
$\hat{Y}$ = 25.00 + 4.87 ln X
r^2 = .928
0
10
20
30
40
50
60
70
80
90
100
mM DMSO

Figure 14. The relationship between peak weight and concentration of DMSO for the third fraction of aspergilline.

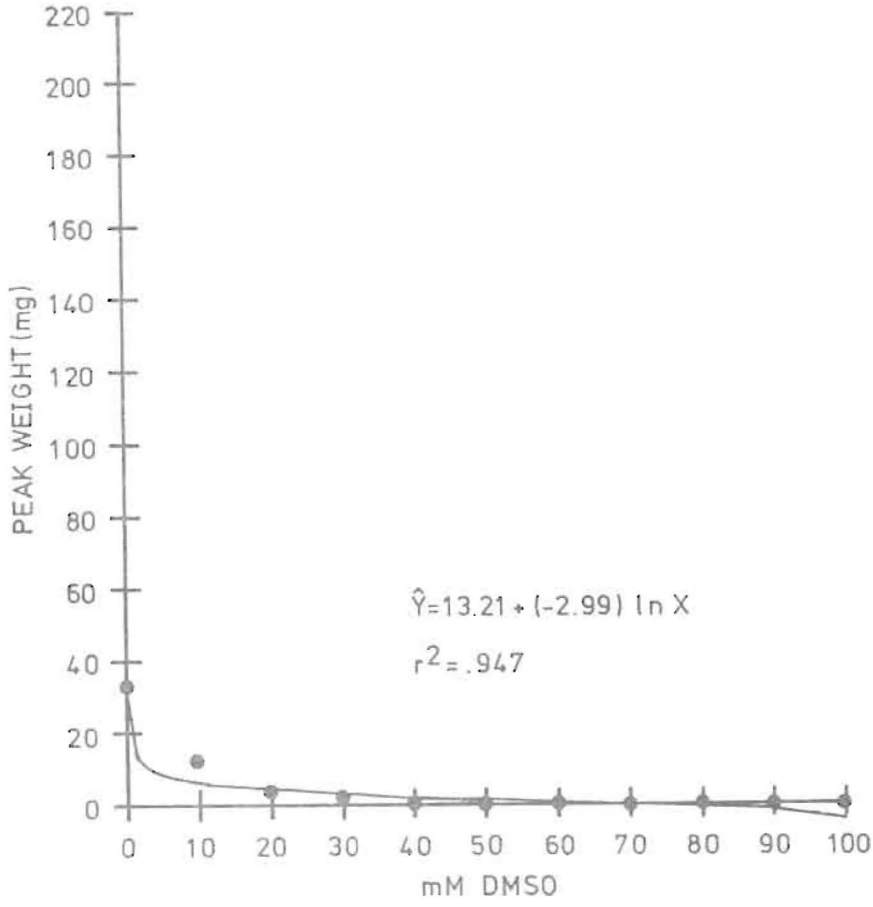

220
200
180
160
140
120
100
80
60
40
20
0
PEAK WEIGHT (mg)
$\hat{Y}$=13.21 + (-2.99) ln X
r^2=.947
0
10
20
30
40
50
60
70
80
90
100
mM DMSO

Figure 15. The relationship between peak height and concentration of DMSO for the first fraction of aspergilline.

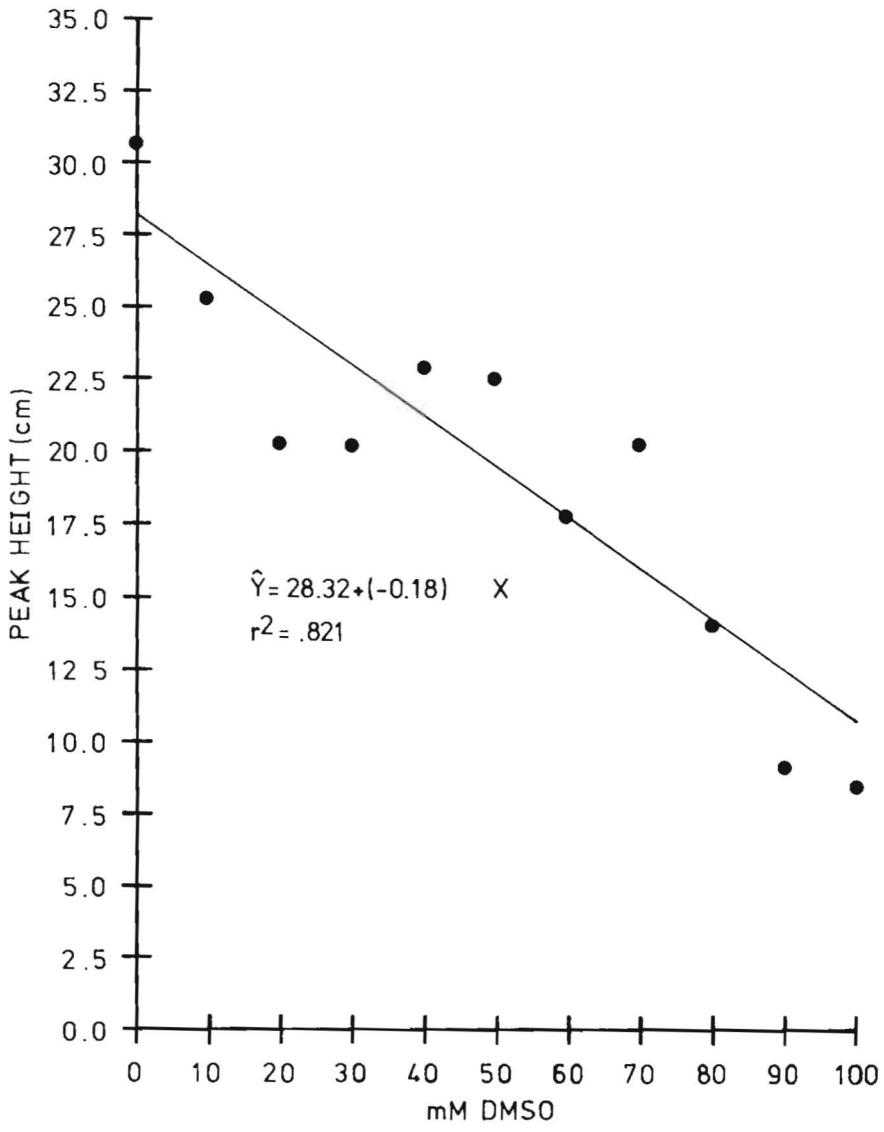

35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12 5
10.0
7.5
5.0
2.5
0.0
PEAK HEIGHT (cm)
$\hat{Y}$= 28.32+(-0.18) X
r^2 = .821
0
10
20
30
40
50
60
70
80
90
100
mM DMSO

Figure 16. The relationship between peak height and concentration of DMSO for the second fraction of aspergilline.

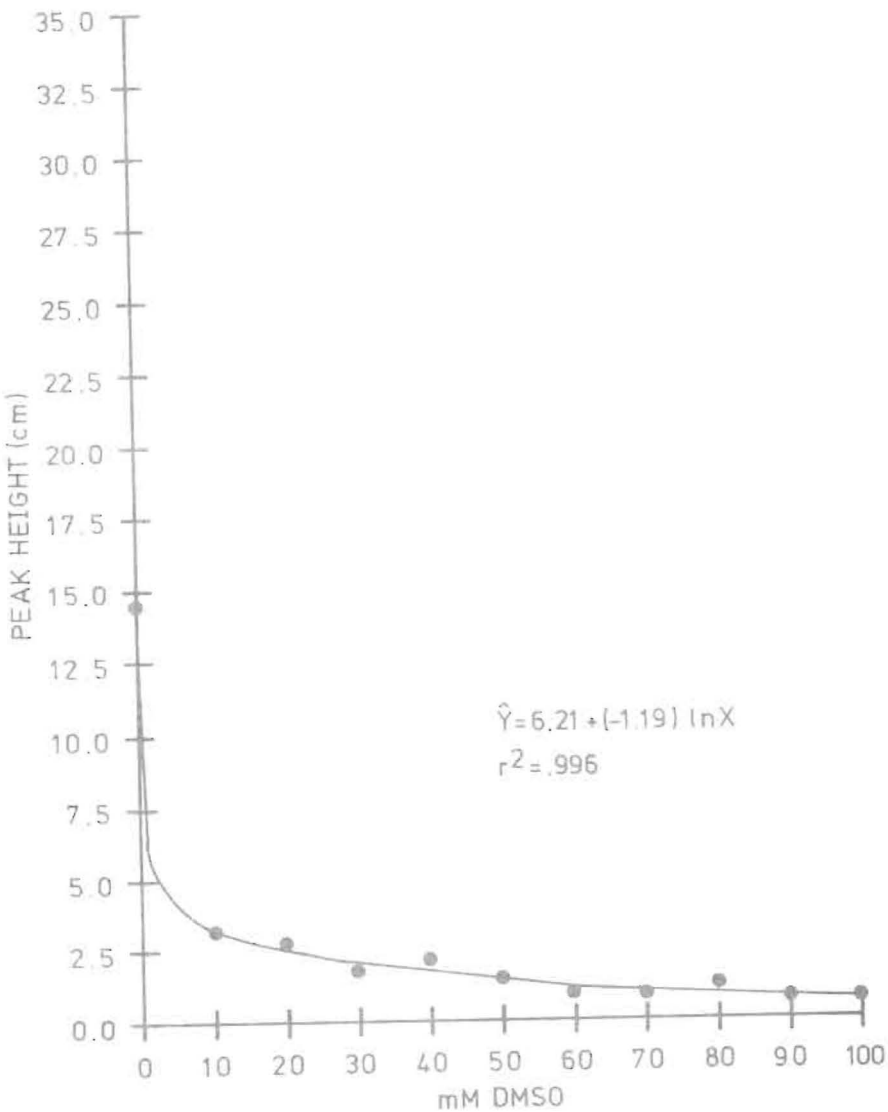
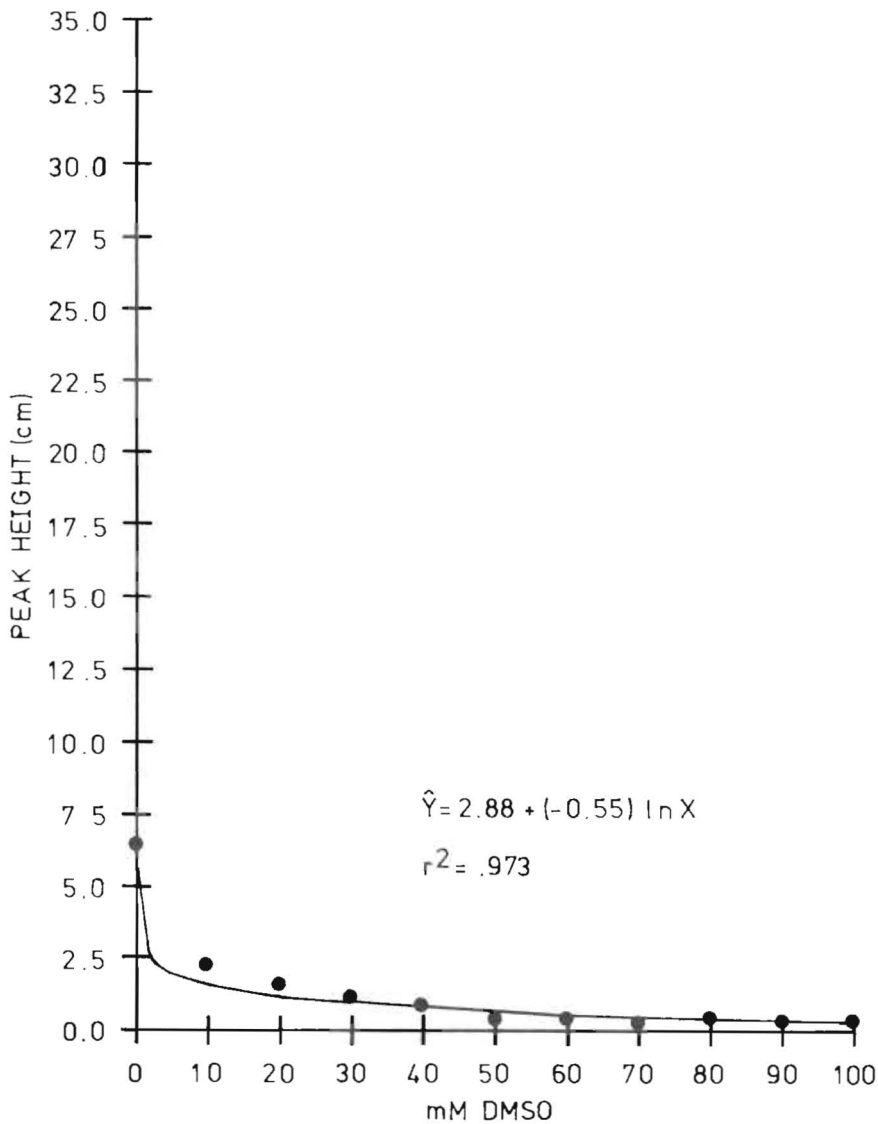

35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
PEAK HEIGHT (cm)
0
10
20
30
40
50
60
70
80
90
100
mM DMSO
$\hat{Y}$=6.21+(-1.19) lnX
r^2=.996

Figure 17. The relationship between peak height and concentration of DMSO for the third fraction of aspergilline.


35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
PEAK HEIGHT (cm)
$\hat{Y}$ = 2.88 + (-0.55) ln X
r^2 = .973
0
10
20
30
40
50
60
70
80
90
100
mM DMSO

research incorporated G-50 Sephadex. It is possible that the smaller pore size of their gel and the more rapid elution rate prevented the final two fractions from being detected. On this basis it would appear that the pigment, aspergilline, found in A. carbonarius is very similar to aspergilline found in A. niger.

The function of aspergilline as a shield against UV irradiation has been previously established. The correlation between the fractional components of aspergilline and their respective function or position in the physiology of the fungus has not been established. Ray and Eakin grew A. niger in the presence of the pigment inhibitors DTP (2,4-Dithiopyrimidine) and DMSO. Conidia grown in the presence of DTP yielded a low molecular weight, green pigment and a relatively high molecular weight brown pigment. Conidia grown in the presence of DMSO yielded a brown pigment similar to that of the DTP treated conidia. They theorized that the green and brown pigments were actually precursors to the higher molecular weight native pigment. However, their investigation was concerned with only the first fraction of the pigment. It is likely that the first fraction is, in fact, the final pigment product and that fractions two and three are the naturally occurring pigment precursors. Further investigation is needed to verify or refute this statement.

Although exclusion gel chromatography was used successfully by Barbetta et al (1967), the technique can be costly and time consuming. In this study a variety of techniques were explored in an effort to establish a more convenient but reliable method for the analysis of aspergilline.

Moving boundary electrophoresis was first introduced in 1892. Until 1937, when the technique was improved, it was given little attention.

During World War II its potential for use in scientific investigations was explored and zone electrophoresis was developed. Zone electrophoresis requires a semisolid or porous supporting medium. Movement of components is accomplished by placing the sample in an electric field. Separation of sample components placed within the field is based on: type and degree of surface charges, molecular shape, medium viscosity, temperature, type of support media, amount of current, pH and concentration of buffer. Supports must be relatively inert to prevent interference with molecular migration. In order to clearly establish individual components of a given sample of separation of bands by approximately one millimeter is required to allow each band to be identified as a unit distinct unto itself. The more bands which must be moved, the greater the distance that must be traveled in order to achieve resolution of individual units. Because the electrophoretic procedures are affected by a wide variety of variables, results can be difficult to predict. Electrophoretic methods are usually established empirically and perfected to give accurate and reproducible results. Advantages to zone electrophoresis include: simplicity, low cost, relatively small time investment and it is both analytical and preparative.

In an effort to establish a procedure utilizing zone electrophoresis which was adaptable to the separation of component fractions of aspergilline, a variety of methods were explored. Only the results of general techniques will be discussed here under headings of the support type used.

Sepraphore III, Cellulose Polyacetate Strips

Sepraphore III was initially selected for its convenience and uniform pore distribution and size. It can easily be cleared for optical scanning

or it can be eluted or dissolved. It has the additional property of reducing running time from the usual 16 to 20 hours on paper to from 20 to 60 minutes.

Cellulose polyacetate strips were prepared according to specifications listed earlier in this paper. Variations were made in buffer pH, buffer type, voltage, application of sample and pigment pH. Although a pattern for bands did emerge the migration did not allow proper identification of component fractions. Best results were obtained with runs at 500 volts utilizing sodium glycinate buffer at pH 10, pigment at pH 5.5 and support at pH 5.5. Three bands were noted moving toward the anode. Streaming occurred between all fractions. The first two fractions did not achieve good separation. The third fraction was a curved band which would not easily lend itself to analysis. Although a number of variations were tried with this method none was successful.

Agarose, type III

Agarose is a medium which allows good separation of component fractions of proteins and other macromolecules. It consists of a purified agar which contains few ionic impurities that could affect sample migration and is held together by a porous support network. This support type separates by charge. It allows the study of larger molecules than other systems.

Agarose for this study was prepared according to standards previously discussed. Variations in procedure were as for cellulose polyacetate strips with an additional variation in gel concentration. Three pigment fractions were noted visually using this method but resolution was poor. However, the bands were more distinct than attempts made with cellulose polyacetate strips.

Agarose-Sephadex Supports

Sephadex is used routinely in exclusion gel chromatography and its properties were discussed earlier in this paper. In an effort to produce better resolution a number of trials were performed with an Agarose gel composed of two per cent Sephadex (w/v). Some separation of component fractions occurred in trials in which barbital buffer was used. Barbital buffer was prepared at pH 8.8 as was the support. The pigment was prepared with barbital buffer at pH 4.5. A voltage of 500 was used. Component separation was not well defined.

Polyacrylamide Gel Supports

Developed in 1959, polyacrylamide gel allows resolution similar to Agarose but has a smaller pore size. Separation occurs by charge density and sieving. Polyacrylamide gel has the benefits of: thermostability, transparency, durability, chemically relatively inert, non ionic and it is easily prepared with a large range of pore sizes.

Methods utilizing Cyanogum-41 polyacrylamide gel were unsuccessful. Separation of pigment fractions into three components did occur in a system which included: sodium glycinate buffer, pH 10; unbuffered pigment, 1 N NaOH; and a 10 per cent Cyanogel prepared with sodium glycinate buffer of pH 6.5. Electrophoresis was continued for 33 minutes. Streaming was prevalent and the gel dried and lifted around the edges. Results were unsatisfactory.

Thin Layer Chromatography Supports

This method utilized a support composed of a powdered cellulose adsorbent on a plastic backing. In paper chromatography, separation occurs as a solvent passes over the adsorbent carrying the vaious molecules present in the sample across the support at different rates. Trials

utilizing this support type in the procedure of zone electrophoresis were also unsuccessful. Limited separation occurred with the buffer and the TLC strips at pH 4.5, pigment at pH 10.0 and a voltage of 500. Two bands moved toward the anode and one toward the cathode. After seven minutes streaming occurred. The bands had not migrated sufficiently to allow distinct separation.

Electrophoresis is a technique which must be adapted to an individual situation. It is often impossible to predict the results of any given set of conditions. This study was unable to demonstrate a procedure which was adaptable to the separation of component fractions of the pigment, aspergilline. Other techniques may provide a more reasonable answer to the problem.

SUMMARY

Chromatographic separation of the components of the pigment, aspergilline, of Aspergillus carbonarius yielded three fractions: a large first fraction, a smaller second fraction and even smaller third fraction. When the fungus was inhibited by DMSO in concentrations from 10 mM to 100 mM a decrease in magnitude of these fractions was observed. The greatest effect was noticed in fraction two and fraction three. Ninety-five per cent or more of those fractions were inhibited while less than 60 per cent of fraction one was inhibited. Little is known about the function of the components of aspergilline. It may be that the first component is a composite of fraction two and fraction three. That is, fractions two and three are precursors to the native pigment found in fraction one.

Gel chromatography can be expensive and time consuming to perform. To establish a more simple method for the analysis of aspergilline utilizing zone electrophoresis, a number of procedures were investigated. Support types, buffer pH, and pigment pH among other factors were varied. Results were unsatisfactory. Other techniques may prove to be more adaptable.

LITERATURE CITED

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Assante, G., L. Carmarda, R. Locci, L. Merlini, G. Nasini and E. Papadopoulos. 1981. Isolation and structure of red pigments from Aspergillus flavus and related species grown on a differential medium. Journal of Agricultural and Food Chemistry. 29(4):785-787.

Barbetta, M., G. Casnati, and A. Ricca. 1967. Aspergillina. Rendiconti. Instituto Lombardo di Scienze e Lettere. A. Scienze Matematiche, Fisiche, Chimiche e Geologiche. 101:75-99.

Carley, H.E., R.D. Watson, and D.M. Huber. 1967. Inhibition of pigmentation in Aspergillus niger by dimethylsulfoxide. Canadian Journal of Botany. 45:1451-1453.

Curtis, C.R. 1970. Comparison of UV-induced delay in germination in pigmented and pigment-inhibited conidia of Aspergillus carbonarius. Radiation Botany. 10(2):125-130.

Keeling, R.P. 1984. Personal communication. Emporia State University.

Linossier, G. 1891. L'aspergilline, pigment des spores de l' Aspergillus niger. C.R. Acad. Sci. 112:489-492.

Lysenko, S.V. and S.P. Lyakh. 1977-78. Protective role of pigments against ultraviolet light in fungi isolated from mesosphere. Microbiology. 46(5):704-712.

Matsumoto, J. 1967. Clinical trials of dimethyl sulfoxide in rheumatoid arthritis patients in Japan. Annals of New York Academy of Sciences. 141:560-568.

Ramirez, E. and S. Luza. 1967. Dimethyl sulfoxide in the treatment of mental patients. Annals of New York Academy of Sciences. 141:655-667.

Rammler, D.H. and A. Zaffaroni. 1967. Biological implications of DMSO based on a review of its chemical properties. Annals of the New York Academy of Sciences. 141:13-23.

Raper, K.B. and D.I. Fennell. 1973. The genus Aspergillus. Robert E. Krieger Publishing Co., Inc., Huntington, NY. 686 p.

Ray, A.C. and R.E. Eakin. 1975. Studies on the biosynthesis of aspergillin by Aspergillus niger. Applied Microbiology. 30(6):909-915.

Reid, W.W. 1950. Yellow pigments of the Aspergillus niger group. Nature. 165(4188):190-191.

Sauter, J.B. 1969. The effects of dimethyl sulfoxide on lipids of Aspergillus niger. M.S. Thesis, Emporia State University. 20 p.

Schiller, R.R. 1967. Cryoprotection by dimethyl sulfoxide in the slow freezing and thawing of selected fungi. Research Project. Department of Biological Sciences. Emporia State University. Unpublished.

Teel, M. 1965. Effects of dimethyl sulfoxide upon physiological and morphological factors of selected molds. Research Project. Department of Biological Sciences. Emporia State University. Unpublished.

Wolf, F.A. 1973. Synthesis of various products, especially pigments, by fungi. Elisha Mitchell Scientific Society Journal. 89(3):184-205.

Zajic, J.B. and H.H. Kuehn. 1962. Biosynthesis of yellow pigments by Aspergillus niger. Mycopathologia et Mycologia Applicata. 17:149-158.

Zhdanova, N.N. and B.D. Pokhodenko. 1973. The possible participation of melanin pigment in the protection of the fungus cell from desiccation. Microbiology. 42(5):753-757.