Avenacin, an Antimicrobial Substance Isolated from *Avena sativa*. I. Isolation and Antimicrobial Activity*

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Earlier studies by E. M. Turner (*J. Exptl. Botany 4*, 254 [1953], *et seqq.*) on host-fungal parasite relations in oats led to the discovery of an antifungal substance accumulated in oat roots. This compound, which has been named avenacin, and which can be observed directly in ultraviolet light as a bright blue fluorescent material in root tips, has been isolated in a pure form. The yield is 155 mg/kg of roots. Avenacin is a potent inhibitor for the growth of sixteen of forty-five microorganisms tested including *Ophiobolus graminis*, *Ceratostomella ulmi*, *Mycobacterium tuberculosis*, *Neurospora crassa*, *Saccharomyces pastorianus*, and *Candida albicans*.

While many antibiotics have been obtained from microorganisms, especially the fungi, observations of antimicrobial activity in preparations from higher plants are less numerous (Nickell, 1959). This report is concerned with the isolation of such a substance from the roots of oat seedlings. As shown by Turner (1956, 1961) this compound, which we have named avenacin, is responsible for the resistance of oats to *Ophiobolus graminis*, the fungus that causes the take-all disease of barley and wheat. The pure substance inhibits the growth of *O. graminis* and fifteen other plant and animal pathogenic microorganisms at levels of 3–50 μg/ml. Avenacin causes hemolysis of red blood cells and is therefore extremely toxic to animals when injected but not when ingested.

Experimental Procedures

Growing Oats.—Roots from oat seeds of different varieties were used throughout the experiments 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 Utsädesforening, Svälof, Sweden), Kanota (California Milling Corp., Los Angeles) or recleaned oats of unknown variety (Ambler Milling Co., San Gabriel, Calif.). Seed was soaked for 30 minutes in tap water and spread on gauze-covered coarse-mesh aluminum grids over plastic pans at the equivalent of 300–400 g of dry seed per 38 × 56-cm pan surface. The pans were filled with tap water to within an inch of the seed bed, equipped with bubblers, and covered with aluminum foil to maintain a moist atmosphere for germination. After about 3 days when the roots extended into the water the foil was removed. The pans were kept with continuous aeration of the water in a greenhouse or on a laboratory bench near a window for 8–9 days, after which the roots were harvested.

Isolation and Purification of Avenacin.—The fresh harvested roots (15 kg) collected from forty pans were immersed in liquid nitrogen and ground to a powder in an electric grinder. This powder was immediately mixed with 15 liters of methanol and agitated in a large Waring Blendor for 15–20 minutes. The resulting slurry was filtered and the solid residue was re-extracted twice with 7.5 liters of methanol-water (80:20, v/v).

The combined extracts were evaporated to dryness *in vacuo* and the remaining powder was extracted with eight 350-ml portions of absolute methanol. Absolute ether (5 liters) was added to the methanol solution, and the precipitate was collected and dried *in vacuo* yielding 56 g of a dry powder: $E^{1\%}_{1cm}$ at 223 mμ = 78 (Fig. 1A). This powder was dissolved in 1680 ml of distilled water and set aside for about 1 hour. Ether (2800 ml) was added and the mixture was shaken thoroughly until a gel had formed. After storage in a refrigerator for several hours the precipitate was separated by centrifugation at 20,000 × *g* for 15 minutes and dried in a vacuum desiccator at room temperature over P_2O_5. Yield: 5.1 g $E^{1\%}_{1cm}$ at 223 mμ = 178 (Fig. 1B). This material was dissolved in a minimum amount of 80% ethanol (50 ml). The avenacin was crystallized by gradual addition of water (100 ml) and was separated, after 24 hours at 0°, by centrifugation. The product was dried in a vacuum desiccator: $E^{1\%}_{1cm}$ at 223 mμ = 211 (Fig. 1C). The solid residue was dissolved in 80% ethanol (100 ml) and treated with washed Darco charcoal (2 g). The charcoal was filtered off and washed with two 50-ml portions of ethanol. After filtration and concentration to a volume of about 20 ml, water (50 ml) was added and the precipitate was collected by centrifugation and dried *in vacuo* over P_2O_5. After two to three additional crystallizations the yield was 2.3 g of chromatographically pure avenacin: $E^{1\%}_{1cm}$ at 223 mμ = 238 (Fig. 1D) mp 248–250° dec. (uncorr), $[\alpha]^{24}_D$ = +35.7° (*c* 1, H_2O) λ_{max} (mμ): 223, 253, and 357 (Fig. 1D). The crystals obtained by adding water to hot saturated 80% ethanol solutions were long filamentous needles that were 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.

Paper Chromatography.—Whatman No. 1 paper was used for qualitative work and three solvent systems were used: (1) methylethylketone–acetic acid–water (30:3:10, v/v); (2) 1-propanol–1% ammonium (3:1, v/v); and (3) 1-butanol–acetic acid–water (100:50:100, v/v). Corresponding R_F values for avenacin are 0.58 (1); 0.75 (2); 0.67 (3).

Bioassay.—*Neurospora crassa*, strain 5912-2A, was used as the bioassay organism. Inoculum material for the assay experiments was prepared by seeding 30-ml portions of minimal nutrient agar (Ryan *et al.*, 1943) with conidiospores from sterile cultures. The seeded agar was poured into 20 × 100-mm petri dishes and incubated from 2 to 3 days at 25° until a thick mycelial growth was formed. These cultures were stored in a refrigerator at 4° and remained usable for several weeks.

Triplicates of samples to be assayed were put into 15 × 150-mm culture tubes and suspended in 4.0-ml aliquots of molten nutrient agar. The contents of the tubes were mixed by shaking and poured into 10 × 50-mm petri dishes. Cylinders (4-mm diameter) were

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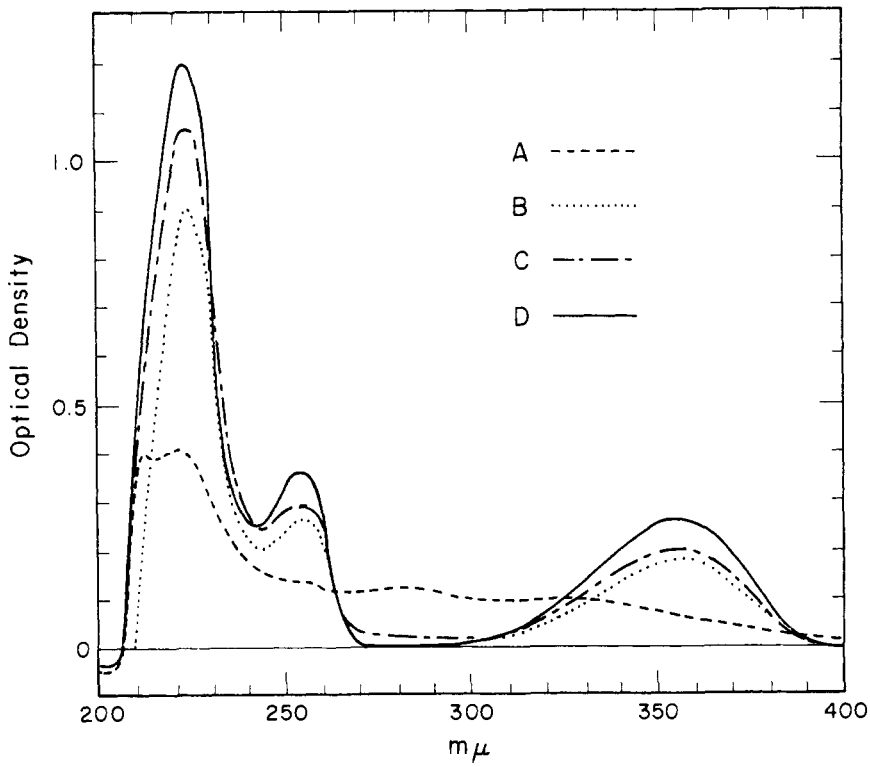

FIG. 1.—Ultraviolet spectra of avenacin at different stages of purification (see text). All curves were obtained with a Cary Model 15 spectrophotometer using a 1 × 1-cm cell and methanol as solvent (concentration 50 μg/ml).

cut from the inoculum plate and placed mycelial side down in the center of the solidified assay plates. After 15 hours incubation at 25° the diameters of cultures were measured to the nearest millimeter. Results were expressed as percentage growth compared to a control which contained no inhibitor.

Purified avenacin when tested in the bioassay gave 50% inhibition at 0.4 μg/ml avenacin. This concentration was defined as equal to one unit of activity. A dose-response curve for various concentrations of avenacin is shown in Figure 2.

Toxicity.—Only limited experiments have been performed to establish the degree of toxicity of avenacin. Avenacin when administered to animals (mice) at 10 mg/kg and higher doses caused, shortly after ip injection, writhing, ataxia, and prostration followed by death. All animals died within 7 days when injected at levels as low as 1.6 mg/kg. Oral administration of avenacin even at levels up to 128 mg/kg showed only slight, if any, effect.

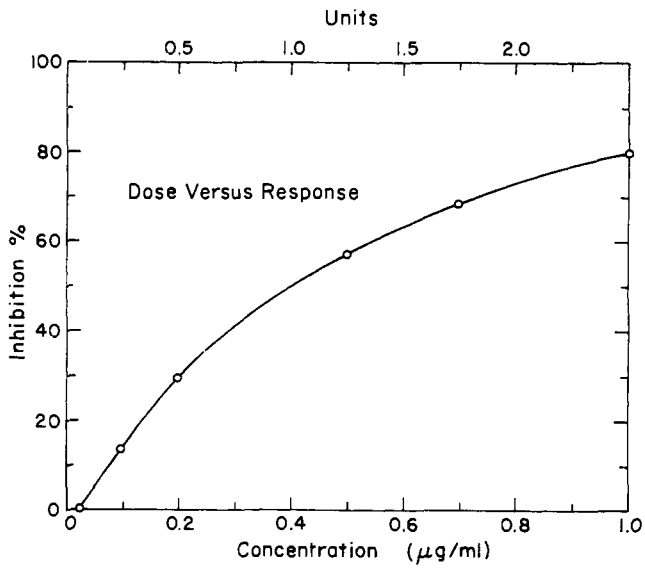

FIG. 2.—Response of *Neurospora* to avenacin.

DISCUSSION

Although several methods have been devised for the purification of avenacin (Turner, 1956; Maizel, 1960) the procedure described here is the simplest and gives much the best yield (155 mg/kg of moist roots). The method takes advantage of the unusual and peculiar property of avenacin to exist in a water-insoluble (from ethanol-water) and in a water-soluble form (from ethanol-ether). This difference is probably due to solvation and the two forms are interconvertible without loss in biological activity.

It has been convenient to use the fungus *Neurospora* for bioassays during purification though many other organisms could have been used. Pure avenacin was tested as an inhibitor on a variety of microorganisms and animals through the courtesy of Dr. W. M. Stark of the Eli Lilly Laboratories and in this laboratory. Results are summarized in Table I. *O. graminis* was used originally by Turner (1960) for bioassay purposes. Determinations of avenacin by chromatography, ultraviolet-absorption spectra, and fluorescence are also convenient and it is highly probable that avenacin is identical with the fluorescent substance purified from oats by Goodwin and Pollock (1954). In the experiments described here both ultraviolet spectra and biological activity were used quantitatively to follow purifications and products were recrystallized to constant properties with respect to these criteria as well as to constant chromatographic behavior and purity.

From the work of Turner (1961) it appears that the accumulation of avenacin in oat roots provides the basis for a natural mechanism of resistance to at least

TABLE I
INHIBITION ACTIVITY OF AVENACIN ON VARIOUS ORGANISMS

Organisms Inhibited by Avenacin at a Level up to 50 μg/ml[a]	
Organisms Tested	100% Inhibition (at μg/ml)
Mycobacterium tuberculosis	12.5
Helminthosporium sativum	3.13
Pelicularia filamentosa	3.13
Ophiobolus graminis	3.13
Alternaria solani	6.25
Ceratostomella ulmi	6.25
Verticillium albo-atrum	6.25
Neurospora crassa	6.25
Botrytis cinerea	12.5
Polyporus ostreatus	12.5
Trichophyton interdigitale	25
Saccharomyces pastorianus	25
Colletotrichum pisi	50
Endoconidiophora fagacearum	50
Candida albicans	50
Pythium irregulare	50

Organisms not Inhibited by Avenacin at a Level up to 50 μg/ml	
Bacteria	Fungal Plant and Animal Pathogens
Staphylococcus aureus	*Aspergillus niger*
Staphylococcus albus	*Colletotrichum lagenarium*
Bacillus subtilis	*Endoconidiophora fagacearum*
Sarcina lutea	*Fusarium oxysporum lytopersici*
Mycobacterium avium	*Penicillium expansum*
Escherichia coli	*Pullularia sp.*
Proteus vulgaris	*Sclerotinia fructicola*
Pseudomonas aeruginosa	*Trichophyton rubrum*
Aerobacter aerogenes	*Glomerella cingulata*
Klebsiella pneumoniae	*Phoma pigmentovora*
Salmonella enteritidis	
Shigella paradysenteriae	
Trichophyton rubrum	
Brucella bronchiseptica	
Vibrio metschnikovii	
Erwinia amylovora	
Erwinia caratovora	
Xanthomonas phaseoli	
Xanthomonas pruni	

[a] An agar-dilution end-point method was used on enriched media and the concentration of avenacin necessary to prevent growth completely in a given time was measured. Concentrations higher than 50 μg/ml were not tested.

some phytopathogenic fungi but the prospects for use of the compound itself as antibiotic are not very good due to toxic properties. However, as may be noted in the accompanying report on the structure of avenacin, the substance represents a new class of compounds that may be of general interest in studies of biological inhibitors.

REFERENCES

Goodwin, R. H., and Pollock, B. M. (1954), *Am. J. Botany 41*, 516.
Maizel, J. V. (1960), thesis, California Institute of Technology, Division of Biology.
Nickell, L. G. (1959), *Econ. Botany 13*, 281.
Ryan, F. J., Beadle, G. W., and Tatum, E. L. (1943), *Am. J. Botany 30*, 784.
Turner, E. M. (1953), *J. Exptl. Botany 4*, 264.
Turner, E. M. (1956), *Ann. Appl. Biol. 44*, 200.
Turner, E. M. (1960), *Nature 186*, 325.
Turner, E. M. (1961), *J. Exptl. Botany 12*, 169.

Avenacin, an Antimicrobial Substance Isolated from *Avena sativa*. II. Structure*

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The structure of the antimicrobial substance avenacin accumulated in oat roots has been analyzed. Avenacin can be hydrolyzed by acid and base into five constituents, three of which are carbohydrates, one is *N*-methylanthranilic acid, and one is a triterpene. The terpene is a pentacyclic triterpene belonging in the class of the Δ^{18}-oleanenes. It has six functional groups, five of which are hydroxyl groups, and one is a keto group. Two of the carbohydrate components are glucose, while the third one has not yet been identified. *N*-Methylanthranilic acid is linked through an ester bond to the triterpene and the carbohydrates are apparently linked to each other and to the terpene through glycosidic bonds.

In the accompanying report (Maizel *et al.*, 1964) on the isolation of avenacin from the roots of oat seedlings it was observed that the compound has a molecular weight of about 1100, it is fluorescent in ultraviolet light, and has a characteristic absorption spectrum. Maizel (1960) identified the absorbing-fluorescing component obtained after hydrolysis as *N*-methylanthranilic acid and also demonstrated the presence of sugars and a large nonpolar component among the hydrolytic products. As a basis for presentation of these and additional extensive details that provide a basis for a provisional structure for avenacin a summary of hydrolytic products is given in Figure 1. As shown, complete hydrolysis yields five molecules. The first is a pentoselike compound not yet fully characterized, 2 and 3 are glucose, 4 is a triterpene called avenagenin, since avenacin is a saponin derivative, and 5 is *N*-methylanthranilic acid. Partial hydrolytic products of particular significance at present are avenamine (from acid hydrolysis for removal of the sugar components) and avenagenol (derived by lithium

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