ISOLATION AND PARTIAL CHARACTERIZATION OF AVENACIN, AN ANTIBIOTIC-LIKE SUBSTANCE FROM OATS

Thesis by

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ABSTRACT

A procedure has been described for isolation and bioassay of a potent antifungal substance from the roots of oat seedlings. The purified substance has been named avenacin. Avenacin gives half-maximal inhibition of Neurospora at a concentration of 0.4 μg/ml and has been found to inhibit a number of plant pathogenic fungi and Mycobacterium tuberculosis at various concentrations less than 50 μg/ml.

Microanalysis of avenacin has given the following composition: C, 59.28%; H, 7.75%; N, 1.41% and O by difference, 31.56%. The molecular weight is 1000 and the empirical formula is $C_{44\pm1}\ H_{78\pm2}\ O_{20\pm1}\ N$. The ultraviolet absorption spectrum of avenacin has maxima at 353, 255 and 222 mμ in methanol.

Alkaline hydrolysis of avenacin liberates the chromophore, N-methyl anthranilic acid, which is esterified to an aglycone in intact avenacin.

Brief acid hydrolysis of avenacin liberates a mixture of oligo- and monosaccharides and a conjugate of N-methyl anthranilic acid with the aglycone. Subsequent basic hydrolysis of the conjugate frees the aglycone and N-methyl anthranilic acid. Further acid hydrolysis of the oligo- and monosaccharide fraction gives a hexose-pentose mixture in the molar ratio of 2:1. Chromatography in methyl ethyl ketone-acetic acid water (30:3:10 v/v) did not separate the pentose from arabinose or the hexose from glucose and galactose. Evidence concerning the structure of avenacin is summarized and biological properties are discussed.

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I. INTRODUCTION

Little is known of the biochemistry of host-pathogen interaction in diseases of higher plants. As a contribution to the knowledge of this field a study has been made of an anti-fungal substance from the roots of oats (Avena sativa). Work of Turner (1), and Mitchell and Turner (2) indicated that such a substance was present. The procedure for isolation and some of the chemical and biological properties are discussed in this thesis. To denote something of its origin and glycosidic nature the name "avenacin" is suggested for the substance. Avenacin may be useful in the treatment of plant and animal microbial diseases.

II. REVIEW OF PERTINENT LITERATURE

Plant Antibiotics

Waksman (3,4), in defining the word "antibiotics," has excluded all substances not of microbial origin. At the same time the name "antibiotic-like substance" was suggested for agents of non-microbial origin in general, and "plant antibiotics" and "phytoncide" were proposed for substances from higher plants. Three reviews on antibiotics (5,6,7) in Annual Reviews of Biochemistry have adhered rigorously to Waksman's definition excluding higher plants while five others (8,9,10,11,12) have disregarded the semantics in question and discussed substances from higher plants.

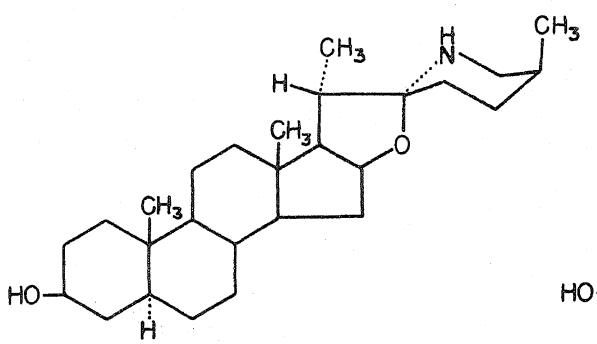
Several reviews dealing especially with antimicrobial substances from higher plants have been written. Kavanagh (13) reviewed the occurrence of antibacterial substances in green plants until 1947. Abraham's (14) chapter on substances from seed plants in the book on antibiotics edited by Florey and others has an enjoyable historical introduction illuminated with excerpts from early herbals on the use of plants for treatment of microbial infections. Also included are a survey of plants for antibiotic substances and the available chemical and biological properties of twenty-five active plant materials. Skinner (15) has listed surveys of higher plants for antibiotic activity up to 1953 along with the methods for preparation and the properties of antibiotically active

materials from seventy-one plants. A book by Köhler (16) on plant antibiotics lists thirty-eight antimicrobial substances and some of their properties. All of the above reviews overlap to a great extent. Skinner's review (15) is the most extensive on the subject of plant antibiotics.

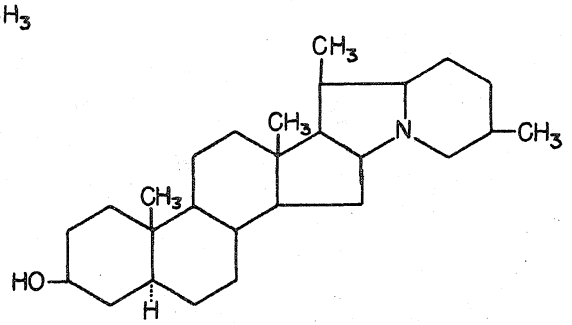
There are numerous papers dealing with observations of antimicrobial substances in extracts, saps, juices, and exudates of plants; but since in all cases there is too little chemical information to show them related to this thesis or they are clearly not related to it, they have been excluded. The paper of Maruzzella and Freundlich (17) surveying the antimicrobial activity of extracts of seeds from drug plants has a good bibliography including recent surveys of plants and seeds.

Several antifungal substances from plants have been found as the result of efforts aimed at understanding the biochemical basis of disease resistance and susceptibility in agricultural plants. For instance, Irving, Fontaine, and Doolittle (18) found that sap from tomato plants inhibited growth of Fusarium oxysporium f. lycopersici, the fungus pathogen of tomato wilt. They first called the material lysopersicin but later changed its name to tomatin (19). Tomatin was found to be active against bacteria, plant pathogenic fungi, and certain human dermatophytes. Its role in wilt resistance, however, is obscure since both resistant and non-resistant varieties showed tomatin activity. The crystalline active substance, given the name

tomatine, was inactive against bacteria but active against several plant and animal pathogenic fungi at concentrations of 0.01 to 0.05 mg/ml (20). From elemental analyses the molecular weight was calculated to be 1050 per nitrogen atom. Acid hydrolysis of tomatine gave an aglycone, named tomatidine, and sugars. The carbohydrate portion of the molecule was estimated to be the size of a tetrasaccharide by difference of the weight of tomatin hydrolyzed and amount of tomatidine isolated. Ma and Fontaine (21) showed that one molecule of tomatine contains one residue each of xylose and galactose and two of glucose. Duggar and Singleton (11)* have reviewed the chemical transformations of tomatidine (I) that show the presence of a steroid nucleus similar to that of demissidin (II).



Tomatidine (I)

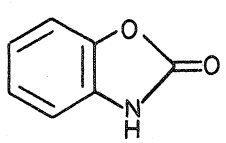


Demissidin (II)

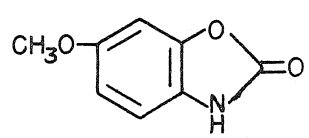
Virtanen and co-workers (22) investigated rye, wheat, maize, alfalfa and red clover for antifungal activity and

*See (53) for an even more recent review.

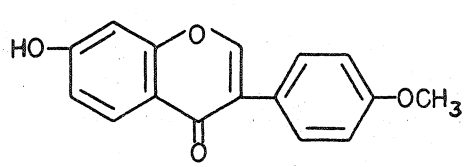
found that juices from all were active against Fusarium nivale and Sclerotina trifolium. The active substance from rye seedlings was shown to be 2(3)-benzoxazolinone (III) by comparison of isolated and synthetic material. Wheat and maize plants were found to have 6-methoxy-2(3)-benzoxazolinone (IV). Both substances are equally active against fungi, giving half inhibition at concentrations from 0.2 to 0.5 mg/ml. The later substance has been implicated also in resistance of corn to European corn borer. The factor from red clover was identified as 7-hydroxy-4'-methoxy-isoflavone (V) (23), and was found to be active at the same order of concentration as the benzoxazolinones.



2(3)-Benzoxazolinone (III)



6-Methoxy-2(3)-benzoxazolinone (IV)



7-Hydroxy-4'-methoxy-*iso*flavone (V)

The substances studied by Virtanen and co-workers can

hardly be called antibiotic-like because the concentrations needed for fungus inhibition are high. Nevertheless, they may play a role in disease resistance since they occur in high concentrations in plants.

Turner (24,25), in studying the nature of resistance of oats to Ophiobolus graminis, the "take-all" pathogen of wheat and barley, found that saps from oat seedlings contained substances that inhibited O. graminis. Ophiobolus graminis var. avenae, capable of infecting oats as well as wheat and barley, was not inhibited. The active material was found to be insoluble in non-polar solvents and soluble in water, acetone, and methanol. Benzene and chloroform washed aqueous extracts of roots were active. A partially purified material was obtained by boiling an aqueous extract of oat seedling leaves, washing the coagulum with ether and ethanol, and extracting with methanol (1). The material obtained from leaf sap was thermostable, base stable, and acid labile. At a level of 10 μg/ml the partially purified material inhibited twenty-one of forty-five fungi tested. Neither growth of bacteria nor respiration of oat and barley roots was inhibited by the same concentration of material. Respiration of fungi was inhibited by concentrations of oat-leaf material ten to twenty times that needed for fifty percent growth inhibition. Glutathione and ascorbic acid had protective action against inhibition of growth and respiration. The semi-pure leaf material was equally effective against O. graminis isolated from oats or wheat in

contrast to oat-root juice which inhibited wheat isolates but not oat isolates. It was not certain that the inhibitory substances in root and leaf juices were identical. Both substances were methanol soluble. Since the methanol soluble fraction of root extracts contained growth stimulating substances the lack of inhibition of oat isolates may have been due to selectively stimulatory substances.

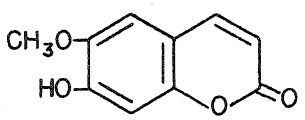
Turner and Mitchell (2) further purified the active substance from plant meristems until fifty percent inhibition could be obtained against O. graminis at 0.2 μg/ml and against Neurospora crassa at 1.0 μg/ml. Turner (26) found that the active substance had chromatographic properties similar to an unidentified blue-fluorescent substance from oat roots studied by Goodwin (27). Likewise, the ultraviolet absorption spectrum was found by Mitchell (28) to be similar to that of Goodwin's substance.

Fluorescent Substances in Plants

Goodwin (29) has reviewed the subject of fluorescent substances in plants and has made an extensive study of their occurrence in roots (30). Roots from 135 species of vascular plants were examined under an ultraviolet lamp. All but six species of ferns were fluorescent and extracts of even those were fluorescent. The fluorescent substances from oat roots were investigated in greater detail than others. The variation of fluorescence with pH was studied on three fractions obtained by adsorption of acetone extracts on alumina followed by elution

with water. Fraction "A" (tightly bound to alumina) had low fluorescence intensity at pH 2, increased to high intensity at pH 4, and remained constant to pH 12. Fluorescence of fraction "B" (weakly bound to alumina) increased steadily from pH 1 to pH 11. Fraction "C" (moderately bound to alumina) fluorescence did not change appreciably with pH.

Goodwin studied the biogenesis of the fluorescent root substances and found that in five days there was a two hundred and fifty-fold increase in total fluorescent substances as compared with dry seed. While the absolute amount of fluorescence increased the relative amount of fraction "A" decreased relative to fractions "B" and "C". Subsequently (31) fraction "B" was identified as scopeletin (VI) (6-methoxy-7-hydroxy coumarin) by comparison of the ultraviolet spectra and pH versus fluorescence curves of the isolated substance and an authentic sample.



Scopeletin (VI)

Goodwin and Pollock (27) showed that fraction "A", which they called "root-tip glycoside," is localized in the root meristem while free scopeletin and its glycoside (the main component of fraction "C") are found only in older cells. The root-tip glycoside was purified by column chromatography on Decalso columns irrigated with eighty percent ethanol, or on paper

using n-butanol-acetic acid-water (100:20.5:50 v/v). The substance was insoluble in dry organic solvents and water but moderately soluble in aqueous organic solvents. Acid hydrolysis in 1.0 N hydrochloric acid for one hour at 100°C gave a fluorescent aglycone and sugars, one of which co-chromatographed with glucose. Tests for nitrogen, sulfur, phosphorus, and halogens were negative. The ultraviolet absorption spectrum of the root-tip glycoside had maxima at 226, 255, and 360 mu. Neither the pH versus fluorescence curve nor the ultraviolet spectrum could be identified with any of a number of coumarin derivatives or other fluorescent biochemicals examined by Goodwin (32,33,34,35).

Eberhardt (36), using fluorescence as a means for identifying roots, studied Goodwin's root-tip glycoside. The only chemical observation reported by Eberhardt was that β-glucosidase did not split the root-tip glycoside though it hydrolyzed scopeletin glycoside.

Although the fluorescent root-tip glycoside was not shown to have antifungal activity by either Goodwin and co-workers or Eberhardt, it is almost certainly avenacin.

Bioassays for Antimicrobial Substances

An excellent review of the methods of assaying plant materials for antimicrobial substances has been given by Skinner (15). Bioassay techniques may be broadly divided into two types: diffusion methods and dilution methods.

Diffusion techniques have been extensively used for screening procedures because they are relatively convenient. Several variations are commonly employed, but all involve measurement of diameter of a circular growth inhibition zone surrounding a localized sample of suspected inhibitor on a uniformly inoculated solid plate. The cylinder-plate method utilizes small glass or vitreous porcelain cylinders set on inoculated agar plates to contain the sample. In the hole-plate method a depression in the agar plate replaces the cylinder as container. Still another alternative is the filter paper disc method in which uniform discs of filter paper are impregnated with the solution to be tested and laid on the plate. Interpretation of any of the diffusion methods can be complicated when the active substance is water insoluble or does not diffuse through agar. Although these methods can be made quantitative, as for example, the cylinder-plate method for penicillin has been, they are not as reliable as dilution methods.

Dilution methods can be used with either solid or liquid media for detection and estimation of inhibiting substances. They involve the direct mixing of the sample into the nutrient medium and comparison of the amount of growth with that of a control. The end-point method uses the concentration of sample necessary to prevent any growth in a given time as a measure of activity.

Ryan, Beadle and Tatum (37) showed that N. crassa could be used in the agar dilution technique as a bioassay for growth

substances. They used Neurospora mutants that lacked para aminobenzoic acid and measured stimulation of the rate of growth in horizontal growth tubes by various materials containing the growth factor. Emerson and Cushing (38) used a similar technique to measure growth responses to sulfanilamide. The competitive inhibition of amino acid metabolism by amino acid analogs was examined by Mitchell and Niemann (39) using the same procedure as Emerson and Cushing. Recently, Rauen, Hess, and Mechery (40), using this procedure, studied a number of forms of inhibition caused by several inhibitors and the release of inhibition by competitive and non-competitive antagonists.

III. METHODS AND MATERIALS

Bioassay Procedure

Neurospora crassa strain 5912-2A was used throughout as the bioassay organism. Inoculum material for the assay experiments was prepared by seeding 30 ml portions of minimal nutrient agar (37) with conidiospores from sterile cultures. The seeded agar was poured into 20 x 100 mm petri dishes and incubated from two to three days at 25°C until a thick mycelial growth was formed. These cultures were stored in a refrigerator at 4°C and remained usable for several weeks.

Triplicates of samples to be assayed were put into clean 15 x 150 mm culture tubes and suspended in 4.0 ml aliquots of molten nutrient agar. Immediately the contents of the tubes were mixed by shaking and poured into 10 x 50 mm petri dishes. Four millilmeter diameter cylinders were cut from the inoculum plate and placed mycelial side down in the center of the solidified assay plates. After fifteen hours incubation at 25°C the diameters of cultures were measured to the nearest millimeter. The petri dishes were inverted during incubation to prevent aerial growth on the inoculum cylinder from falling on the agar surface and causing uneven growth. Usually the perimeter of the culture was uniform, but in cases where irregular growth occurred several diameters were measured and an average recorded. Results were expressed as percentage growth compared to a control which contained a reagent blank when

necessary. In most cases the percentage inhibition was also calculated by subtracting percentage growth from one hundred.

When samples to be assayed were in volumes larger than 0.1 ml or contained toxic solvents the procedure was modified. By mixing the sample with 0.3 ml of two percent sucrose solution and evaporating to a dry, amorphous glass over phosphorus pentoxide *in vacuo* materials that tended to cling to the tube walls were readily dispersed in agar and gave uniform results.

Application of this bioassay to purified avenacin will be discussed in the section on biological properties of avenacin. For this thesis a unit of activity is defined as the amount of substance necessary per milliliter of assay agar to give fifty percent inhibition.

Procedure for Growing Oats

Oats, *Avena sativa*, were used throughout as the source of avenacin. There were no apparent varietal differences in yield or nature of avenacin from the following types of oats: Victory (Sveriges Utsädesferening, Svälof, Sweden), Kanota (California Milling Corporation, Los Angeles, California), or recleaned oats of unknown variety (Ambler Milling Company, San Gabriel, California). Germination was most uniform with Victory but germination yield was good from all types. In the most satisfactory method of production, dry seed wrapped in gauze was soaked in running tap water for thirty minutes prior to planting. The moist seed was then allowed to germinate and

grow hydroponically on polyethylene baby bath pans (14 x 38 x 56 cm) (Sears Roebuck and Company, Pasadena, California) covered with gauze supported by a single piece of expanded aluminum sheet (Aluminum Expand-X 3/4" #018 from Ducommen Metals and Supply Co., Los Angeles, California) cut to fit snugly near the top of the pan. Pieces of Rutger's gauze were spread over the aluminum metal and draped into the bottom of the pan. The gauze served to support the seeds and by its wick action helped keep the seeds moist during germination. The pans were filled with tap water to within an inch from the seed bed, equipped with bubblers to pass water-saturated air through the pan, and covered with aluminum foil. The foil covering was kept on the pans for four or five days until the tops of the seedlings were about two inches in length and the roots reached into the water. Passage of air through the pans was essential to provide oxygen for growth and to keep the seedlings moist until roots reached the water. Since seedling growth was always very dense, air bubblers were needed even after the aluminum foil covers were removed. Figure 1 shows a cutaway view of an assembled pan with one week old seedlings. The pans were kept in a greenhouse or on laboratory benches by a north window. After seven to ten days the roots were harvested and frozen to await extraction. Roots were used because of their higher content of avenacin and lower content of lipoidal material and carbohydrates compared with tops and seeds. About four hundred grams of dry

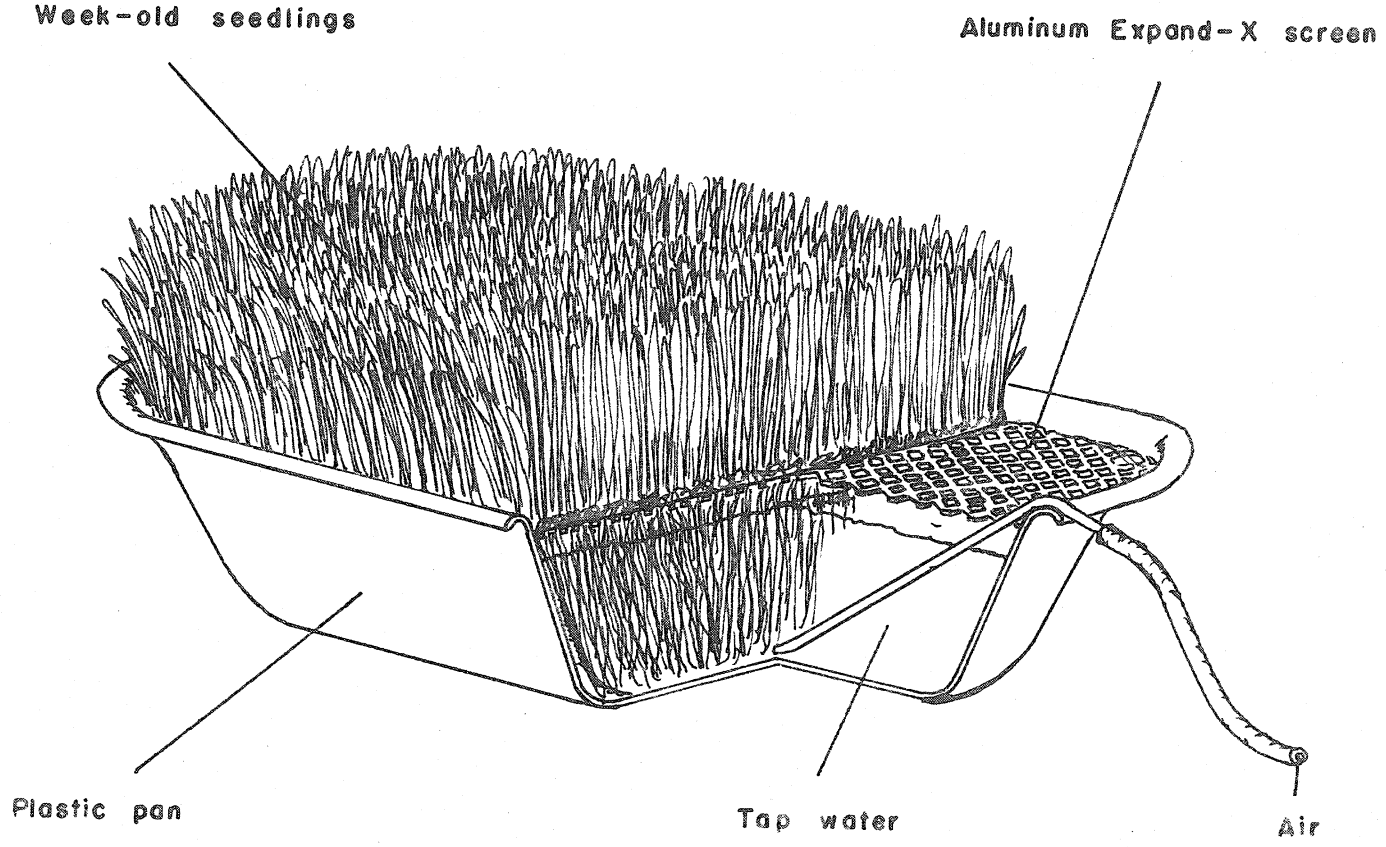


FIGURE 1

Cutaway View of a Pan for Growing Oats

seed was soaked and planted per pan, usually yielding between three and four hundred grams fresh weight roots. The section on biological properties of avenacin gives a further account of the distribution of activity in the plant at various ages.

Isolation Procedure

Isolation procedures for avenacin evolved through many modifications. Only the most successful procedure is included here. Methanol was chosen as the extracting solvent because it was obtainable in high purity and easy to remove from extracts, and because work of Mitchell and Turner (2) showed that active material was methanol extractable. Chromatographic techniques were not convenient for bulk isolations because of their low capacity. Solvent fractionation proved the simplest and most convenient method.

Frozen roots mixed with two to three times their weight of Dry Ice were ground to a fine powder in an electric grinder (Quaker City Mill Mod F-3E, Fischer Scientific Company). Overnight storage in a freezer at -15°C removed most of the Dry Ice. The finely divided roots were mixed with one liter of reagent grade methanol per kilogram of ground roots and extracted in a blender (Waring Model CB-3, Los Angeles Chemical Company) for fifteen to twenty minutes. The slurry was filtered through gauze and the solid residue extracted with one-half liter of eighty percent methanol per fresh weight kilogram of roots. After clarification by filtration through

a one to two centimeter thick celite pad on a Büchner funnel the combined filtrates were evaporated in a flash evaporator to about one-fiftieth the original volume. The residue contained little avenacin; it was discarded.

The biologically active substance is always associated with a water insoluble fraction in concentrated extracts. Subsequent isolation steps utilized this property along with the ether insolubility of avenacin to remove water-soluble and lipoidal material from the extracts. Solubility of avenacin in methanol and hot n-butanol enabled the removal of large amounts of material insoluble in either solvent. The flow chart in figure 2 shows the procedure for obtaining crystalline avenacin. Several arbitrary conditions, found by experience rather than logic, were used to make the scheme satisfactory. For instance, instead of distilled water one-tenth molar potassium chloride was used to prevent the loss of avenacin that occurred when precipitates were washed with water. High-speed, rather than clinical, centrifuges were used in many of the operations because flocculent precipitates could be compacted easily making washing and crystallization steps more efficient.

Chromatographic Methods

Paper chromatographic techniques were used throughout for qualitative studies and as a test for purity of purified avenacin. The solvents most frequently used and working

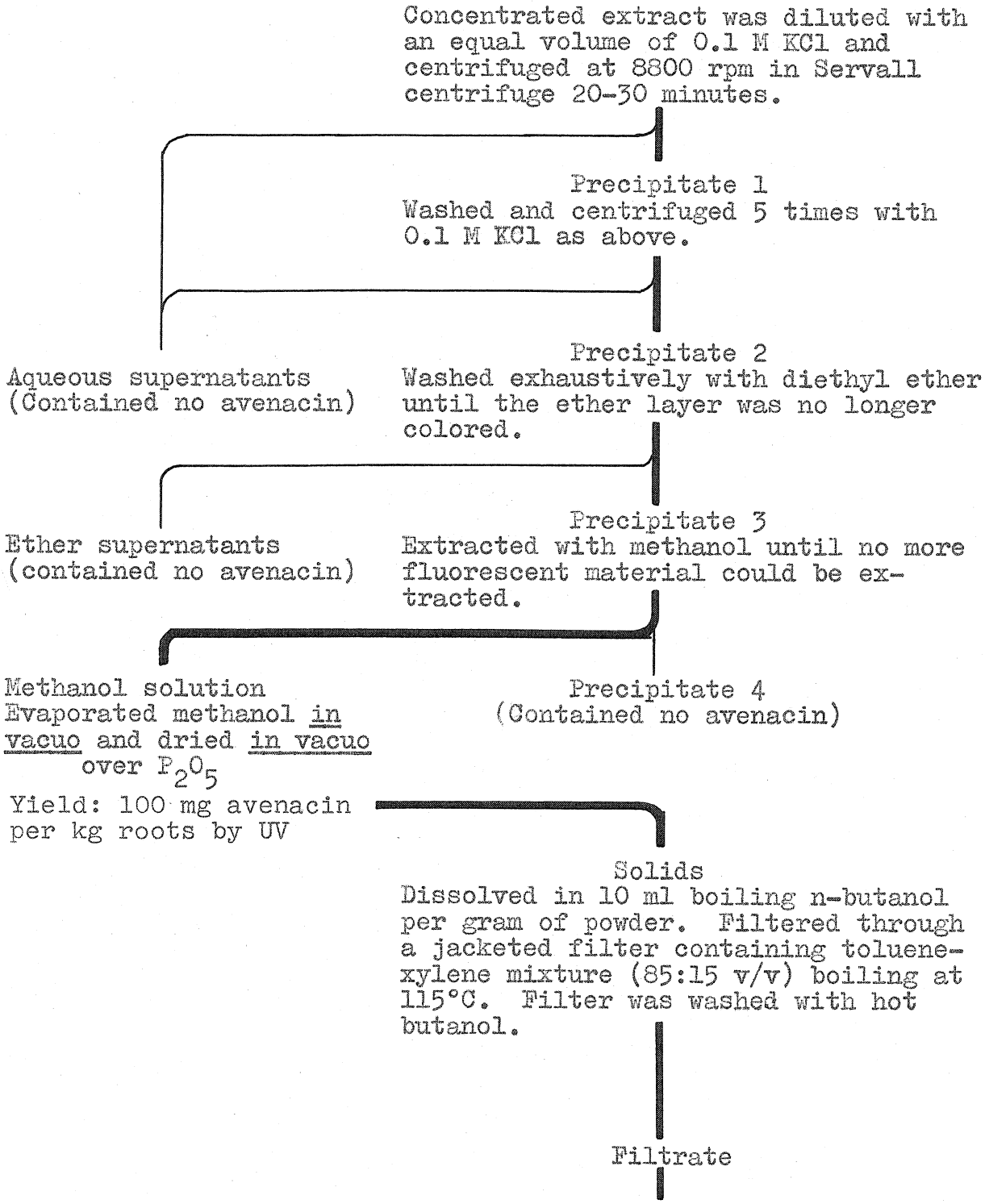


Figure 2

Flow diagram of purification procedure.

Filtrate
Filtrate cooled and butanol decanted after centrifugation 10 minutes in clinical centrifuge.

Precipitate 5
Dissolved and reprecipitated 3 times from a minimal amount of butanol.

Butanol supernatants (contained some avenacin)

Precipitate 6
Washed with ether and dried to remove butanol.

Precipitate 7
Dissolved in a minimal amount of hot 80% ethanol and crystallized by adding water to the heated solution dropwise until the first insoluble material appeared then cooled slowly. The mixture containing crystals was centrifuged 15 minutes at 25,000 rpm in a Spinco Model L centrifuge. Crystallization was repeated 4 times.

Precipitate 8
Dried 24 hours at 100°C <u>in vacuo</u> over P_2O_5.

Ethanolic supernatants (contained some avenacin) combined with the butanol supernatants of the previous page and mixed with 3-4 volumes of ether. Centrifuge in clinical centrifuge

Pure avenacin
(Contains about 50% of the avenacin estimated by UV absorption to be in the methanol extract of ppt. 3)
Yield: 50 mg/kg roots

Partially purified avenacin

Supernatants
(no avenacin)

Figure 2 (cont.)

numbers to designate them in this thesis are given below.

TABLE I

Chromatographic Solvents

Solvent Designation	Composition
Solvent 1	Methyl ethyl ketone: acetic acid: water (30:3:10 v/v)
Solvent 2	n-propanol: 1% ammonium (3:1 v/v)
Solvent 3	n-butanol: acetic acid: water (100:50:100 v/v)

Whatman #1 paper with ascending irrigation was used for qualitative work. Schleicher and Schull 470-A heavy paper washed exhaustively with eighty percent ethanol was used for preparative work.

IV. EXPERIMENTAL STUDIES AND RESULTS

Identity of Avenacin with the Ultraviolet Light Absorbing Material

Before a biologically active material can be characterized it must be determined that the activity is associated with the bulk of the material and is not caused by a trace of very potent substance. Unambiguous synthesis is the ultimate proof but the structure must first be determined. To show that the purified avenacin used in this work is identical with a potent antifungal substance several kinds of data were accumulated: data to show that the ratio of anti-Neurospora activity to ultraviolet extinction at 353 mμ was constant at various stages of purity and data to show that changes of the properties of the fluorescent, ultraviolet absorbing substance paralleled changes in biological activity under various conditions.

Table II shows the results for a comparison of anti-Neurospora activity per unit concentration and per unit ultraviolet extinction value at various stages of purification. The ratios of activity to concentration and ultraviolet absorption to concentration vary considerably; however, the ratios of biological activity to ultraviolet extinction value are the same to within experimental error.

When dissolved in dilute acid, avenacin was transformed to a chromatographically and spectrophotometrically similar

TABLE II

Comparison of Biological Activity with Ultraviolet Extinction

Material examined	Biological activity (units)/(mg/ml)	$E^{1\%}_{1cm}$ at 353 mμ in methanol	Ratio of activity to $E^{1\%}_{1cm}$
Dried methanol extract of precipitate 3 (see figure 2)	740	21	35
Purified avenacin (stock 6/8/59B)	1900	51	37
Purified avenacin (stock F)	2100	58	36
Twice chromatographed avenacin	2000	49	41

Explanation of Table II.--Activity was determined as described in the Methods section. The symbol $E^{1\%}_{1cm}$ means the extinction equivalent to that of a one centimeter layer of solution containing 10 mg/ml. Dried methanol extract is the first stage of purification at which the avenacin spectrum is recognizable. Stock 6/8/59B used for most of the chemical studies was about 90% pure compared to stock F. "Twice chromatographed avenacin" was obtained by chromatography of 49.4 g of purified avenacin (similar to stock 6/8/59B) with solvent 1 on S&S 470-A paper followed by elution and rechromatography with solvent 2. A suitable blank was considered for impurities eluted from the paper. Eighty-seven percent was recovered.

substance that no longer had anti-Neurospora activity. The new substance crystallized in an entirely different form and, while no quantitative technique was found to estimate the change in form, loss of activity was coincident with the change to the new form. Thirty minutes hydrolysis in N KOH at 105-110°C completely hydrolyzed avenacin and completely destroyed the biological activity. The detailed behavior of avenacin in acids and base will be discussed in following sections.

Chemical and Physical Properties of Avenacin

Solubilities.--Avenacin, as shown in Table III, was found to be insoluble in all non-polar organic solvents tried and in highly aqueous solvents except for acids. Eighty percent ethanol or methanol gave highest solubilities and were the only solvents from which avenacin would crystallize. Evaporation of an absolute ethanol or methanol solution always gave an amorphous solid. The crystals obtained by adding water slowly to hot saturated 80% ethanol solutions and cooling were long filamentous needles that were soft and highly hydrated but showed strong dichroism under a polarizing microscope. When such crystals were dried they lost their form completely indicating a high degree of hydration.

Chromatography.--The solvents most used for avenacin and its derivatives are described in Table I of the Methods section. Solvent 1, acidic and containing methyl ethyl ketone, was used

TABLE III

Solubility of Avenacin in Various Solvents

Very Soluble	Soluble	Insoluble
(50 to 100 mg/ml)	(10-50 mg/ml)	(less than 1 mg/ml)
Methanol-water (80:20%)	N HCl	water
ethanol-water "	methanol (abs.)	N KOH
n-propanol-water "	ethanol (95%)	ether
	n-butanol (115°C)	n-butanol (0°C)
	glacial acetic acid	benzene
		carbon tetra-chloride
		chloroform
		hexane

for most chromatograms because it gave very well defined avenacin areas, even when amounts used were as large as 200 μg/cm at the origins on Whatman #1 paper and as high as 800 μg/cm at the origin when S & S 470-A heavy paper was used. The R_f in solvent 1 was 0.58 with avenacin confined in a region about 0.1 R_f unit wide even at the high concentration. The R_f in solvent 2 was 0.75 and in n-butanol acetic acid-water (10:3:5 v/v) was 0.67. The latter solvents gave much more spreading of spots than did solvent 1. Avenacin was usually detected on chromatograms by its fluorescence under either a germicidal ultraviolet lamp (principal wavelength 3650A) or

a Mineralite (principal wavelength 2537A). Periodate (41,42) and permanganate (42) reagents were also used but were much less sensitive than fluorescence.

Elemental analysis.--Analyses were performed on two samples of avenacin. In each case the sample was dissolved in alcohol and filtered through Permutit to remove traces of ammonia, then dried at 100°C over phosphorus pentoxide. One sample, a portion of stock 6/10/59A, recrystallized after filtration through one gram of Permutit then dried eighteen hours gave the following microanalysis:* C, 55.58%; H, 8.19%; N, 1.90%. Another sample, a portion of stock F, filtered through about two grams of methanol washed Permutit, evaporated under a stream of nitrogen then dried for sixty hours gave the following microanalysis:* C, 59.28%; H, 7.75%; N, 1.40%. The latter analysis is likely to be more accurate because the increased drying time and elimination of the recrystallization step gave less chance for contamination and because stock 6/10/59A was estimated by ultraviolet spectrum to be 90% as pure as stock F. Since avenacin gave no ash and qualitative sodium fusion showed the absence of sulfur or halogens and phosphate was not detected by a molybdate test on paper (43) the unaccounted 31.56% was assumed to be oxygen.

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*Microanalyses performed by Elek Micro Analytical Laboratories, Los Angeles, California.

Taking the percent nitrogen as 1.40% a value of 1010 is found as the molecular weight per nitrogen atom, in good agreement with other estimates of the molecular weight. Very great reliability cannot be entrusted to the nitrogen analysis, however, because the percent nitrogen is very low.

Saponification.--Carefully weighed two to six milligram samples of avenacin were saponified by heating with 0.04 ml of N KOH in ethanol at 105-110°C in sealed tubes. After eleven to fifteen hours the tubes were crushed in beakers containing 1.00 ml of 0.0531 N HCl and titrated with 0.0943 N KOH using a Radiometer titrimeter. A blank was titrated and the difference in volume of standard acid to titrate the blank and the sample was used to calculate the saponification equivalent. Values for four samples were 870, 935, 895, and 873 with an average of 893.

Ultraviolet spectrum.--The ultraviolet absorption spectra of avenacin in acidic, basic, and neutral conditions determined with a Cary 11 MS spectrophotometer is shown in figure 3. Spectra of methanolic or pH 7 aqueous solutions of avenacin resemble very closely the spectrum of methyl N-methyl anthranilate (44), however, the extinctions at the various wavelengths are much lower for avenacin than for methyl N-methyl anthranilate. ($E^{1\%}_{1cm}$ at 353 mμ is 58.2 for avenacin and 357 for methyl N-methyl anthranilate). Assuming the molar extinction coefficients to be the same, which seems reasonable

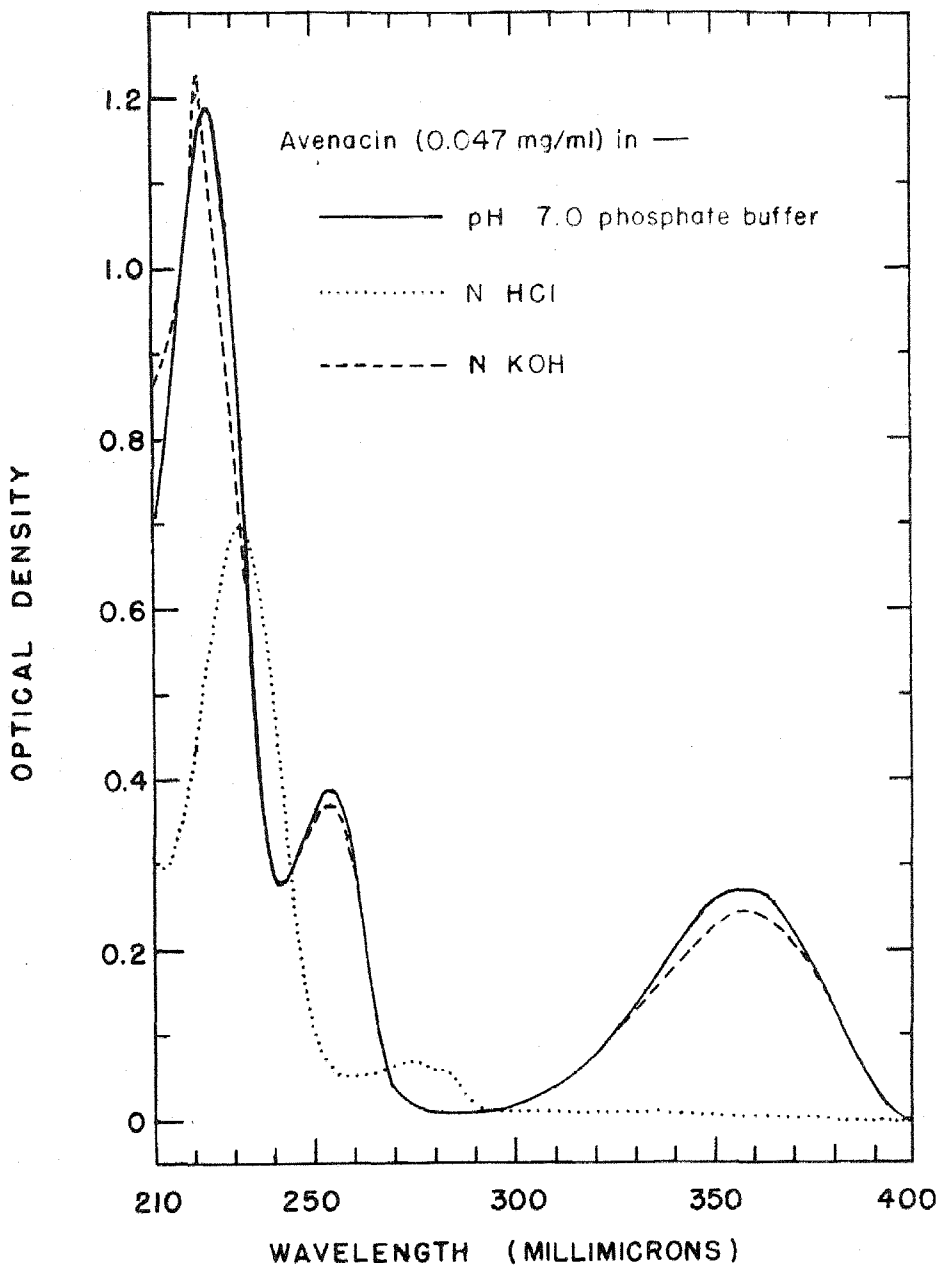

FIGURE 3

Ultraviolet Spectra of Avenacin Under Various Conditions

because of the similarity of shape of spectrum and because of chemical similarities to be discussed in following section, an estimate of the molecular weight of avenacin was made. The value obtained from this estimate was 1020 in good agreement with the value calculated from the nitrogen content.

Fluorescence versus pH.--As mentioned in the Introduction, Goodwin described the pH versus fluorescence curves of a large number of substances including the fluorescent substances from oat root tips. Goodwin's procedure (34) was applied to avenacin and methyl N-methyl anthranilate with the results shown in figure 4. A Farrand Fluorometer Model A was used for the determination. It is apparent that avenacin and methyl N-methyl anthranilate behaved identically. The decrease in fluorescence with lowered pH parallels the change in ultraviolet spectrum in the same conditions, both changes being caused by ionization of the strongly auxochromic methyl amino group (45).

Infrared spectrum.--The infrared absorption spectrum of avenacin pressed in a KBr disc determined with a Perkin-Elmer Model 21 spectrophotometer is shown in the Appendix. Spectra of paraffin oil and hexachlorobutadiene mulls agree completely with the KBr disc results. Detailed analysis of the spectrum is not possible because of the large size and complexity of avenacin; the OH (2.8 μ), N-H (2.9 μ), C-H (3.4 μ), and C=O (5.5-6.0 μ) regions of the spectrum all show strong absorption.

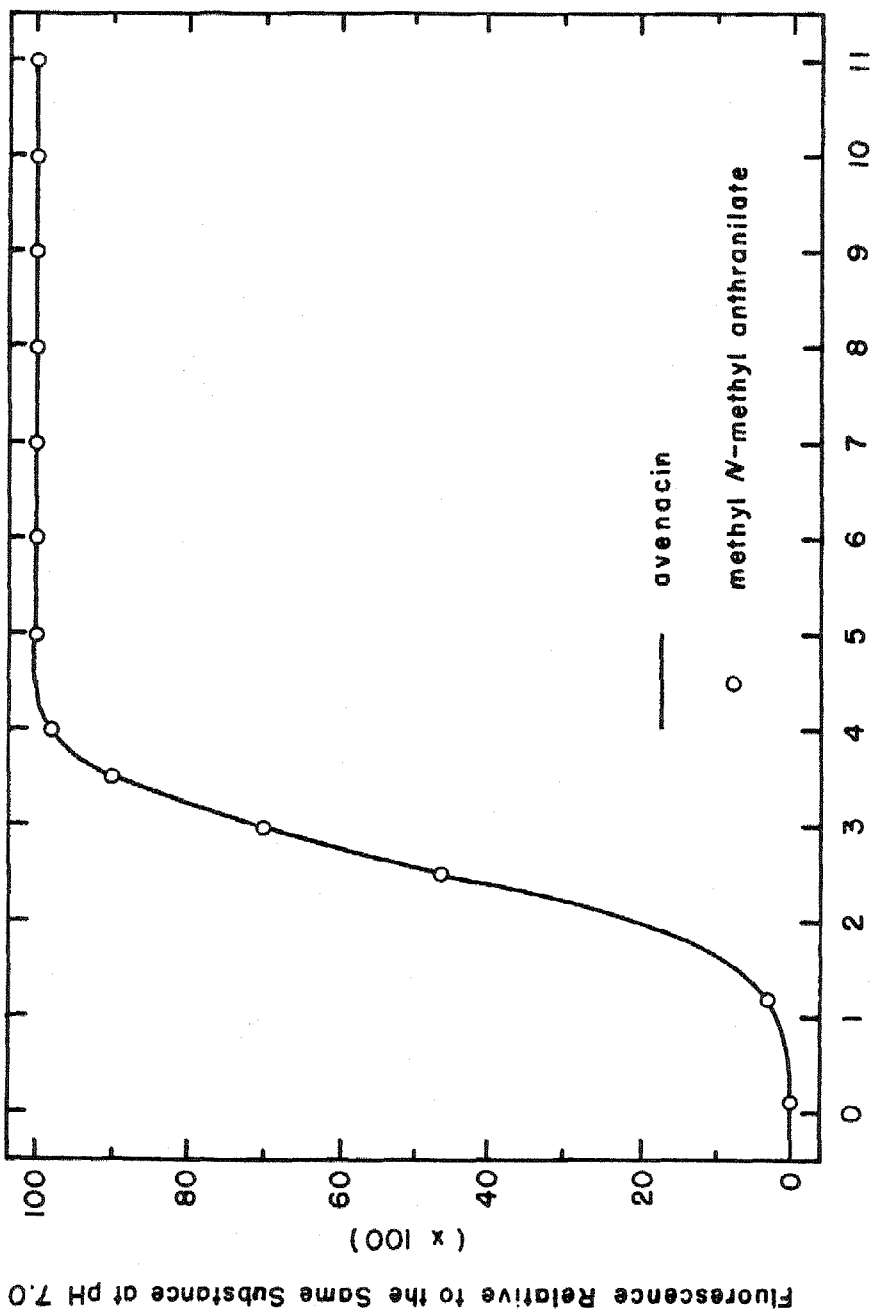

FIGURE 4

Fluorescence Versus pH Curves

Molecular weight determination.--Two direct physical methods were used to estimate the molecular weight of avenacin. The Rast method (46) using camphor as solvent gave molecular weights of 1050 and 1100 for two samples with an average of 1070 ± 100. The thermometric method, a technique utilizing vapor pressure lowering in a modification of the method of Müller and Solton (47), was also employed. In that method the equilibrium temperature differential between a drop of pure solvent and a drop of solution in an atmosphere saturated with solvent vapor is measured with two thermistors in a wheatstone bridge circuit. The temperature difference, measured as resistance needed to balance the bridge circuit using an electronic voltmeter, is proportional to the vapor pressure lowering. Very small temperature differences are measured and a very carefully controlled constant temperature bath is needed. Methanol was used as the solvent for avenacin with a standard curve established using known concentration of benzoic acid in methanol. A difference in resistance of 550 ohms was measured for a 0.2 M solution of benzoic acid with an average deviation of ±10 ohms in all readings at a bath temperature of 25.95°C. An avenacin solution containing 6.0 mg per 0.15 ml gave an average difference of 125 ± 5 ohms which corresponded to 0.037 M, or a molecular weight of 1100 ± 100. A repeat determination at half the concentration agreed. This method was used primarily because there was no chance of decomposition

of avenacin under the conditions used.*

Melting point.--Avenacin does not have a sharp melting point but rather decomposes of a wide range starting at 240 to 250°C. The decomposition point was of no use for checking purification.

Biological Properties of Avenacin

Response of Neurospora to avenacin.--Neurospora was used as bioassay organism for this work because it was conveniently available and because it had previously been shown to be inhibited by preparations containing avenacin (2). Purified avenacin when tested in the bioassay scheme previously outlined (see Methods) gave fifty percent inhibition at 0.4 μg/ml avenacin. This concentration was defined as equal to one unit of activity. A log dose-response and a dose-response curve for various concentrations of avenacin are shown in Figure 5. At degrees of inhibition greater than eighty percent the curves were not usable since the growth on the assay plate was so small that it could not be measured precisely. There was a threshold at 0.025 μg/ml below which no response was detected. In the range 0.03 μg/ml to 0.2 μg/ml the response was linear with concentration while from 0.2 μg/ml to 1.0 μg/ml response

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*I am very grateful to Dr. Gordon Alderton of the Western Regional Laboratory, USDA, Albany, California, for introducing me to this method and for allowing me to use the apparatus assembled by him during his stay at the Institute.

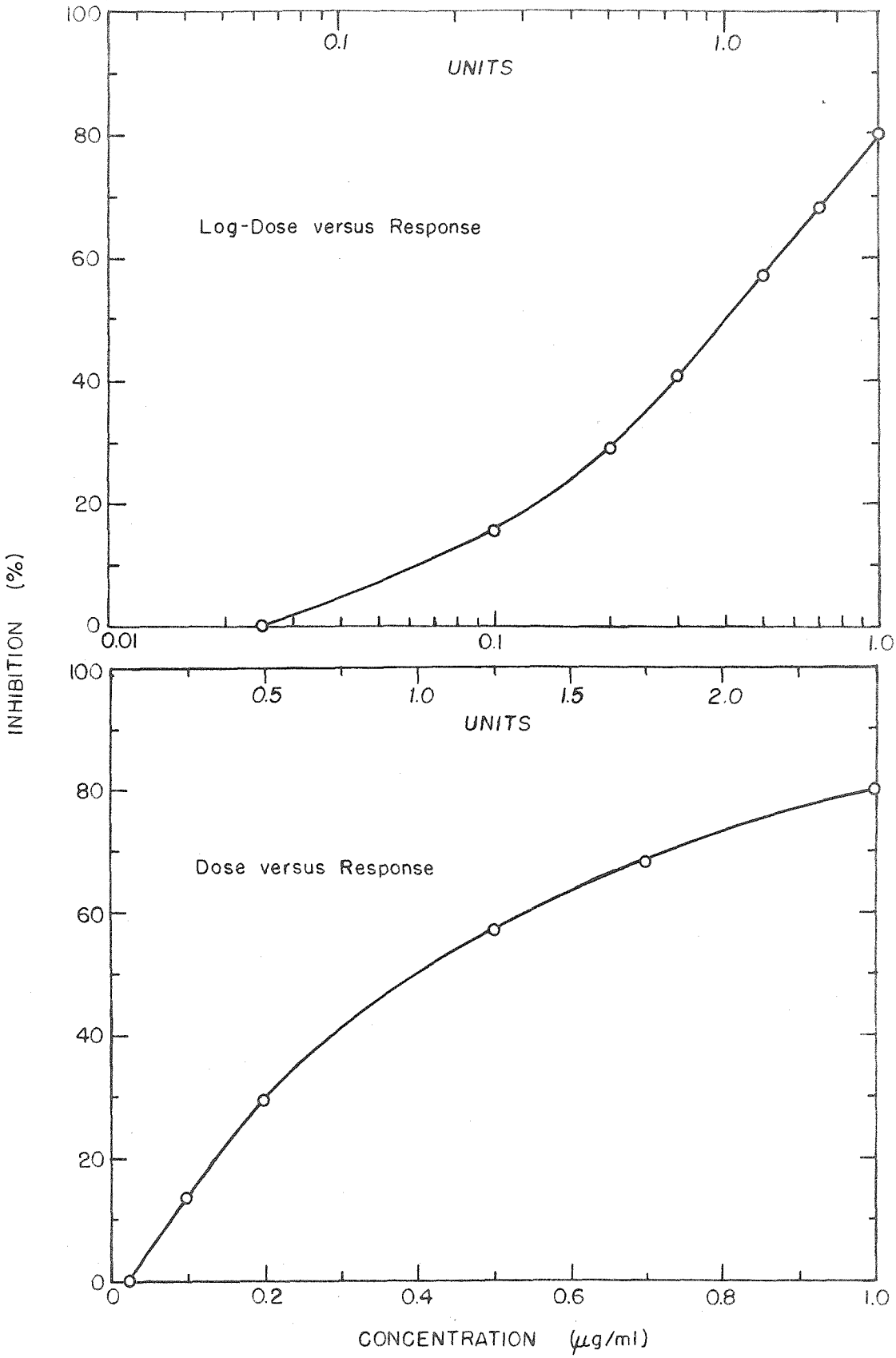


FIGURE 5

Response of Neurospora to Avenacin

was proportional to the logarithm of the concentration. The time factor in the Neurospora bioassay was very critical. Although a detailed study was not conducted it became apparent during this work that as incubation time increased beyond fifteen hours the percent inhibition at all concentration decreased. This factor and the biological variability of the mold probably contributed to the variation in the actual position of the curves at various times even though the shape of the curve was constant.

Effect of avenacin on other organisms.--As was mentioned in the Introduction, Turner (1) tested materials containing avenacin against a number of fungi and found growth and respiration of a number to be inhibited. Purified avenacin has been tested against a number of bacteria and plant pathogens.* An agar dilution end-point method was used on enriched media and the concentration of avenacin necessary to completely prevent growth in a given time was measured. Concentrations higher than 50 μg/ml were not tested. Of thirty-eight organisms tested ten were inhibited. The ten organisms and the lowest effective concentration for each in μg/ml were: Mycobacterium tuberculosis (#607) (12.5), Saccharomyces pastorianus (25), Candida albicans (50), Alternaria solani (50), Ceratostomella ulmi (12.5), Colletotrichum phomoides (50), Helminthosporium

*I am very grateful to Dr. W. M. Stark and Eli Lilly and Company for performing these tests and making their results available. Organisms not inhibited by 50 μg/ml or less are listed in the Appendix (p. 61).

sativum (6.25), Pelicularia filamentosa (3.13), Pythium irregulare (50), and Verticillium albo-atrum (6.25). The last seven organisms are plant pathogenic fungi.

Distribution of Anti-Neurospora Activity in Oat Seedling

Oat seedlings of various ages were grown as described in the Methods section and when possible the tops, seeds and roots were harvested and extracted separately at the desired age. Seedlings were harvested at one, two, three, six, eight, ten, and fifteen days after planting. The one and two day old seedlings could not be separated into tops and roots and the three day old seedlings had but a few roots and no tops. Table IV summarizes the results of application of the bioassay to extracts of the various materials.

There was nearly a seventy-fold increase in anti-Neurospora activity from the first day of germination to about the eighth day followed by a decline in activity as the seedlings got older. Although the roots had the highest activity per gram dry weight seeds and tops also had high activity.

It is possible that some of the activity of seeds was due to contamination with roots since the separation of seeds and roots on this scale was not clean-cut. Chromatographed seed extracts showed some fluorescence at the avenacin region. Leaf extract chromatograms showed very little fluorescence at the avenacin region. No special effort was made to study the leaf or seed inhibitors but one attempt to isolate avenacin was made with a leaf extract using the avenacin isolation scheme.

TABLE IV

Distribution of Anti-Neurospora Activity

in Oat Seedlings of Various Ages

Age (days)	Plant part	Fresh wt (grams)	Dry wt (grams)	Total Units	Units/gram dry wt
1	whole	470	280	390	1.4
2	whole	455	250	1100	4.3
3	Seed & tops	475	220	1700	7.7
	Roots	6	0.42		
	(Sum)	481	220.42		
6	Tops	25	2.5	2000	800
	Seeds	606	194	4000	24
	Roots	57	10	1200	120
	(Sum)	688	206.5	7200	
8	Tops	245	22	4500	200
	Seeds	787	181	13000	72
	Roots	143	10	9300	930
	(Sum)	1175	213	26800	
10	Tops	295	29	13500	460
	Seeds	800	175	2000	11
	Roots	183	7.2	1600	220
	(Sum)	1278	211.2	17100	
15	Tops	202	19	620	33
	Seeds	446	121	288	2.3
	Roots	140	5.6	3900	700
	(Sum)	788	145.6	4808	

Tops consisted of leaves, stems and the remains of coleoptiles. Seeds consisted of short segments of tops and roots attached to the remains of the endosperm and seed coat. Roots consisted of all the roots that could be harvested by cutting as close as possible to the gauze seed bed on which the seedlings were supported.

The experiment was abandoned when no material passed the "hot butanol soluble-cold butanol insoluble" stage of the scheme. The leaf inhibitory substance or substances cannot be avenacin.

Degradations of Avenacin

In studying the structure of a complex substance the customary procedure is to degrade the large molecule to smaller, identifiable substances. For instance, in the case of suspected glycosides acid hydrolysis is usually performed to separate aglycone from sugars. With avenacin both basic and acidic hydrolyses provided interesting information and products.

Hydrolysis by alkali.--Avenacin is easily hydrolyzed in hot dilute alkali. After ten minutes in N KOH in a sealed tube at 105-110°C followed by chromatography in solvent 1 (acidic) no fluorescence remained at the avenacin region while nearly all the fluorescence was at the solvent front. Thirty minutes at 105-110°C was needed for complete hydrolysis in 0.1 N KOH and in 0.01 N KOH about half of the fluorescence remained at the avenacin region after one hour at 105-110°C. Chromatography of a complete base hydrolyzate in the solvent 2 (basic) showed all of the fluorescence in a region centered around Rf 0.71. Nothing could be extracted from an alkaline hydrolysis mixture with ether but when a basic hydrolyzate of 2.0 mg of avenacin was acidified, extracted with ether and the ether evaporated a small amount of blue fluorescent, crystalline

acidic substance less than a milligram in weight was deposited.

In order to obtain a reasonable quantity of the fluorescent acid for characterization 110 mg of avenacin was hydrolyzed in 6.0 ml of 0.25 N KOH in an open tube at 98 to 100°C. At first the avenacin was insoluble but gradually the solution cleared and after 45 minutes the solution was clear but viscous. At 15 minute intervals small aliquots were removed from the reaction mixture, spotted on the origins of 0.75 x 7 inch strips of Whatman #1 paper, the KOH neutralized with a drop of glacial acetic acid, excess acetic acid evaporated, and the chromatograms irrigated with solvent 1. After hydrolysis for one and one-half hours no fluorescence could be seen at the avenacin region and the reaction was stopped by cooling and acidifying to about pH 3. Five extractions of the acidified mixture with 12 ml portions of ether removed all but a trace of fluorescent acid. After evaporation of the ether and drying over phosphorus pentoxide in vacuo for three hours 12.8 mg of solid acid resulted. In an attempt to determine the melting point on a hot stage melting point apparatus the material rapidly sublimed so that at 145°C no more crystals remained. Since, when applicable, sublimation is a convenient and effective way of purification the acid was sublimed onto a Dry Ice-methyl cellosolve chilled cold finger in vacuo in a micro-sublimation apparatus heated three hours at 80 to 90°C in a water bath.

The neutral equivalent was determined by titration of samples of the acid with standard base using a Radiometer

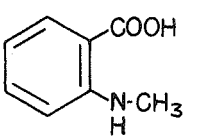
recording titrimeter (Radiometer, Copenhagen, Denmark). Two samples weighing 1.43 ± 0.02 mg and 2.14 ± 0.02 mg were titrated with 0.0460 N KOH and gave as their neutral equivalent 152 and 162 respectively, with an average of 157. To determine if the substance was a mono- or poly-carboxy acid estimates of the molecular weight were made by two direct methods; the Rast (46) and thermometric (47) methods. The Rast method using camphor gave an average molecular weight of 172 and the thermometric method using absolute ethanol as solvent and benzoic acid as standard gave an average of 141. Because the neutral equivalent was more accurate than either of the physical methods it was taken as the molecular weight.

An infrared spectrum of a saturated carbon tetrachloride solution in a 0.1 mm NaCl cell was determined on a Perkin-Elmer Model 137 spectrophotometer. The solid line of figure 6 shows the usable portion of the spectrum. The strong absorption at 6.05 μ (1650 cm^{-1}) and the broad absorptions centered about 3.80 μ (2650 cm^{-1}) and 11.11 μ (900 cm^{-1}) are suggestive of a hydrogen bonded carbonyl stretching and hydroxyl stretching and deformation frequency, respectively, of an aromatic carboxyl group (46). A single sharp peak of medium intensity at 2.95 μ (3400 cm^{-1}) is like that of a secondary amine.

A study of ultraviolet absorption of the acid in various solvents showed that it was similar in many respects to the chromophore of avenacin. In methanol absorption maxima

occurred at 218 mμ (ϵ = 25,000),* 255 mμ (ϵ = 8800) and 342 mμ (ϵ = 4200), with absorption minima at 235 mμ (ϵ = 5100) and 280 mμ (ϵ = 400) and no absorption above 400 mμ. In water the two maxima at lowest wavelength were unchanged but the 342 mμ peak was shifted to 330 mμ (ϵ = 2600). In N HCl the spectrum was identical with that of avenacin but with higher extinction at all peaks. The maxima were at 228 mμ (ϵ = 10,000) and 274 mμ (ϵ = 1400). In N KOH the changes in spectrum were more pronounced than those of the avenacin spectrum. The shortest wavelength peak was replaced by a strong end absorption below 230 mμ, the 252 mμ (ϵ = 7600) peak was nearly unchanged and the long wavelength peak shifted to 328 mμ (ϵ = 3000).

Consideration of the molecular weight (neutral equivalent), and infrared spectrum and similarity of the ultraviolet spectrum (43) implied strongly that the isolated substance was N-methyl anthranilic acid (VII)



N-Methyl anthranilic acid (VII)

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* ϵ = molar extinction coefficient.

Comparison of the isolated acid with authentic N-methyl anthranilic acid (Eastman) leaves no doubt that they are identical. Similarity of the infrared spectrum as shown in Figure 6 alone is strong proof of identity for a molecule of this size (MW 151) (49). In addition to infrared the ultraviolet spectra of both materials was found to be identical in all respects in water, methanol, N HCl, and N KOH as was the melting point and mixed melting point (m.p. isolated substance, 177-178°C; m.p. commercial substance, 179-180°C; literature, 179-182°C (50); mixed m.p., 177-178°C). Both the isolated and commercial acids behaved identically on paper chromatography in solvents 1 and 2.

The aqueous phase of the alkaline hydrolyzate was neutralized and evaporated to dryness in vacuo over phosphorus pentoxide. Trituration of the salt-containing residue with 1.0 ml of methanol gave a solution containing very little ultraviolet absorption except for a weak N-methyl anthranilic acid spectrum and slight end absorption at 210 mμ. Chromatography of aliquots of the material showed a non-fluorescent material at R_f 0.47 in solvent 1, R_f 0.32 in solvent 2 and R_f 0.59 in n-butanol-acetic acid-water (25:7:20) that could be detected by periodate and permanganate reagents but not o-aminobiphenyl reagent, a reagent which detects reducing sugars pyrolizable to furfural derivatives (42).

Acid hydrolysis.--Avenacin was easily hydrolyzed in hot dilute acids to give products different than those from basic

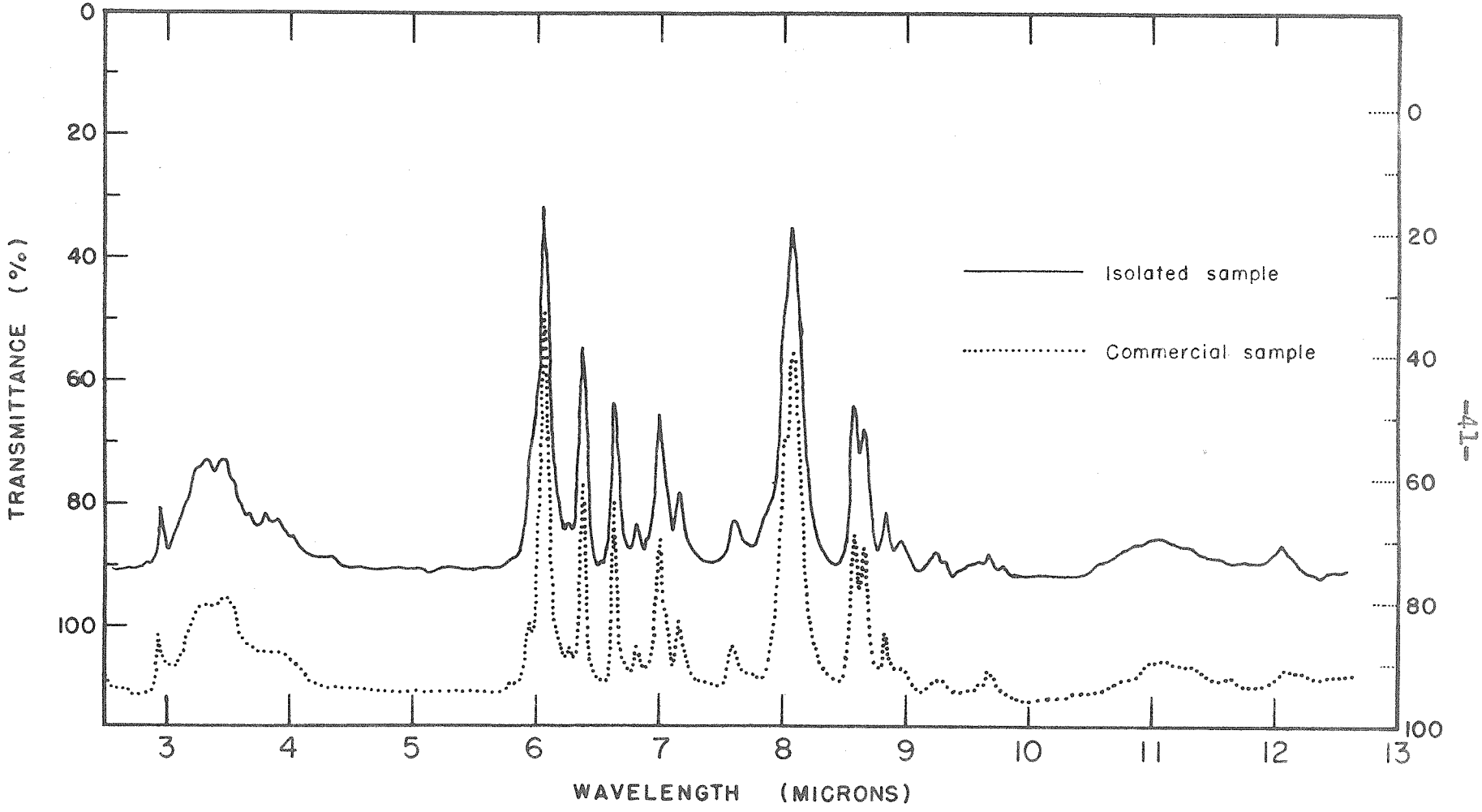


FIGURE 6

Comparison of IR Spectra of Isolated and Commercial *N*-Methyl Anthranilic Acid in CCl_4

hydrolysis. Ten minutes in N HCl at 105-110°C or thirty minutes at 105-110°C hydrolyzed avenacin to give a fluorescent substance moving with the solvent front after chromatography in either solvent 1 or solvent 2 and materials at lower R_f that reacted with carbohydrate reagents. The slowly migrating material from short time hydrolysis was complex. There were distinct but weak spots at R_f 0.25 and 0.3 as well as a faint trail of material reactive to o-aminobiphenyl reagent extending from the origin to R_f 0.2 after chromatography in solvent 1. The spots were not intense enough to account for the amount of material applied at the origin. When chromatograms were treated with periodate or permanganate, however, the streaked material from the origin to R_f 0.2 as well as the spots at R_f 0.25 and 0.3 gave strong reactions.

In order to obtain some of the hydrolysis products for further study eighty milligrams of avenacin was dissolved in 4.0 ml of 0.75 N HCl. After heating ten minutes at 98-100°C in a water bath the solution suddenly became milky white. Aliquots taken periodically and chromatographed in solvent 1 showed that no intact avenacin remained after two hours. Centrifugation of the hydrolysis mixture gave a white precipitate and a clear colorless supernate. The precipitate was washed with 1.5 ml of water and dried over phosphorus pentoxide in vacuo at room temperature for four hours giving a dried powder weighing 42.1 mg. The washings and the supernate after combination and lyophylization to dryness over solid sodium

hydroxide weighed 37.7 mg. Recovery of material was nearly 100%.

The ultraviolet absorption spectrum of a solution of the precipitate was identical with unhydrolyzed avenacin with the exception that $E^{1\%}_{1cm}$ at 355 mμ was 92.4. This material was called "N-methyl anthranilate-aglycone conjugate" or "N MA-conjugate." An infrared spectrum of the material in a KBr disc is shown in the Appendix. The spectrum shows among others the following absorption maxima: 2.95 μ (3400 cm^{-1}), O-H and N-H stretching; 3.45 μ (2900 cm^{-1}), C-H stretching; many peaks from 5.9 μ to 8.7 μ that are similar to the N-methyl anthranilic acid spectrum; 8.9 μ (1120 cm^{-1}), could be ester C-O stretching; and 9.6 μ (1040 cm^{-1}) probably due to O-H deformation vibrations of alcohols. The NMA-aglycone conjugate when hydrolyzed in N KOH at 105-110°C for one hour gave a blue fluorescent substance that chromatographed like N-methyl anthranilic acid in solvents 1 and 2. From the yield of NMA-aglycone (42.1 mg) from avenacin (80 mg) and assuming the molecular weight of avenacin to be about 1000 the molecular weight of the NMA-aglycone conjugate was estimated as 530. Estimation from the ultraviolet absorption assuming the molar extinction coefficient is the same as methyl N-methyl anthranilate ($\epsilon = 5900$) gives a molecular weight of 630.

Further studies on the water soluble material from short time hydrolysis of avenacin showed that it was a mixture of oligosaccharides and monosaccharides as was suggested by the

fact that o-aminobiphenyl reagent did not give as intense reactions on chromatograms as periodate and permanganate reagents. A quantitative test in solution for sugars using the orcinol reagent (51), a reagent that detects oligo- and monosaccharides, resolved some of the anomaly concerning amount of sugars. In the test aldopentoes give green colored products and aldohexoses give greenish-brown products which can be distinguished by spectrophotometric examination. Thus hexoses and pentoses can be estimated simultaneously in mixtures by measuring the absorbance at two different wavelengths and solving the suitable simultaneous equations in two unknowns. The absorption curve of the reaction with the water soluble components of short time hydrolysis could be represented by a 2:1 molar ratio of hexose to pentose (glucose and ribose were used for standards). Furthermore, the sugars were determined by this method to constitute 47% of the weight of avenacin. If avenacin has a molecular weight of 1000 the theoretical percentage sugar for one molecule of pentose and two molecules of hexose per molecule of avenacin is 46%. It seems likely that the three sugars are linked to each other and are linked to the rest of the molecule by a single glycosidic linkage. If that be the case, short time acid hydrolysis should liberate only about one reducing group per molecule of avenacin. The reducing power of the water soluble short-time hydrolysis products was determined quantitatively by a ferricyanide reduction method (52) and was found to be 0.53 mole of reducing

power per mole of avenacin. This method is very sensitive (standards contained one to ten micrograms glucose) but does not detect non-reducing oligosaccharide, even oligosaccharides as labile as sucrose. Intact avenacin showed no reducing power even at a level of 50 μg.

When an aliquot of the water soluble short-time hydrolysis products was hydrolyzed eighteen hours at 105-110°C in 0.5 N sulfuricacid, neutralized with calcium carbonate and chromatographed in solvent 1 next to an equivalent amount of short-time hydrolyzate a remarkable result appeared. Instead of a feebly positive reacting streak of material from the origin to R_f 0.3 there were only two strong spots in the long-time hydrolyzate, easily detectable with o-aminobiphenyl reagent. A greenish-brown spot appeared at R_f 0.24 and a slightly less intense reddish-brown one appeared at R_f 0.03. The sensitive periodate reagent failed to detect any other sugar-like substances on the chromatogram. A chromatogram with hydrolyzates and several known sugars, prepared by allowing solvent 1 to ascend the paper three successive times with intermediate drying, separated the two spots cleanly with the greenish-brown spot at R_f 0.36 and the reddish spot at 0.45. The o-aminobiphenyl reagent gives red colors with pentoses and green-brown colors with hexoses. The green-brown spot was chromatographically similar to glucose and galactose but different from fructose and the reddish spot was similar to arabinose but unlike xylose and ribose.

Basic hydrolysis of material previously hydrolized in acid.--Basic hydrolysis of the NMA-aglycone provided a simple means for getting the aglycone since it was likely that the NMA to aglycone linkage was the same as in avenacin, probably an ester.

Thirty-one milligrams of the NMA-conjugate was mixed with 2.0 ml of 0.5 N KOH and heated for six hours at 98-100°C after which time the solid had not all dissolved and only a small fraction of the conjugate was hydrolyzed to N-methyl anthranilic acid as shown by paper chromatography in solvent 2. The mixture was dried and the entire solid residue was dissolved in 2.0 ml of n-propanol: ethanol: water (1:1:1 v/v) mixture and reheated in a sealed tube for three hours. Hydrolysis was complete at that time. The solvents were evaporated under nitrogen and the residue was triturated with 4.5 ml of water. The water insoluble precipitate was dissolved in 0.5 ml of ethanol and transferred to a dried, weighed tube. After drying under nitrogen and over phosphorus pentoxide in vacuo four hours the yield was 9.4 mg of solid (melting point about 250°C with decomposition) which was soluble in methanol and ethanol but insoluble in chloroform, carbon tetrachloride, benzene, carbon disulfide, diethyl ether, methyl ethyl ketone, water, 5% KOH, and 5% HCl. This material, the "aglycone," at a concentration of 0.1 mg/ml in methanol showed no absorption from 210 mμ to 400 mμ except for a trace (equivalent to a few percent) of unhydrolyzed NMA-conjugate. An infrared spectrum of the

aglycone in a KBr disc is shown in the Appendix. Strong absorption is shown in the region of O-H, N-H, C-H, and C=O stretching and at 9.2-10.0 μ, where O-H deformation vibrations occur. The peak that suggests C=O stretching is at 5.95 μ (1700 cm^{-1}) which is just below the aliphatic ketone region (1725-1705 cm^{-1}) (46), but the presence of hydroxyls in the molecule may permit some hydrogen bonding, especially in the solid, which could lower the absorption frequency. Since the infrared spectrum suggested a ketone an attempt was made to make a 2,4-dinitrophenyl hydrazine derivative by reacting a small quantity of the aglycone overnight with the reagent (44) in a sealed capillary at room temperature. A small amount of aglycone when chromatographed in solvent 1 and treated with permanganate reagent gave a positive reaction from R_f 0.6 to 0.9.

Cold acid treatment of avenacin.--When avenacin was dissolved in N HCl at room temperature and the solution neutralized immediately to pH 7.0 avenacin did not precipitate. When methyl ethyl ketone was added very fine white needles crystallized from solution. They were of entirely different form than avenacin crystals. Such material no longer showed anti-Neurospora activity. Recombination of the crystals with the aqueous and methyl ethyl ketone phases did not restore activity. Since the ultraviolet absorption and chromatographic behavior in solvent 1 are similar to those of avenacin the

material has been given the working name "isoavenacin." The infrared spectrum in a KBr disc (shown in the Appendix) is generally similar to that of avenacin but there is some difference in the 5.9 μ - 6.3 μ region that is as yet uninterpreted.

Miscellaneous Results

Reduction.--Attempts were made, before the nature of the chromophore of avenacin was known, to reduce avenacin with hydrogen and palladium on charcoal or hydrogen and platinum oxide. All reactions were done in glacial acetic acid in a microhydrogenation apparatus at atmospheric pressure and avenacin in all cases failed to take up hydrogen. The catalyst was always tested with cinnamic acid after the attempts and in all cases the cinnamic acid rapidly took up one or four moles of hydrogen, depending whether Pd/C or PtO_2 was used.

Optical rotation.--One attempt was made to observe the optical rotation of avenacin at a concentration of 10 mg/ml in methanol in a 0.4 ml, one decimeter polarimeter tube. The rotation was very slightly positive at the limit of detectability with $[\alpha]_D^{27°C} = +3.5$ (C = 0.010 g/ml in methanol). This experiment should be done with higher concentrations of avenacin. Eighty percent ethanol could be used to obtain a satisfactory concentration.

V. DISCUSSION

Biological

If the general mechanisms of disease resistance in plants are ever to be fully understood on the molecular level the nature, origin and mode of action of the biochemicals involved must be studied. A logical approach to the biochemistry of disease resistance in plants is to study plant substances that are known to inhibit plant pathogens. Avenacin is such a substance; a potent plant antibiotic, especially inhibitory to fungi, that gives half-inhibition of Neurospora at concentrations of about 4×10^{-7} M (0.4 µg/ml). Tested in a similar manner, 2,4-dinitrophenol (DNP), a well-known poison of oxidative energy metabolism causes similar inhibition at concentrations between 5×10^{-5} M and 5×10^{-4} M (Author, unpublished).

From Turner's work and from the data of Table IV mentioned earlier, there seem to be at least two different inhibitors in oat seedlings. The amount of avenacin obtained from roots estimated from anti-Neurospora activity as compared with the amount of avenacin in the extracts of roots estimated by ultraviolet absorption agrees fairly well, indicating that avenacin is the chief anti-fungal, or at least anti-Neurospora, substance in oat roots. On the other hand the amount of avenacin in leaves, if there is any, is much too small to

account for the extractable anti-Neurospora activity. The nature of the leaf substance or substances remains to be investigated. Another question is whether or not purified avenacin shows the specificity against various Ophiobolus isolates that was found by Turner in oat root juices (24,25). The results of Turner's experiments with partially purified material from plant juices are difficult to interpret because one stage in the purification scheme involved boiling the juice. Considering the usual acidity of plant juices and the lability of avenacin to such treatment it seems unlikely that avenacin could have survived the isolation. This question can be answered now by testing purified avenacin against the various isolates.

With the knowledge that avenacin is an active in vitro inhibitor an obvious question that arises is, "What is the function of avenacin in the plant?" No specific studies have been devoted to this question, but some information that bears on the question can be obtained from the amounts of avenacin obtained from seedlings. The concentration in the root can be estimated to be about 0.1 mg/ml from the known yield of 100 milligrams of avenacin per kilogram of roots. Even considering the possible errors of such an order of magnitude estimate, concentrations of avenacin must occur in vivo that are able to give very strong in vitro inhibition. As is suggested by Goodwin's evidence (27) as well as visual observation of the fluorescence of intact roots the tip may

contain a very high concentration of avenacin. For instance, if all the avenacin is in a tip approximated roughly by a cylinder 0.5 x 2 mm and there are 10^5 tips per kilogram of roots, the concentration would be about 2.5 mg/ml, a very toxic concentration indeed. Even though these figures do not prove that avenacin is responsible for disease resistance in oats they show that concentrations sufficient to be highly inhibitory exist in the plant and hence strongly suggest that it is a protective substance. Comparative biochemical and genetic studies with oats types of different susceptibilities would be of great interest in dealing with this aspect of avenacin studies.

Chemical Nature of Avenacin

Although the complete structure for avenacin cannot as yet be written enough information is available to describe some of its most conspicuous features. The elemental composition given by the best analysis is C, 59.3%; H, 7.75%; O, 31.6%, and N, 1.4%; giving 1000 as the molecular weight, a value that agrees exactly with the estimate from ultraviolet absorption and is bracketed by all other estimates. At best an approximate molecular formula can be given as $C_{49\pm1}\ H_{78\pm2}\ O_{20\pm1}\ N$, assuming reasonable errors in the analysis to be $\pm$0.2% for C and H, $\pm$0.3% for N, and $\pm$0.5% for O.

Comparison of the ultraviolet absorption of the N-methyl anthranilate moiety with that of avenacin shows that it is the only chromophore of avenacin. The base lability of the bond linking N-methyl anthranilate to the rest of the molecule and the similarity of ultraviolet absorption and fluorescence versus pH behavior of avenacin with that of methyl N-methyl anthranilate show that the acid is linked to the remainder of the avenacin molecule in an ester linkage. Short-time (one hour) acid hydrolysis, in contrast to base hydrolysis, does not give the free acid, but rather a water insoluble substance with spectrum similar to avenacin but constituting only 53% of the weight with the remaining 47% as a water soluble carbohydrate mixture. Longer (18 hours) acid hydrolysis converts the short-time hydrolyzed water soluble material from a complex mixture to a simple mixture containing pentose- and hexose-like

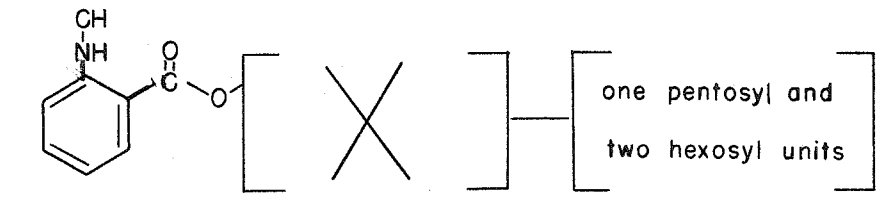
substances. Chromatographically the pentose was not distinguished from arabinose and the hexose was not distinguished from glucose or galactose in the solvent used. A quantitative orcinol reaction, which detects oligo- and monosaccharides, shows that the short-time hydrolyzate also contains pentose and hexose, in the amount of one molecule of pentose and two molecules of hexose per molecule of avenacin. A ferricyanide reducing sugar determination revealed no reducing power in avenacin and somewhat less than one mole of reducing power in the water soluble short-time acid hydrolyzate. Thus the simple sugars are apparently linked in glycosidic linkage to each other and to avenacin. Furthermore, since acid hydrolysis frees the carbohydrate portion while leaving the N-methyl anthranilate still bound, the acid must be attached to some part of the avenacin molecule other than the three sugars.

Figure 7 shows a semi-schematic formula summarizing the above discussion of the chemical nature of avenacin. The identity of N-methyl anthranilic acid as the chromophore is certain. There is good evidence that the carbohydrate portion of avenacin consists of an oligosaccharide containing one pentosyl and two hexosyl units linked to each other and to the rest of avenacin in such a way that all reducing groups are covered. The complete identity of the monosaccharides, their arrangement and linkage in the oligosaccharide and their configuration have yet to be determined. The most important

Avenacin

$C_{49\pm1}H_{78\pm2}O_{20\pm1}N$

MW~1000



$C_8H_8O_2N$ $C_{24\pm1}H_{40\pm2}O_{3\pm1}$ $C_{17}H_{30}O_{15}$

MW= 150 MW~400 MW= 457

FIGURE 7

Semi-schematic Formula for Avenacin

missing link in the structure of avenacin is "X" in figure 7. If "X" and the so-called aglycone isolated from alkaline hydrolyzates of the water insoluble acid hydrolysis product are the same, something is known of its properties. Its solubility is reminiscent of avenacin itself with the aglycone being soluble in alcohols but not in strongly polar or non-polar solvents. Infrared spectroscopy indicates the presence of hydroxyls and a carbonyl function in the material. The absorption frequency (1700 cm^{-1}) of the carbonyl is similar to those of several types of functional groups (conjugated aldehydes and ketones, aryl ketones, aliphatic aldehydes and

some amides) (48). All but two types of those remaining can be eliminated by other evidence. Saponification of avenacin indicates only one saponifiable group per molecule; that must be the one involved in the N-methyl anthranilic acid ester linkage. Similarly amides and lactams are eliminated because there is only one nitrogen atom. Of the remaining types all but two are conjugated systems and because the aglycone has no strong ultraviolet absorption above 210 mμ, all are eliminated but aliphatic aldehydes and ketones (45). A weak but positive 2,4-dinitrophenyl hydrazine reaction also suggests aldehyde or ketone presence. Although it is difficult to determine weights of fractions accurately with small amounts of material there was no apparent unaccounted for loss of large portions in the degradation reactions so if such groups exist they probably have molecular weight less than 100. If isoavenacin is the result of an isomerization as suggested in the working name or even if it is the product of splitting off a small fragment by hydrolysis the transformation could very well result from some alteration in the "X" portion of the molecule. Indeed, it is difficult to imagine a change involving sugars or N-methyl anthranilic acid that would have the characteristics of the avenacin-isoavenacin transformation. Explanation of the formation of isoavenacin as well as elucidation of the complete structure of avenacin await further studies. The availability of an isolation technique

for avenacin and knowledge of the hydrolytic behavior of avenacin make possible the obtaining of additional amounts of material needed for extensive chemical and biological studies.

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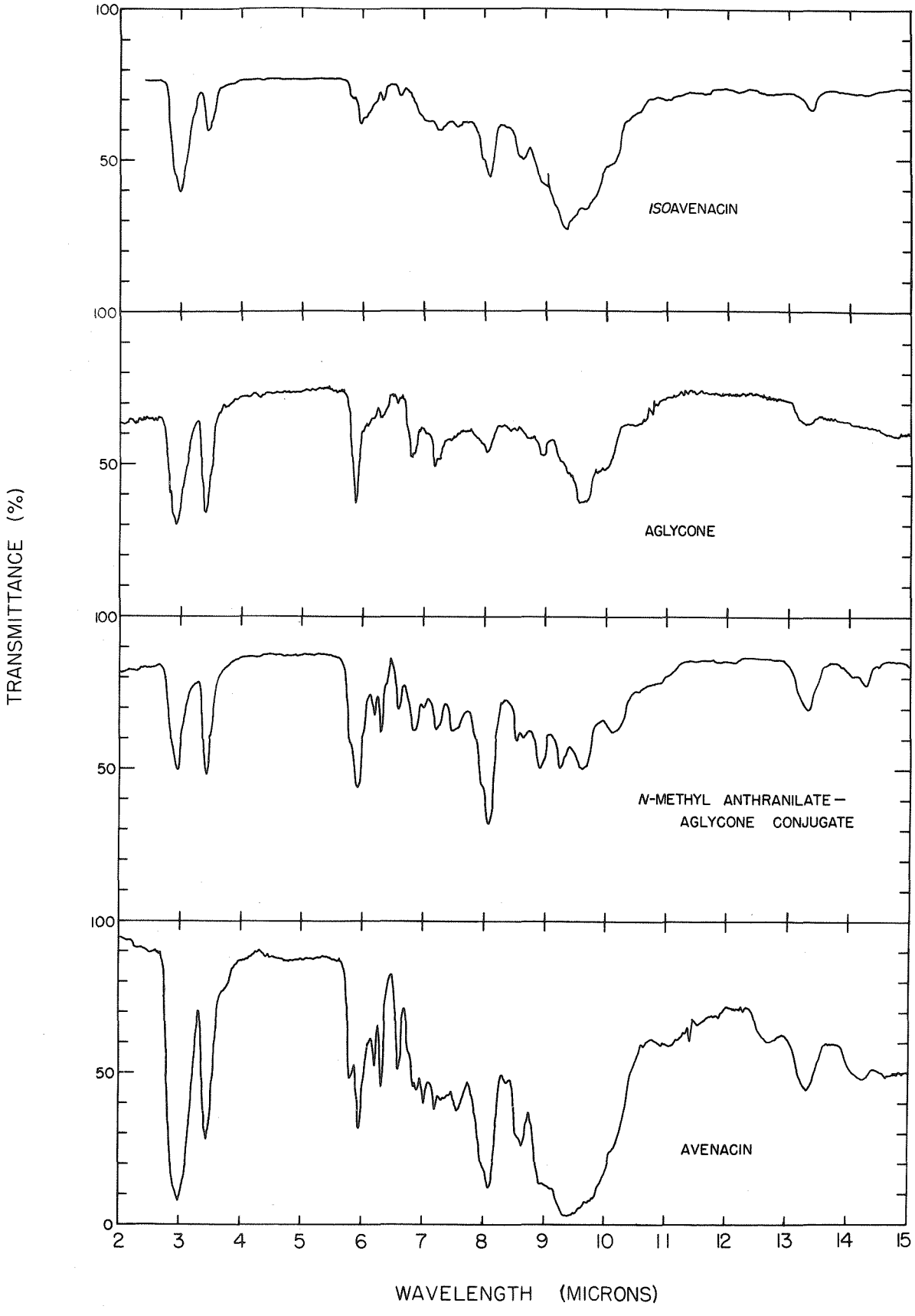
TRANSMITTANCE (%)
100
50
ISOAVENACIN
100
50
AGLYCONE
100
50
N-METHYL ANTHRANILATE—
AGLYCONE CONJUGATE
100
50
0
AVENACIN
2
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

APPENDIX

Organisms Not Inhibited by Avenacin at a Level of 50 µg/ml

Bacteria

Staphylococcus aureus
Staphylococcus albus
Bacillus subtilis
Sarcina lutea
Mycobacterium avium
Escherichia coli
Proteus vulgaris
Pseudomonas aeruginosa
Aerobacter aerogenes
Klebsiella pneumoniae
Salmonella enteritidis
Shigella paradysenteriae
Trichophyton rubrum
Trichophyton interdigitale
Brucella bronchiseptica
Vibrio metschnikovii

Bacterial Plant Pathogens

Erwinia amylovora
Erwinia caratovora
Xanthomonas phaseoli
Xanthomonas pruni

Fungal Plant Pathogens

Aspergillus niger
Colletotrichum lagenarium
Endoconidiophora fagacearum
Fusarium oxysporum lycopersici
Glomerella cingulata
Penicillium expansum
Pullularia sp.
Sclerotinia fructicola

APPENDIX