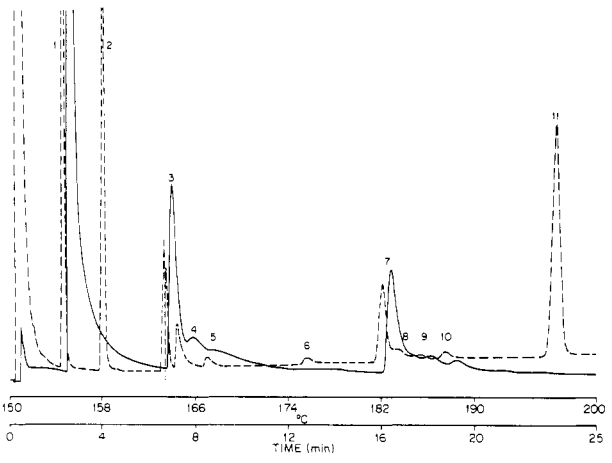

Figure 1. GLC of cigarette smoke condensate amines as tosylamides, 10 ft × 2 mm i.d. 5% SE 30/Gas-Chrom Q (80–100), 150 to 200 °C at 2 °C/min: (peak 1) nicotine; (peak 2) acenaphthene (internal standard); (peak 3) dimethylamine; (peak 4) methylamine; (peak 5) methylethylamine; (peak 6) *n*-butylamine; (peak 7) pyrrolidine; (peak 8) unknown; (peak 9) morpholine; (peak 10) piperidine + Δ^3-piperidine; (peak 11) benzyloxynaphthalene (internal standard); F.I.D (- - -); C.E.C.D. (—).

including the cyclic amines pyrrolidine (Mysliwy et al., 1974) and morpholine (Sander et al., 1968).

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Isolation and Biological Activity of the Pigments of the Mold *Epicoccum nigrum*

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Besides flavipin and humic acid, new types of pigment (pigments A and B) have been isolated from cultures of the mold *Epicoccum nigrum*. Pigments A and B are alcohol-soluble dark orange-red solids which are yellow in dilute solution. Ethanol solutions show a very characteristic absorption maximum at 429 nm and a shoulder at 450 nm. Acidification results in a decrease in absorbance and a shift in the maximum to 390 nm. In aqueous solution the pigments interconvert, A appearing more stable, and give rise to fluorescent products. Exposure of solutions of the pigments to direct sunlight causes a rapid bleaching and the formation of fluorescent products. Flavipin strongly inhibited the growth of *Chlorella pyrenoidosa* while pigment A and humic acid showed no effect. Pigment A strongly inhibited the growth of *Bacillus megaterium* while flavipin and humic acid had little effect.

Epicoccum nigrum is a red pigment-producing mold which grows on and spoils a variety of agricultural products. The carotenoids β-carotene, γ-carotene, rhodoxanthin, and torularhodin (Gribanovski-Sassu and Foppen, 1967) and the yellow pigment flavipin (3,4,5-trihydroxy-6-methyl-*o*-phthalaldehyde) (Bamford et al., 1961) have been identified among the pigments produced by the mold. In addition, brown polymeric "humic acids" consisting of phenolic substances and amino acids have also been isolated from *E. nigrum* cultures, especially from old cultures (Foppen, 1969; Martin et al., 1967). Naumann (1911) described a purple-red pigment produced by this mold, when grown on rice, which was soluble in ethanol and methanol, difficultly soluble in water, and insoluble in the less polar solvents, and which showed a weak absorption band at 448–468 nm and end absorption in the

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blue and violet. In solution the pigment turned yellow upon the addition of acid and red upon the addition of base. On standing, the purple-red pigment changed to a brown-red pigment which no longer changed color on the addition of acid and base. The brown-red pigment appeared to be the same brown-red pigment obtained from older cultures of the mold, which showed a weak band at 461–491 nm and end absorption. The latter pigment was also soluble in the more polar organic solvents and almost insoluble in the less polar solvents. Foppen (1969) carried out an extensive investigation on the pigments of this mold. He found that only on a glucose–yeast autolysate medium did he get dark-red mycelial growth and a wine-red fermentation broth, and only when the inoculum was finely divided rather than in clumps, the latter giving rise to brown pigmentation. The pigments were extractable from the mycelia with ethanol, methanol, or acetone, but it was observed that, when these extracts were concentrated in vacuo, they gave rise to offensive smelling viscous brown-red oils which could only be partially redissolved in the original solvents. Foppen (1969) also observed that the original red pigment was yellow in acid, red at pH 8–9, and yellow again at higher pH values. Foppen (1969) isolated a red pigment fraction from the mold culture fluid by ammonium sulfate precipitation followed by gel filtration or by dialysis followed by gel filtration of the nondialyzable fraction. This material had a molecular weight of ca. 4200 and was shown to contain amino acids and phenols. This material turned yellow in acid and red at pH 7 but showed no pronounced absorption in the visible and ultraviolet region except for end absorption below 240 nm and no pronounced spectral differences at pH 1, 7, and 10. Foppen (1969) concluded that the red pigment produced by *E. nigrum* is an unstable pigment which, during growth of the mold, changes to a brown "humic acid".

Antifungal activity was ascribed to the mold by Campbell (1956) and Eka (1970), and both Bamford et al. (1961) and Raistrick and Rudman (1956) have shown the antifungal activity of flavipin. Bamford et al. (1961) also reported a weak phytotoxic effect of flavipin using pea stems and cress roots. Foppen (1969), however, was unable to demonstrate any significant antibiotic or antifungal activity being produced by his strain of *E. nigrum* against 15 microorganisms, including bacteria, molds, yeasts, and algae.

Our interest in *E. nigrum* was aroused when an extract of a moldy feed sample inhibited the growth of *Bacillus megaterium*. One of the fungi isolated from this material which produced inhibitory metabolites was *E. nigrum*. When a study of these metabolites was undertaken, it soon became apparent that at least part of the activity was due to the pigments produced by the mold. This paper presents some of our findings with regard to the nature of the pigments of *E. nigrum* and their biological activity.

MATERIALS AND METHODS

Organism Used. The *E. nigrum* strain was isolated from a moldy feed sample obtained from H. A. Davis, Analytical Services Laboratory, New Hampshire Agricultural Experiment Station, University of New Hampshire, and identified by J. J. Ellis, Northern Regional Research and Development Division, U.S. Department of Agriculture, Peoria, Ill. It was maintained on a malt extract (2%)–dextrose (2%)–peptone (0.1%)–agar (2%) medium.

Assay Methods. The *Bacillus* paper disk assay was carried out according to the method of Jayaraman et al. (1968) using agar plates seeded with spores of *Bacillus megaterium* (UNH strain) or *Bacillus subtilis* (Difco Laboratories) and 6-mm paper disks (Difco Laboratories). Twenty microliters of solution was applied to each disk.

The *Chlorella* paper disk assay was carried out according to the method of Ikawa et al. (1969) using buffered agar plates seeded with *Chlorella pyrenoidosa* (UNH strain), *Chlorella pyrenoidosa* (Strain 15-2070 from the Carolina Biological Supply Co., Burlington, N.C.), or *Chlorella vulgaris* (Carolina strain 15-2075) and 6-mm paper disks. Twenty microliters of solution was applied to each disk.

Glucostat Spray. For the detection of glucose on thin-layer chromatograms the chromatograms were sprayed with glucostat reagent prepared by dissolving 1 mg of glucose oxidase (Worthington), 0.5 mg of peroxidase (Sigma), and 20 mg of *o*-dianisidine diHCl in 100 ml of distilled water.

Thin-Layer Chromatography (TLC). Thin-layer chromatograms were carried out using either Eastman 6061 Chromagram silicic acid sheets or EM Reagent silicic acid plates (Brinkman Instruments).

Isolation of Flavipin. Flavipin was isolated using the procedure of Bamford et al. (1961) from liquid cultures of *E. nigrum*. The product was purified by recrystallization from acetone and vacuum sublimation at 140 °C (Raistrick and Rudman, 1956) and the pure product obtained as a yellow microcrystalline powder. Approximately 0.53 g of crude flavipin was obtained from 3000 ml of culture filtrate. This material yielded 47% of pure flavipin after sublimation with a melting point of 229–231 °C dec. This compares favorably with 233–234 °C dec reported by Raistrick and Rudman (1956) and 228–229 °C dec reported by Bamford et al. (1961). Anal. Calcd for flavipin ($C_9H_8O_5$): C, 55.1; H, 4.1; N, 0. Found: C, 54.7; H, 4.2; N, 0.

The compound also gave a precipitate with 2,4-dinitrophenylhydrazine and a steel blue color with ethanolic $FeCl_3$ as reported by Raistrick and Rudman (1956) for flavipin. The principal peaks of the infrared spectrum (Burge, 1972) corresponded with the principal peaks for flavipin reported by Raistrick and Rudman (1956).

Isolation of Humic Acid. Humic acid is an ill-defined dark brown polymeric substance containing phenols and amino acids generally resulting from the decomposition of organic matter. The mold was grown in a medium containing glucose (10 g) and yeast extract (5 g) per liter. Test tubes containing 5 ml of medium were inoculated from slants and incubated at 25 °C under fluorescent lighting for 5 days. This was used to inoculate 100 ml of medium in 500-ml Erlenmeyer flasks. The flasks were incubated at 25 °C for 3 days on a rotary shaker under fluorescent lighting. At the end of the growth period the culture was filtered through Whatman No. 1 paper and the filtrate collected and lyophilized to minimize decomposition. The residue was taken up in 50 mM Tris buffer (pH 7.5) and the solution dialyzed in the cold against the same buffer for 3 days. The nondialyzable material was placed on a Sephadex G-25 column and the brown band collected and lyophilized. The residue from lyophilization was taken up in 10 ml of distilled water, dialyzed against distilled water for 3 days, and lyophilized. The resulting nondialyzable water-soluble brown pigment showed a molecular weight of 4350 ± 350 by thin-layer gel filtration using Sephadex G-50. The ultraviolet and visible spectra in water showed only end absorption below 240 nm and a slight shoulder at 280 nm. The properties correspond to those reported for "humic acid".

Isolation of Alcohol-Soluble Pigments from Malt Extract–Agar Cultures. The mold was cultured on a

Table I. Yields and Activities of the Alcohol-Soluble Pigment Fractions Obtained from Malt Extract-Agar Cultures of *E. nigrum*

Fraction	Yield per flask, g	Test solvent	Net zone of inhibition, mm,[a] at concn (mg/ml)			
			10	1	0.1	0.01
Crude pigment mixture	1.3-1.9	MeOH	7.5	0		
Crude pigment mixture		MeOH	12[b]	3[b]		
Fraction I	0.12-0.19	$CHCl_3$-MeOH (95:5)	9	0		
Fraction II	1.2-1.7	MeOH	3.5	0		
Fraction II-A-1	0.13	MeOH	0			
Fraction II-A-2	0.35	MeOH	7.5	0		
Fraction II-A-3	0.42	MeOH	9	0		
Fraction II-A-4	0.28	MeOH	13	Trace		
Active pigment mixture	0.05	MeOH		4.5		
Pigment A	0.01	MeOH		7.2	3.1	0
Pigment B	0.006	MeOH		7.2		

[a] Total diameter of inhibition zone less disk diameter against *B. subtilis*, the organism used to follow the fractionation. [b] From a culture grown in the dark.

solid medium consisting of malt extract (Difco) (20 g), peptone (Difco) (1 g), glucose (20 g), and agar (Difco) (20 g) in water (1000 ml). Two hundred milliliters of the medium was poured into each 2.5-l. low-form culture flask, autoclaved, and inoculated with 5 ml of washings from an agar slant of the mold. Cultures were incubated for 10–14 days at 25 °C under fluorescent lighting or in the dark. At the end of the growth period each flask was extracted overnight with 500 ml of ethanol. This was followed by two 3-h extractions with 300-ml portions of ethanol. The combined ethanol extracts were concentrated to dryness in vacuo to yield the crude *pigment mixture*. Pigment mixture from five flasks was extracted with 500 ml of $CHCl_3$–MeOH (95:5). This was followed by two further extractions with 250-ml portions of the same solvent. The $CHCl_3$–MeOH soluble fractions were combined, filtered, and taken to dryness in vacuo to yield fraction I. The $CHCl_3$–MeOH insoluble residue was dried and extracted with 300 ml of ethanol. The extract, after filtering, yielded fraction II. Fraction II (from five flasks) was placed on a 3.8 cm diameter alumina column prepared by packing 120 g of alumina (80–200 mesh, Fisher Scientific Co.) into the column and washing with 500 ml of ethanol. The column was developed batchwise with the following solvent sequence: 1000 ml of 95% ethanol (fraction II-A-1); 1000 ml of 95% methanol (fraction II-A-2); 3000 ml of 95% methanol (fraction II-A-3); 1000 ml of 80% methanol (fraction II-A-4). Fraction II-A-4 had the highest level of activity. It was found that absorption at 429 nm was associated with the highest activity. Therefore absorption was routinely used in the purification of the pigment. Fraction II-A-4 (100–300 mg) was dissolved in 1–2 ml of methanol and applied to a silicic acid column (2.2 cm diameter) prepared by adding a slurry of 50 g of silicic acid (Fisher Scientific Co., activated at 105 °C for 1 h) in $CHCl_3$–MeOH (70:30) to the column and washing with 300 ml of the same solvent. The column was developed with $CHCl_3$–MeOH (70:30) and 6-ml fractions were collected. Each of the yellow fractions was chromatographed on EM Reagent TLC plates in acetone–MeOH (1:1) and sprayed with glucostat reagent. Those yellow fractions which were free of glucose were combined and taken to dryness in vacuo to give the *active pigment mixture*. The *active pigment mixture* was further fractionated by TLC on either the Eastman or EM Reagent plates by applying 5 mg per plate and developing in acetone–MeOH (1:1). The yellow-orange bands were scraped off the plates, the pigments eluted with ethanol, and the ethanol extracts concentrated to dryness in vacuo to give pigment A (the more abundant slower band) and pigment B (the less abundant faster band). A column procedure was employed to separate the pigments. Silica gel H (EM Reagent, Brinkmann) was first prepared by washing with acetone and then methanol, drying in vacuo, and activating by heating in an oven at 105 °C for 3 h. A column (0.6 cm diameter) was filled with the dry support and tightly packed by tapping the sides and applying pressure to the top with a glass rod. The *active pigment mixture* dissolved in a minimum of acetone–MeOH (1:1) was applied to the top of the dry column and developed with 40 ml of acetone–MeOH (1:1) to yield the faster moving yellow-orange pigment band (pigment B). Subsequent development with 20 ml of methanol eluted the more abundant slower moving yellow-orange band (pigment A).

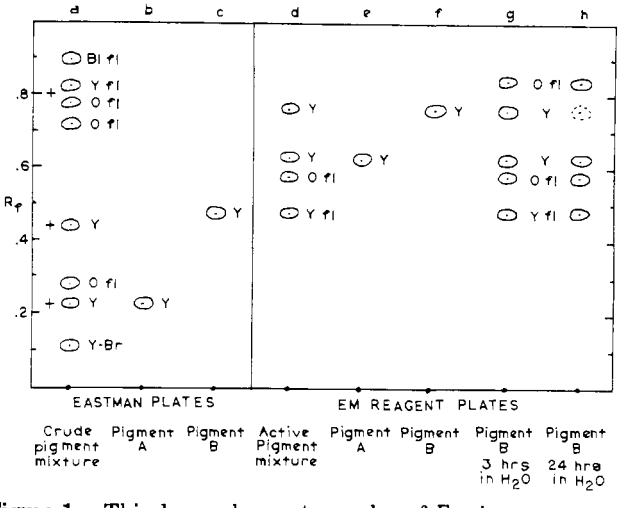

Figure 1. Thin-layer chromatography of *E. nigrum* pigment fractions; solvent, acetone–MeOH (1:1); abbreviations: Y, yellow; O, orange; Bl, blue; Br, brown; fl, fluorescent; +, active against *B. subtilis*.

RESULTS AND DISCUSSION

Pigment Production on Malt Extract–Agar. *E. nigrum* appeared to grow rapidly and reproducibly on the malt extract–agar medium. After 10 days, a heavy brown-red mycelial mat had developed on the surface of the medium, red pigments had diffused into the agar, and small beads of intense orange-red solution were observed on the surface.

Results of the fractionation procedure are shown in Table I. Ethanol extraction of the culture medium gave

an orange-red extract containing the crude pigment mixture. TLC of this extract showed a large number of pigment and fluorescent components (Figure 1a). When zones from the TLC plates were scraped off and eluted with ethanol, and the extracts tested against *Bacillus subtilis*, inhibitory activity was found to correspond with the two yellow bands at R_f 0.23 and 0.44. Inhibitory activity was also observed in the R_f 0.8 region. Extraction of the crude pigment mixture with $CHCl_3$–MeOH (95:5) solubilized about 10% (fraction I) while the greater part was subsequently extracted by ethanol (fraction II). A small amount of material did not redissolve in the ethanol. It was nondialyzable and probably "humic acid" in nature. Although fraction I was more active on a weight basis than fraction II, the bulk of the activity was in fraction II. TLC of fraction II gave a complex chromatogram similar to the crude pigment mixture. Activity against *B. subtilis* was localized at R_f 0.23 and 0.44 (Figure 1a system). When fraction II was chromatographed on the alumina column, the highest activity corresponded with a strong yellow-orange band (fraction II-A-4) which was eluted with 80% methanol. Fraction II-A-4 was found to be highly contaminated by glucose from the medium. Chromatography on silicic acid removed the glucose and gave an active pigment mixture consisting of two pigments and some fluorescent decomposition products (Figure 1d). The fluorescent products were more visible on the EM Reagent plates than on the Eastman plates. Fractionation of the active pigment mixture by preparative TLC or on silica gel H columns gave pigment A (more abundant) and pigment B as dark orange-red soft amorphous powders. Fresh solutions were shown to be chromatographically pure (Figures 1b, c, e, f).

The mold also grew well on cooked rice, producing a complex pigment mixture which could be extracted with alcohol and from which pigment A could be isolated by a series of silica gel chromatographic steps (Burge, 1972).

Properties of Pigments A and B. Pigments A and B are readily soluble in polar organic solvents such as ethanol, methanol, and acetone, or in aqueous base, and are only slightly soluble in nonpolar solvents such as hexane or benzene, or in water or aqueous acid. An elementary analysis shows approximately 58% C, 8% H, and no N, halogen, or S. The pigments therefore contain a high percentage of oxygen as one might suspect from their solubility behavior.

It was found that the pigments are unstable under aqueous conditions. When a fresh aqueous solution of pigment B was allowed to stand in the dark, the gradual appearance of pigment A and several fluorescent substances was noted (Figure 1g). On longer standing pigment B disappeared almost completely (Figure 1h). When a fresh aqueous solution of pigment A was allowed to stand, it was observed that a small amount of pigment B and the same fluorescent products were formed. This suggests that pigments A and B are in equilibrium with each other, with the equilibrium favoring the formation of A and that these in turn give rise to fluorescent decomposition products. The pigments appeared to be more stable in methanol in the dark, but when methanol solutions were exposed to direct sunlight, they rapidly faded and fluorescent products were formed.

Pigments A and B both showed the same absorption spectrum with a maximum at 429 nm and a shoulder at 450 nm (Figure 2 shows the pigment A spectrum). Log a at 429 nm (for 1% solutions in ethanol) was generally 2.84–2.94 for the best preparations. The 429-nm peak is so prominent and characteristic that it is evident even in

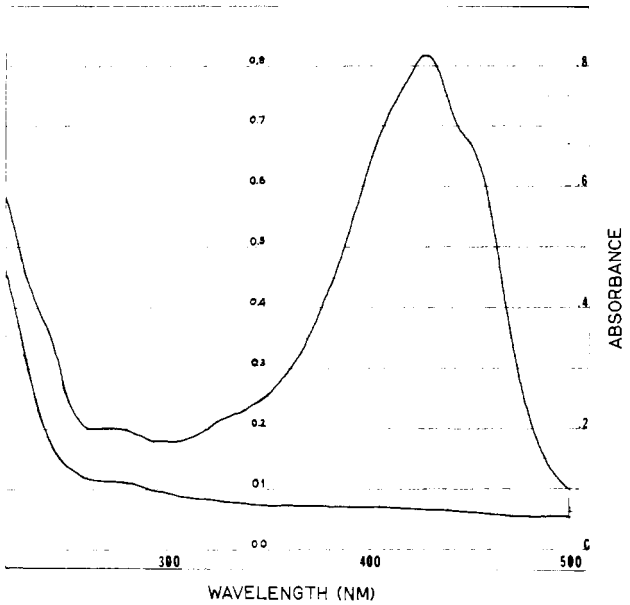

Figure 2. Ultraviolet and visible spectrum of pigment A in ethanol (10 μg/ml). (The lower trace is solvent only in the sample cell.)

crude mixtures containing these pigments. The spectrum of these pigments closely resembles, except for the position of the peak, the absorption spectra of such polyene macrolide antibiotics as dermostatin (Narasimhachari and Swami, 1970) and flavomycoin (Schlegel and Thrum, 1968) and the carotenoid ethyl β-apo-8'-carotenoate (Vetter et al., 1971), each of which contains a polyene system conjugated to a lactone or ester function.

When a neutral aqueous solution of pigment A or B was adjusted to pH 12, the solution remained yellow and no change in the absorption spectrum was noted. However, when the pH was adjusted to 2 the color changed from a yellow to a tan, the absorbance dropped to about half its original value, and the absorption maximum shifted down to 390 nm. When the pH of the acidified solution was brought back up to 12, the original spectrum was restored. These pigments are, therefore, clearly different from the alcohol-soluble red pigments described by Naumann (1911) and Foppen (1969) which were yellow in acid and red in base, and they represent a new class of biologically active pigments which have not heretofore been described for *E. nigrum*.

Infrared spectra of the pigments taken in KBr pellets showed very little fine structure. Spectra of films deposited on NaCl plates from an acetone solution gave sharper peaks, but the sharpest most reproducible spectra were obtained when the pigments were spread directly on NaCl plates (Figure 3). Pigments A and B appear to give the same infrared spectrum. A carbonyl function is indicated by the band at 1730 cm^{-1}, and the band at 1270 cm^{-1} may be due to C–O stretching in an acid or ester. Hydroxyl groups are indicated by the broad band at 3300 cm^{-1}, and the prominent bands in the 1200–1000-cm^{-1} region may be due to C–O stretching in alcohols or ethers. A conjugated double bond system is indicated by the broad band at 1600 cm^{-1}, but the absence of strong bands in the 900–650-cm^{-1} region would indicate the absence of an aromatic system. CH_3 groups are indicated by the band at 2970 cm^{-1} and C–CH_3 by the band at 1380 cm^{-1}. CH_2 groups are indicated by the bands at 2930, 2860, 1460, and 730 cm^{-1}.

The amorphous nature of the pigments, their instability, and the difficulties encountered in their purification have

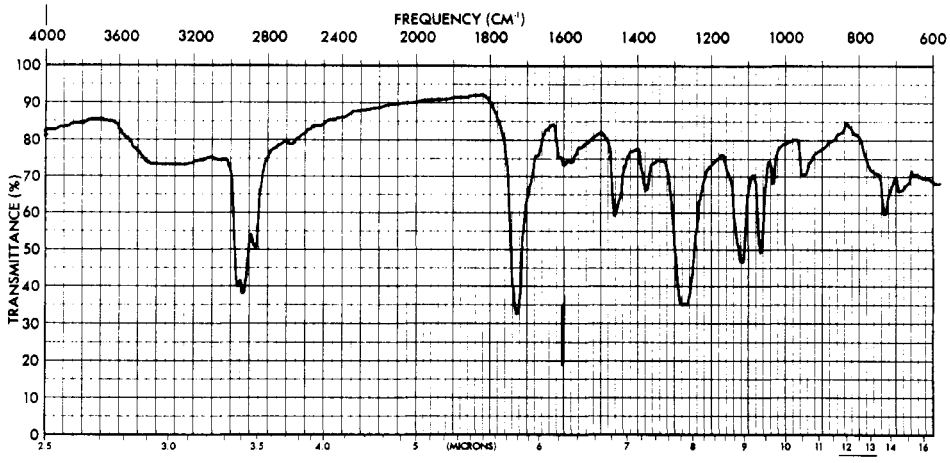

Figure 3. Infrared spectrum of pigment A (contains some B). Taken of a thin film smeared on a sodium chloride plate on a Perkin-Elmer Model 710A infrared spectrophotometer. Calibration point is at 1601 cm^{-1}.

Table II. Growth Inhibitory Activity of *E. nigrum* Pigments against *Chlorella* and *B. megaterium*

Pigment	Organism	Test solvent	Net diam. inhibition zone, mm,[a] at concn (mg/ml) 1	0.1	0.05
Flavipin	*C. pyrenoidosa* (UNH)	Ethanol	15.6	3.7	1.4
	C. pyrenoidosa (Carolina)	Ethanol	17.5	5.0	2.3
	C. vulgaris (Carolina)	Ethanol	3.4	0	
	B. megaterium	Ethanol	3.4		
Humic acid	*C. pyrenoidosa* (UNH)	Water	0		
	B. megaterium	Water	0		
Pigment A	*C. pyrenoidosa* (UNH)	Ethanol	2		
	B. megaterium	Ethanol		8.2	4.8

[a] Total diameter of inhibition zone less disk diameter. Average of three or more determinations.

impeded the characterization of these interesting compounds. Further work is in progress.

Activity of the *E. nigrum* Pigments against *Chlorella* and *B. megaterium*. Table II shows the activity of the various *E. nigrum* pigments against *Chlorella* and *B. megaterium*. Flavipin appears to be very active against *C. pyrenoidosa*. Not many substances have been found to inhibit growth at 0.1 mg/ml (Sullivan and Ikawa, 1972). *C. vulgaris* was considerably less sensitive to the compound. The diminished sensitivity of *C. vulgaris* as compared to *C. pyrenoidosa* to a number of compounds has been previously noted (Sullivan and Ikawa, 1972). Flavipin thus appears to have some phytotoxic effects. Humic acid and pigments A and B showed little or no inhibitory activity against *C. pyrenoidosa*.

Against *B. megaterium* flavipin showed weak inhibitory activity and humic acid no activity at 1 mg/ml. On the other hand pigment A appeared to be highly active and comparable in activity to a number of other toxic fungal metabolites (Buckelew et al., 1972). It is also noted that pigment A appears to be more active against *B. megaterium* than *B. subtilis*.

Pigment A also exhibits toxicity to brine shrimp (*Artemia salina*) and weak antifungal activity (McGrattan, 1975).

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