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The Biological Activity of Phytotoxic Extracts from Corynebacterium sepedonicum

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THE BIOLOGICAL ACTIVITY OF PHYTOTOXIC EXTRACTS FROM *CORYNEBACTERIUM SEPEDONICUM*

by

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INTRODUCTION

Isolation of a phytotoxin from the ring rot pathogen of potatoes, Corynebacterium sepedonicum, had previously been reported by Strobel (21). The first phase in our study was to investigate more extensively the general nature of this compound. The phytotoxin could possibly be a specific compound associated only with the ring rot pathogen. However, the possibility that the substance could be obtained from sources other than the bacterium had not been completely eliminated.

In an attempt to answer this question, three extracts were purified and compared. First, compounds were isolated from a broth culture of bacteria in the manner described by Strobel (21). Second, since the culture medium consisted of yeast extract, the possibility that similar types of compounds could be obtained from this medium was considered. From this work, much information could be gained on the nature of these substances. Third, materials that possessed wilting activity were isolated from whole bacterial cells which had been subjected to sonic oscillation. This procedure eliminated impurities present in extracts from broth cultures and was applied to the purification of similar extracts from other bacteria. Upon isolation of these three extracts, the physical properties and biological activity were compared to determine their degree of similarity.

An investigation was also made of the type of compound which was present in the extracts and capable of inducing biological activity. Preliminary work had shown the presence of nucleic acid in the extracts. Nucleic acid, as a constituent of the biological active materials, had previously been unrecognized.

LITERATURE REVIEW

Potato Ring Rot - The pathogen, Corynebacterium sepedonicum (Spieck) Dows., incites an economically destructive disease known as ring rot of potato. The causal organism is a gram positive bacterium characterized by a pleomorphic rod-shaped morphology. Pathogenicity is specifically restricted to potato hosts. Related bacteria, Corynebacterium michiganense and Corynebacterium insidiosum, have identical morphology and gram reactions but are strictly pathogenic to tomato and alfalfa, respectively.

If potato plants are subjected to conditions such as those which occur during a warm summer, infection by Corynebacterium sepedonicum may produce chlorosis and marginal wilting of the leaflets. Later in the season, stunting and collapse of stem tissue may be evident. Internal sectioning may reveal browning of the vascular elements. In tubers, the first sign of disease is usually a light yellow vascular discoloration. Later development may produce a breakdown of parenchyma tissue giving rise to large hollow cavities. Cankers and lesions which provide areas through which saprophytes may enter are commonly formed on the surface of the tuber (25). Excessive wilting of infected plants is seldom observed until late in the growing season. Symptoms develop slowly and very rarely shorten the life of the plant by more than fourteen days (19).

Therefore, detection of the disease by means of symptoms is difficult unless infection occurs in a large number of plants.

Wilting mechanism - Wilting induced by pathogenic attack indicates an impairment of the water economy of the plant. The loss of water through transpiration is greater than the rate of absorption through roots. Losses of cellular fluids coincide with a reduction in turgor pressure. Tissues become flaccid with ultimate results showing cell collapse and death.

One mechanism by which a pathogen may incite wilting is the stimulation of enzyme activity. Enzymatic degradation of plant cells may result in the deposit of pentosans, gums, and various mucilaginous substances in the water conducting elements. Such deposits could cause an obstruction of the water transport system. An increase in the viscosity of tracheal fluid might provide a decrease in the supply of water to cells either through blockage of vessels or a retarded rate of water movement (4). Enzymes might act by a second mode, also. Quantities of pectic enzymes associated with a pathogen may incite an alteration in the cellular permeability, allowing leakage of cell fluid. Plant fluid would subsequently be more readily transpired through the stomates and cuticle.

Paquin and co-workers in 1960 (17), growing Corynebacterium sepedonicum on synthetic media, were unable to detect quantities of pectic enzymes. Pectin methylesterase, depolymerase, polygalacturonase and protopectinase were not found in cultures. None of the enzymes were present in detectable quantities in heavily infected potatoes showing ring rot symptoms. The authors speculated that a toxin secreted by the bacteria, but differing in nature from the pectinases, might be responsible for the wilting.

Polysaccharide materials produced by pathogens have been associated with several wilt diseases. Husain and Kelman (11) correlated slime production with the degree of pathogenicity of Pseudomonas solanacearum. Weakly pathogenic and avirulent strains secrete small quantities of the carbohydrate. Highly pathogenic bacteria were associated with sizable concentrations of the slime. Viscous polysaccharides have also been isolated from Xanthomonas phaseoli by Leach, etc. (14).

Spencer and Gorin (18) in 1961 reported the isolation of toxic gums from aerated broth cultures of Corynebacterium insidiosum and of Corynebacterium sepedonicum. Purified polysaccharides were recovered at an average yield of 2.5 grams/liter for Corynebacterium sepedonicum. The assay for toxicity was young alfalfa cuttings. Plants placed in solutions of purified toxins wilted within ten to fifteen minutes. Chromatographic analysis of acid hydrolyzates through a

cellulose column revealed the presence of D-glucose, D-galactose, and L-fucose residues. The ratios of these sugars were 1.55 : 0.93 : 1, respectively.

Chemical analysis of phytotoxin - Large amounts of non-biologically active materials were eliminated by Strobel's modification (21) of the purification procedure used by Spencer and Gorin. Culture broth was passed through ion exchange resin followed by precipitation in acetone. The precipitated compound was then subjected to chromotography through a Sephadex G-200 column. This filtration yielded two peaks of material differing in molecular weight. Values of molecular weight were determined by membrane osmometry, equilibrium centrifugation, and column chromatography with Sephadex G-200. The first peak, eluted in the void volume of the column, contained a polysaccharide that had a molecular weight greater than 2×10^5. It was water soluble but possessed no specific physiological activity. The polysaccharide in peak two yielded an average molecular weight of 21,450. This material induced wilting in young tomato cuttings as described by Hodgson, etc. (10).

Chemical analysis of the phytotoxic product in peak two was made by Strobel (21). Elemental analysis showed an empirical formula of $C_{48}H_{96}O_{48}N$. The infrared spectrum possessed absorption bands typical of polysaccharides: 3300 cm^{-1} and 1100 cm^{-1} for OH^- groups and 2900 cm^{-1} for -CH-.

Another band at 1615 cm^{-1} was not assigned. The general spectrum was characteristic of a compound acidic in nature.

Constituents of sugar residues were determined by gas chromatography of silyl ether derivaties (24). The most predominant sugar was glucose which comprised 49% of the total material. Mannose was present at 12.8%. L-Fucose was detected only in 1% quantities. Trace amounts of other sugars were also evident.

An organic acid was isolated by passing an acid hydrolyzate through a Dowex 1, acetate form, column; the acid fraction was held by the resin. The fraction was removed from the column by elution with 5 N acetic acid. The dried weight showed that the anion fraction made up 6% of the total material. Infrared absorption analysis strongly suggested the presence of a carboxylic acid. The compound showed a maximum ultraviolet absorbance at 330 mμ when reacted with o-phenylenediamine reagent, a reaction indicative of alpha-keto acids. An absorption maximum at 549 mμ with the thiobarbituric acid method was obtained. This reaction identified the material as a 2-keto-3-deoxyaldonic acid or a related derivative. In further studies, R_F values of the unknown in four solvent systems were identical with 2-keto-3-deoxygluconic acid.

The possible presence of a peptide moiety was also investigated by Strobel (24). The following test results supported the concept that the isolated material partially

consisted of protein: a) the copper salt micromethod produced a complex with an ultraviolet absorption maximum at 221 mμ, b) a yellow reaction product with ninhydrin, c) an ultraviolet absorption band of the toxic material between 245 and 250 mμ, and d) an aniline blue-black reaction product. Upon acid hydrolysis, nine free residues of amino acids were recovered. These residues accounted for 4.4% of the phytotoxic material (24).

Analysis of NH_2-terminal amino acids showed the occurrence of alanine to glycine in a 3/2 ratio. The presence of two amino acids in terminal positions suggested multiple fragments or a branched peptide moiety in the molecule. A covalent union between peptide and carbohydrate moieties was also proposed by Strobel (24). A ninhydrin positive area migrated at the same rate as a $AgNO_3$ positive zone when subjected to electrophoresis, and no other zones were detected. Furthermore, residues of amino acids were released only upon acid hydrolysis. Such results are likely to be obtained from a compound with a glycopeptide molecular structure.

Other physical properties were determined by Strobel (21). When subjected to high voltage electrophoresis, the material did not migrate in 2.0 M acetate, 1.5 M formate, pH 2.1 after 3 hours. The lack of mobility at this particular pH value indicated it was the approximate isoelectric point for the compound. Other properties were an intrinsic viscosity of

0.125 and an S_{20W} of 0.76. The specific optical rotation was $(alpha)_D^{30} = + 30$.

Biological activity of the phytotoxic material - Hodgson, Riker, and Peterson in 1947 first recognized the use of the tomato, Lycopersicon esculentum, as a biological assay of wilting. Symptoms were induced by submerging excised ends of plants in solutions of bacterial polysaccharides. Plants, two to three weeks old and twelve to eighteen inches in height, were selected for uniform size and leaf area (9). Age and condition of the tomato plants influenced considerably the type and severity of symptoms. For example, young cuttings wilted faster and showed more extensive stem and petiole damage when compared with older plants. Variable conditions such as temperature, light intensity, and relative humidity during the treatment period influenced the reaction. To provide reliability of the test, these conditions were defined.

Other workers have also used young tomato cuttings as an assay method for wilt-inducing polysaccharides. Goodman and Lewis studied the toxic agent of Colletotrichum fuscum (15). In 1951, Feder and Ark assayed purified polysaccharides derived from Erwinia carotovora, Xanthomonas phaseoli and Agrobacterium tumefaciens (5).

For determination of phytotoxic activity, a modification of the tomato plant assay was adopted by Strobel (24). Plants of the variety Earliana were grown in vermiculite under

continuous illumination. Shedding of the seed coat prior to use was necessary as plants with the coat attached to the primary leaves were less responsive to wilt. Test cuttings were excised at an age of eight to ten days; the primary leaves were removed, and the seedlings were submerged in test solutions. Wilting was allowed to take place in an illuminated chamber at 24°C. A water reservoir on top of the chamber provided cooling for the light source. Average values of stem strength were determined by a wilt-o-meter for each treatment (12). The plant stem was positioned in a holder located on a turntable. As the turntable rotated, it brought the tomato stem in contact with the recording needle. As the needle was moved by contact with the stem, tension upon the needle increased linearly with distance. At a certain point, the stem would bend and slip under the needle. A numerical value was obtained at this point from the calibration scale. This value was a measurement of the flaccidity of the stem.

In contrast to the occlusion induced with large molecular weight compounds, stem strength was reduced by the glycopeptide from Corynebacterium sepedonicum. Strobel and Hess (22) for this reason, proposed that the mode of phytotoxic action was an alteration in the cellular membranes rather than the commonly held theory of physical occlusion (18). In 1969, a series of experiments were presented to reveal the apparent

mode of action of the phytotoxin. Three treatments of dextran, water, and phytotoxin were conducted on assay cuttings. Dextran was used as a high molecular weight standard to show the amount of wilting caused by a blockage of the vessel elements. Cuttings placed in water were assumed to be standards in which there was no alteration in the transportation of water. The movement of acid fuchsin dye through the stems was observed after the three assay treatments. In water and phytotoxin assay plants, dye reached terminal leaflets within five to six minutes. Dye movement through dextran treated plants was irregular and required 35 minutes. This delay was presumably due to vessel obstruction.

The leakage of cellular fluids into conducting tissues was measured by two methods. The degree of electrolyte leakage from cells was determined by the conductivity of washes of distilled water flushed over test cuttings. The conductance of water and dextran treatments were similar, but in contrast, a much higher value was obtained from the phytotoxin -plant reaction. The second method involved the take-up of tritiated water prior to the three treatments. The plants were treated and placed in a chamber containing anhydrous P_2O_5. Water escaping from the leaves was trapped in the desiccant. Therefore, the radioactivity count in the P_2O_5 was an indication of the water loss by plant transpiration. The greatest loss of tritiated water was acquired from the phytotoxin treated cuttings.

In the study by Strobel and Hess (22), root systems of two to three week old tomato plants were placed in water, phytotoxin, and dextran solutions. At the end of specified time periods, the roots were placed in a hypertonic solution of 0.5 M mannitol. If membranes had been damaged, cells would not plasmolyze in this solution. Within one hour after exposure to the toxin, root hairs lost their ability to plasmolyze, but dextran and water treated roots did not show the effect until after three hours. The results of this test indicated that damage to the membrane was induced by the toxin.

Observations made of the ultrastructure of toxin treated tomato cuttings showed cellular membrane as well as mitochondria and chloroplast membrane damage (22). Destruction was not confined to a particular tissue layer or cell type. However, extensive tissue degradation was present in the stem, an observation in agreement with earlier autoradiography studies (21). Infected potato cultivars of Russet and Norgold Russet varieties also showed a significantly large number of alterations of cellular membranes in comparison to non-infected plants (8). The separation of the middle lamella from the cell wall was a distinctive feature observed in sections obtained from plants which were infected with the pathogen. Intracellular spaces were readily observed at the site where three cell walls had previously been united in the tissue.

From the results obtained by Strobel and Hess(22), the phytotoxin apparently induced an alteration in the cellular tissue which resulted in an increase in leakage of fluids from the tomato cuttings. Evidence from a study of the ultrastructure of plants infected with the pathogen also suggested that an alteration of the cellular membranes does occur. To see if this alteration occurred with other types of cells, Johnson and Strobel in 1970 (12) studied the effect of the phytotoxin upon the membranes of various animal cells. The following types of cells were used: a) guinea pig red blood cells suspended in 0.7% saline, b) 72 hour monolayer cultures of Madin Darby bovine and monkey kidney, and c) Chang liver and embryonic bovine trachea. Upon a treatment with 0.5% solutions of toxin, a decrease in any of the cell populations was not observed. Microscopic examination up to 72 hours showed no visible evidence of damage to the cells.

Site of attachment of phytotoxin - If membrane systems are altered, a possible toxin-cell component complex would be expected. Evidence of such a complex has been presented by Strobel and Hess (22). A plant extract from a ^{14}C-toxin treated assay plant was chromatographed on a Sephadex G-200 column. Labeled material having a molecular weight greater than 200,000 was isolated. The complex was disassociated by an adjustment of the pH to 1.5. Subsequent gel filtration of the disassociated product yielded material having chromatographic properties of the phytotoxin. The plant component

from the complex was not identified. Recovery of 90% of the intact ^{14}C-toxin from plant extracts suggests that breakdown of the phytotoxin does not occur prior to formation of the complex (22).

The active site of biological activity was examined by Johnson and Strobel (12). The phytotoxin was altered in the following manner: a) the NH_2-terminal groups on the peptide fragment were blocked by dansylation, b) the -OH groups and -NH- groups were blocked on the sugar and amino acid residues by exhaustive methylation, c) the COO- groups were converted to methyl esters, and d) the peptide moiety was treated with pronase. After chemical alteration in each of the above manners, the physiological activity of the material was assayed. The only modification resulting in a blockage of wilt inducing activity was the conversion of the carboxyl groups on the molecule to methyl esters. Pronase digestion was conducted to rule out the possibility that methylation of glutamic and aspartic amino acids was destroying the activity. The capacity to induce wilting was present in the pronase-digested material.

Serodiagnostic test - Serodiagnosis of potato ring rot was first attempted by Katznelson and Sutton in 1956 (13). An antiserum was made to whole cells of strain C5850 of C. sepedonicum. Agglutination properties of the serological reaction were combined with culture-microscope techniques

for final verification of infected hosts. The variability of the serological tests made it difficult to formulate conclusions from these test results alone.

In 1968, Strobel and Rai designed a serodiagnosis test for the detection of the phytotoxic glycopeptide in plant tissues (23). The presence of the toxin within the plant was shown to be specifically related to the presence of the pathogen. It was diffused systemically throughout the plant and occurred in detectable amounts prior to wilt development. These factors provided additional advantages over the agglutination test.

An antiserum was prepared in a New Zealand white rabbit to the bacterial glycopeptide by intramuscular injections. Four injections, each containing ten mg. of material emulsified in Freund's incomplete adjuvant, were made at weekly intervals. Maximum titers occurred during the third and fourth week after the last injection; a titer of 1 : 512 was obtained against an anigen concentration of 2.5 mg/ml. (24). The antigen used in this case was the purified phytotoxin obtained from bacterial cultures. When tested with this antiserum, the phytotoxins extracted from infected plants had an identical dilution end-point as that from culture broth. An identical or similar antigenic relationship was established with an Ouchterlony double diffusion test. Test material also cross-reacted with antiserum to phytotoxin from Corynebacterium michiganense.

For routine diagnosis of potato ring rot in the field, test antigen was extracted from fresh stem tissue or the vascular area of the tuber. The crude juice was decanted and passed through a small column of Dowex 50 (200-400 mesh, H^+ form). Cold acetone was added, and the precipitate was resuspended in water. Upon another centrifugation, the supernatant was collected and mixed with undiluted antiserum. The solution developed a distinct cloudiness if the tissue had been infected with the pathogen.

METHODS AND PROCEDURES

Two bacterial extracts were prepared for this study. The biological activity of the extracts was defined by the method described in the latter part of this section. The methods used to determine the chemical constituent which was responsible for wilting are also presented. These methods include the determination of the extinction coefficients, electrophoresis, and enzyme treatment.

Culturing methods - California and Montana isolates of Corynebacterium sepedonicum used in this study were obtained from Dr. Gary A. Strobel, Montana State University. The cultures were grown on the medium described by Spencer and Gorin (18): 1.5% glucose, 1.0% yeast extract dialyzate, and 0.5% calcium carbonate. Yeast extract was dialyzed for twelve hours in cellulose tubing three inches in width. All bacterial inoculations were made from stock cultures held under refrigeration. Cell-free extracts were prepared from non-aerated cultures incubated for six days at 25°C in 100 ml. quantities in 250 ml. Erlenmeyer flasks plugged with disposable sponge stoppers. Cell extracts were acquired from 500 ml. of liquid medium incubated in 1 liter flasks on a shaker at an identical temperature and time period. Disposable sponge stoppers were also used to plug the 1 liter flasks.

Cell-free extracts - A substance was extracted from fluid of a bacterial culture by the procedure of Strobel (21). Cells were removed from the culture fluid by centrifugation at 16,000 g. The liquid (500 ml.) was passed through a 2.5 cm. diameter by 5 cm. long column of 200-400 mesh Dowex 50 (H^+), followed by a 20 ml. rinse of distilled water. The effluent was given a repeat treatment by passage through another Dowex 50 column of identical dimensions, followed by a 20 ml. rinse. The liquid was concentrated by flash evaporation at 50°C to 10% of its original volume. Four parts of acetone at -15°C were combined with the concentrate. The solution was allowed to cool for ten minutes at 0°C. Following cooling, the precipitate was collected by centrifugation at 7,700 g and was resuspended in 1 ml. of distilled water. The resuspended material was stored in the refrigerator for twelve hours; whereupon, the insoluble material was pelleted at 27,000 g. The material present in the supernatant was termed "crude bacterial cell-free extract".

Sephadex G-200 was prepared in distilled water and was allowed to swell for three days. After swelling, a column, 1.4 x 24.5 cm., was prepared. A sample of the crude cell-free extract was then chromatographed on this column using distilled water for elution. Samples of 1.5 ml. were collected and analyzed by ultraviolet absorption at a 260 mμ

wavelength. Material eluted between 34 and 43 ml. was collected and dried over P_2O_5. This substance was termed "purified bacterial cell-free extract".

Bacterial cell extract - Bacterial cells (approximately 0.6 - 0.8 grams of dry weight per liter) from three liters of broth cultures were collected by centrifugation at 16,000 g. The supernatant was discarded, and the cells were resuspended in 40 ml. of saline (0.85% NaCl). The suspension was centrifuged at 12,000 g, and the supernatant discarded. This procedure was repeated two more times using 40 ml. of saline per wash. After the final wash, the cells were resuspended in 20 ml. of saline and allowed to remain at 4°C for twelve hours. Prior to sonic oscillation, the cell suspension was decanted into a stainless steel test tube from the calcium carbonate sediment which had been retained with the cells from the culture medium. The cells were cooled continually in ice during the disintegration with a Sonifier Cell Disruptor.[1] The microtip which had an output setting of 70 to 80 watts was used. During sonic oscillation the instrument was on for 20 seconds, then off for 20 second intervals to allow cooling. The total time of exposure to sonic oscillation was six minutes. Cell fragments were removed from the liquid suspension by centrifugation at 27,000 g. The pellet

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[1]Branson Sonic Power Company, Plainview, New York.

was washed with 10 ml. of saline and centrifuged. The supernatant fluid was immediately added to four parts of cold acetone (-15°C), and cooled at 0°C for 15 minutes. The precipitate was pelleted at 7,700 g and resuspended in 30 ml. of distilled water. The material stood for a twelve hour period at which time it was centrifuged at 27,000 g to remove the insoluble fraction. The supernatant was then passed through a 2.5 x 5 cm. Dowex 50 (H^+) 200-400 mesh column prepared in distilled water. The column was rinsed with 20 ml. of distilled water, and the rinse was added to the effluent. This liquid was concentrated by flash evaporation and termed "crude bacterial cell extract". One ml. of the crude extract was chromatographed over a Sephadex G-200, 1.4 x 24.5 cm., column prepared in distilled water. Aliquots of 1.5 ml. were collected, and the ultraviolet absorption was recorded at a 260 mμ wavelength. The volume between 34 and 43 ml. was collected and dried over P_2O_5. This substance was termed "purified bacterial cell extract".

Extinction coefficients - To estimate the percentage of nucleic acid in the extracts, extinction coefficients were determined for each substance. The wavelength of maximum ultraviolet absorption was determined with the Cary 14 Spectrophotometer recorder. Extinction coefficients were calculated from maximum absorption values at 260 mμ. Dry weight determinations were made on material dried over P_2O_5 in a

vacuum desciccator jar. Four experimental values were obtained for the following: crude cell-free extract, crude cell extract, and purified normal broth extract.

Electrophoresis - Each purified bacterial extract was subjected to electrophoresis to determine if more than one compound was present in each solution. Such a study would enable one to determine the purity of the extracts.

Six cellulose acetate strips, 1 x 6.75 inches, were placed in the electrophoresis chamber at one time. Sample sizes of 200 µg of purified cell-free extract and 600 µg of purified cell extract were used. Electrophoresis was performed at 2 milliamp., 150 volt, current for 90 minutes in 0.01 M sodium phosphate buffer, pH 6.5.

Staining solutions of 0.01% buffalo blue, aniline blue black, and ninhydrin were prepared in 65% ethanol (6). The 65% ethanol solutions were used to permit only a limited amount of elution of the water soluble sample from the paper. A 0.01% acridine orange solution in McILvanines buffer, pH 4.0 was used to detect nucleic acids (2). Strips remained in each staining solution for ten minutes, followed by a ten minute wash in 65% ethanol (6).

For ultraviolet absorption studies, cellulose acetate strips were washed for 10 seconds in an ultrasonic bath prior to use. This step removed any water soluble materials which could otherwise interfere with detection of zones derived

from the samples. Strips were cut into sections of 0.5 cm. in width proceeding electrophoresis. Material from each section was eluted into one ml. of distilled water. The elution was accomplished by a ten second agitation with a vortex stirrer. Zones were detected with a spectrophotometer setting at 260 mμ.

Nuclease reactions - An attempt was made to alter the biological activity of the extracts by treatment of the material with nucleases. Solutions of purified extracts were concentrated by flash evaporation to one ml. volumes prior to enzyme treatment. Ribonuclease was added at a concentration of 10 μg/ml.; deoxyribonuclease was added as 100 μg per ml., in 10^{-3} M $MgSO_4$. The solutions were held at room temperature for twelve hours at which time four parts of cold acetone (-15°C) were added. Following a ten minute cooling, the solution was subjected to 7,700 g centrifugation. The precipitate was resuspended in water and eluted through a Sephadex G-200 column, 1.4 x 24.5 cm. Equilibration and elution were made with distilled water. The elution volume between 34 and 43 ml. was tested for biological activity after drying over P_2O_5. In later studies, the gel filtration and drying procedures were omitted prior to testing with assay plants.

Biological assay - Tomato seedlings were used for the biological assay in the manner described by Strobel (21).

Plants of the variety Earliana were grown in vermiculite at 24°C under 1,200 foot-candles of light for a twelve hour day. Eight to ten day old plants were excised seven cm. from the primary leaves, and the cuttings were placed in short test tubes containing purified extracts. Test solutions for assay trials consisted of 0.5% extract material in 0.05 M potassium phosphate, pH 7.0. Nucleic acid was buffered in a similar manner at a pH of 7.0.

For wilt inducement, cuttings were placed under a constant environment for a specified period of time. A specially equipped incubator provided controlled conditions. Temperature was set at 24°C, and the relative humidity was maintained at 48%. A light source consisting of two fluorescent lamps and one 100 watt incandescent bulb was set 30 cm. above the plants. A water reservoir, consisting of a plexiglass box, was placed directly under the light source to provide cooling.

At the end of one hour, the cuttings were taken from the incubator, and the primary leaves were removed. The relative stem strength was determined with a wilt-o-meter similar in construction to that of Johnson and Strobel (12). Figure 1 shows the design of the machine used in this study. The maximum distance through which the tomato stem could push the needle prior to folding was recorded. This distance was recorded in wilt-o-units. Eight to ten cuttings were used for each experimental treatment with three values obtained for each stem. An identical number of cuttings was placed

in buffer alone as controls. In each treatment, an average value of stem strength was obtained for the plants in the control and also for those in the extract solution. To determine if the two means were identical, the values were subjected to the t test as described by Steel and Torrie (20). If the two means were statistically non-identical (95% confidence limit), the percentage of reduction in stem strength was calculated:

$$\% \text{ Reduction of Stem Strength} = \frac{\text{Control value - Extract value}}{\text{Control value}}$$

Nucleic acid - Ribosomal ribonucleic (r-RNA) and double stranded deoxyribonucleic (DNA) acids from Gynura aurantiaca were obtained to determine if the wilting of assay cuttings was a response induced by nucleic acids in general. Solutions used in the plant assay were buffered in 0.05 M phosphate buffer, pH 7.0.

Double stranded deoxyribonucleic acid was broken into single strands by heating the solution for ten minutes in a boiling water bath and plunging it into an ice bath to provide rapid cooling.

Diphenylamine reaction - The diphenylamine reaction was used to detect the presence of deoxyribonucleic acid in the extracts. The reaction was conducted according to the procedure reviewed by Ashwell (1). 500 µg of extract was used in each test.

FIGURE 1: A top and angle view of the wilt-o-meter are shown. The calibration scale was divided into wilt-o-units which were arbitrarily set. The turntable mechanism rotated clockwise. When the stem was properly placed in the holder, movement of the turntable brought the tomato stem in contact with the needle. The machine was designed after the model constructed by Johnson and Strobel (12).

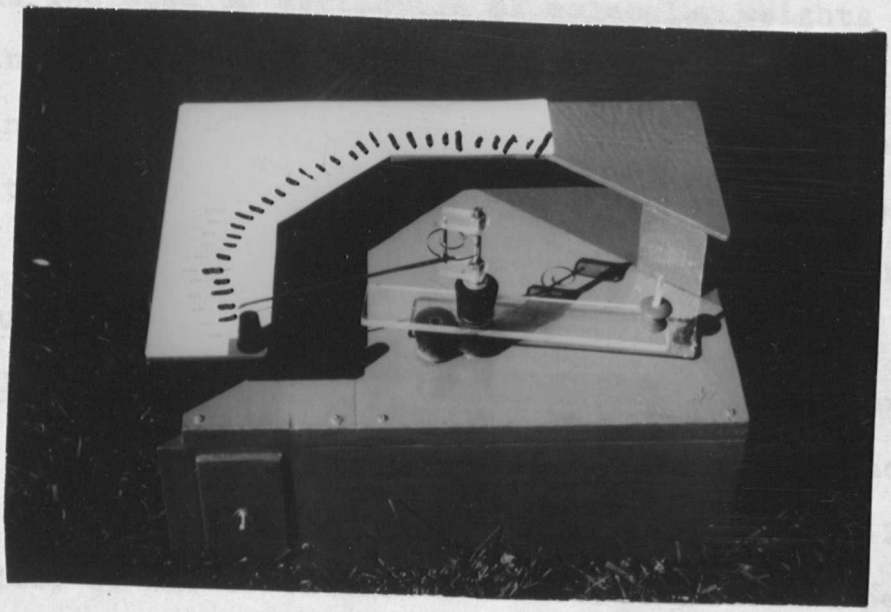
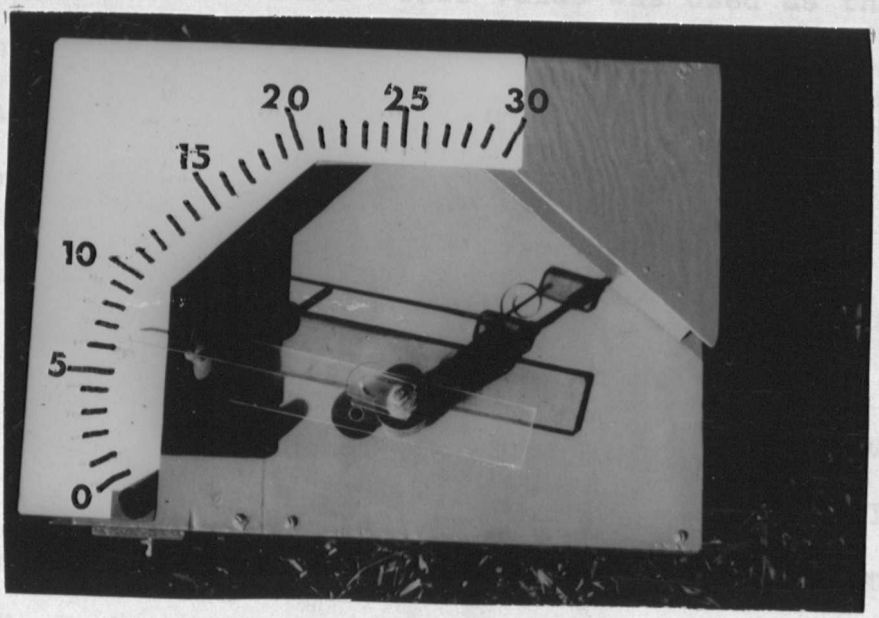


FIGURE 1



RESULTS

A chemical analysis of the cell and cell-free extracts was made to establish their degree of similarity. The analysis included an estimation of molecular weights and determination of nucleic acid content. As shown by the following results, nucleic acid appeared to be a major constituent of each extract, and for this reason, the role of nucleic acid with respect to wilting in assay plants was also studied.

Molecular weight - Chromatography of cell-free extract through Sephadex G-200 yielded one peak between 34 and 48 ml. with no quantities of material showing absorbance at 260 mμ until after 30 ml. of elution volume (Fig. 2). The peak had a maximum absorbance value at 40 ml. of elution with ± 1 ml. of experimental variance. This value was used as the sample elution volume in the estimation of the molecular weight.

The molecular weight was determined by the following equation formulated for Sephadex G-200 (27):

$$\frac{V_e}{V_t} = 3.20 - 0.58 \log \text{Molecular Weight}$$

V_t equals the total volume of the column which was 44.2 ml. in our experiments. V_e equals the sample elution volume minus the void volume of the column. Calculations showed V_e to be equal to 28.5 ml. for the cell-free

extract peak. From these values, the molecular weight was estimated to be 25,420 for the purified bacterial cell-free extract.

Gel filtration of the cell extract, prior to elution on Dowex 50, produced two peaks (Fig. 3). Peak No. 1 had a maximum absorbance value at 16 ml. of elution. The elution maximum occurred 4.5 ml. after the passage of the void volume which was 11.5 ml., illustrating that the peak contained materials of high molecular weight. The ultraviolet scanning spectrum was typical of nucleic acids, a maximum at 260 mμ and a minimum at 240 mμ. Solutions obtained from peak No. 1 were concentrated by flash evaporation and treated with nucleases. After 12 hours of incubation, four parts of cold acetone (-15°C) were added, and the precipitated material collected. All degradation products were removed with the acetone. Upon Sephadex G-200 elution, results showed that the substance in peak No. 1 had been degraded by individual treatments of each of the two nucleases.

When cell extracts were passed through Dowex 50, the material in peak No. 1 was removed from the effluent by attachment to the cation exchange resin. Sephadex G-200 chromatography of the effluent from Dowex 50 yielded products only in the region of peak No. 2. This peak had an elution volume between 34 and 48 ml. with a 40 ml. average peak maximum. Using 40 ml. as the sample elution volume, this peak had a molecular weight

FIGURE 2 AND 3: Sephadex G-200 elution of cell-free extract (Fig. 2), cell extract (Fig. 3) in comparison with the elution of a control broth extract.

FIGURE 2

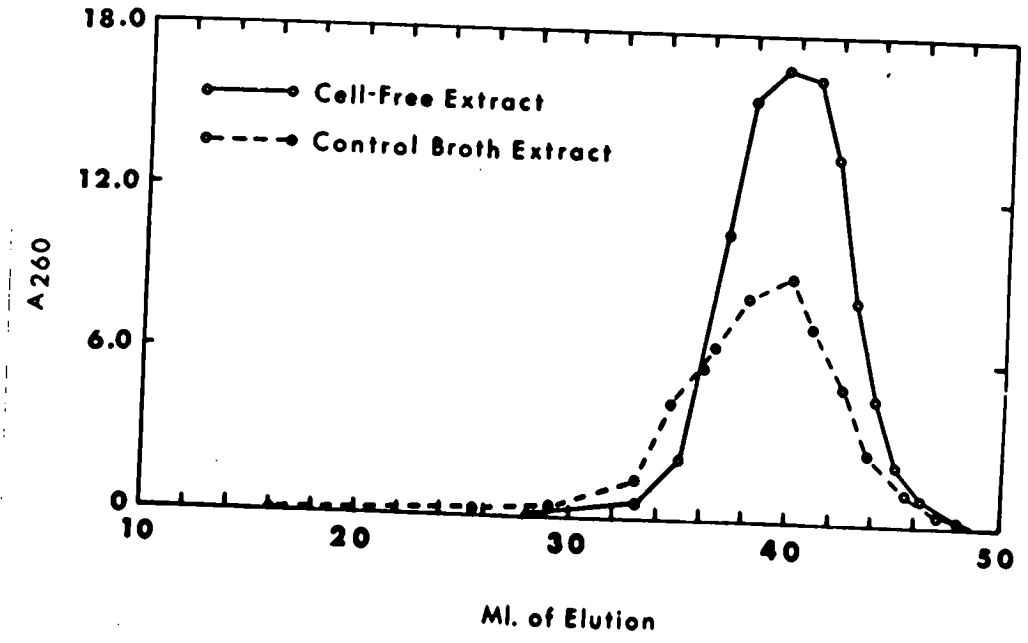
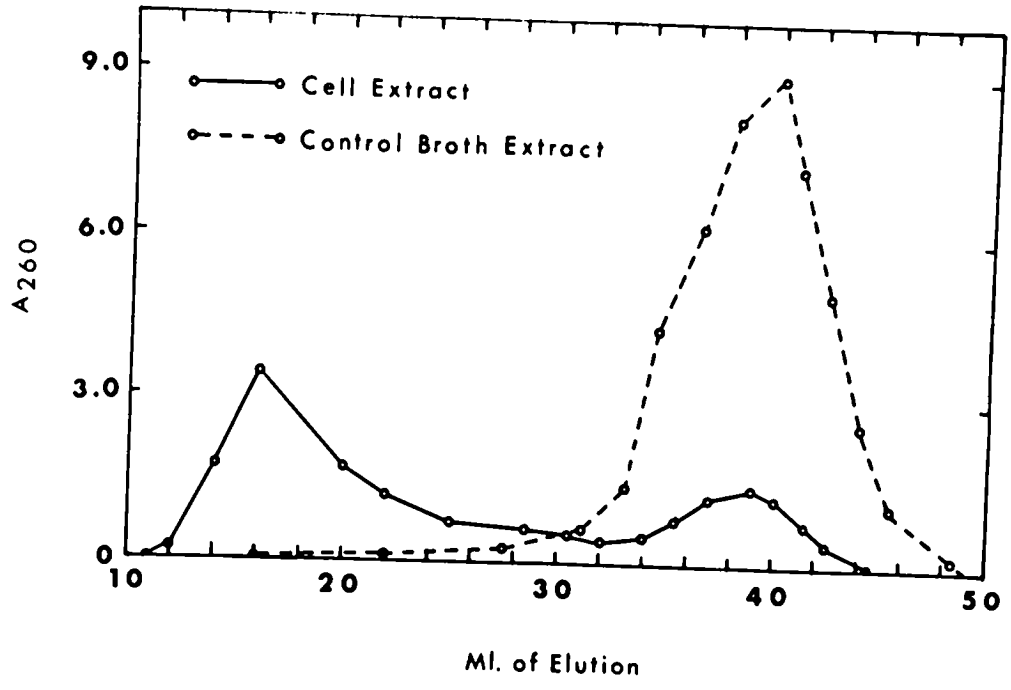


FIGURE 3



estimation of 25,420, an identical value in comparison with that of the cell-free extract.

Culture medium, which was not inoculated with bacteria, was subjected to the cell-free extraction procedure. The extract obtained from the procedure was termed "control broth extract". Chromatography with Sephadex G-200 yielded one peak in an elution zone comparable with peaks of cell-free and cell extracts (Fig. 2 and 3). An average peak maximum at 40 ml. of elution volume was obtained. The molecular weight estimation was 25,420 as calculated by the Sephadex G-200 formula.

To minimize the quantity of normal broth substances in the bacterial extracts, three inch dialysis tubing was used to prepare yeast extract for all culture medium. Upon twelve hours of dialysis, the larger tubing (3 inch) allowed less extract to pass into the dialyzate when compared with one inch tubing. Control broth extracts from 300 ml. of culture medium were chromatographed over Sephadex G-200; extracts from the large tubing showed a peak absorbance of 4.0 in comparison with a peak maximum at 9.0 for the small tubing. The small tubing allowed more than two times as much material to pass through. The biological activity of these substances is presented in a later section.

Nucleic acid content - Purified cell, cell-free, and control broth extracts exhibited similar scanning spectra with

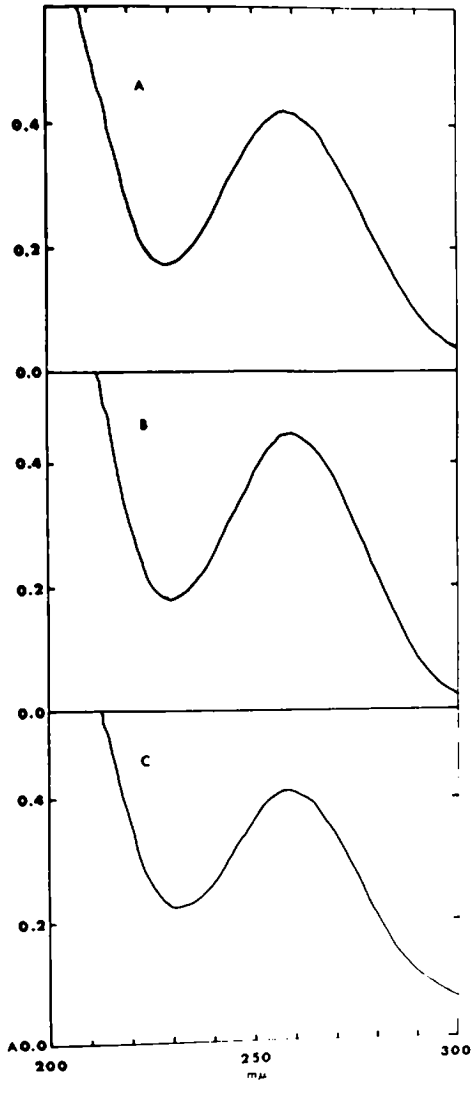
a maxima at 260 mμ and a minima at 230 mμ (Fig. 4). A spectrum of this nature is indicative of nucleic acid (3). A product with an absorption at 595 mμ was also obtained with the diphenylamine reagent, which indicates the presence of deoxyribonucleic acid.

Extinction coefficients (A_{260}/mg/ml) were determined to obtain an estimation of the percentage of nucleic acid present in the extracts. In all cases, the extracts showed a linear relationship between concentration (mg/ml) and absorbance at 260 mμ(A_{260}), (Fig. 5). Extinction coefficients were calculated as: control broth - 6.5/mg/ml., cell-free - 5.5/mg/ml., and cell - 2.7/mg/ml. An accurate characterization of the nucleic acid contained in the extracts was not made. Depending upon the type under consideration, the extinction coefficients of pure nucleic acid may vary from 20 to 30 A_{260}/mg/ml. For this reason only a range of the percentage of composition may be given: control broth - 22-33%, cell-free - 18-28%, and cell - 9-14%.

Multiple components - Electrophoresis on cellulose acetate paper was conducted to determine if more than one compound was present in each of the bacterial extracts. Such results would allow one to determine their purity.

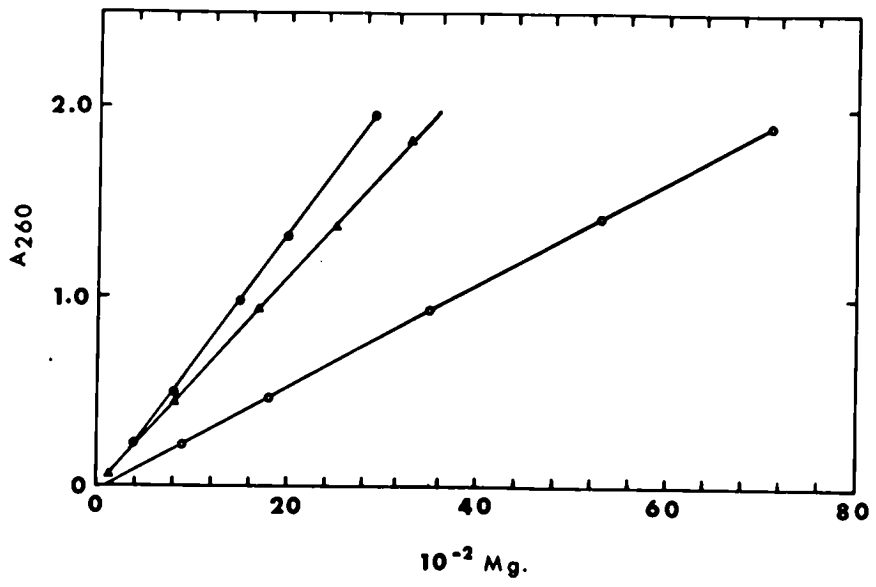
An attempt to detect protein and nucleic acid in cell-free extracts by means of staining solutions was unsuccessful. No zones were observed when strips were individually stained with

FIGURE 4



Ultraviolet scanning spectra of: A) Bacterial cell-free extract; B) Control broth extract; and C) Bacterial cell extract.

FIGURE 5



The relationship between absorbance at 260 mμ and concentration (mg/ml) of the following:

● Control broth extract

△ Bacterial cell-free extract

○ Bacterial cell extract

aniline blue black, ninhydrin, and buffalo blue black. A dull red zone appeared after placing strips in a 0.01% solution of acridine orange. The zone was fluorescent under ultraviolet light and showed migration in the direction of the anode, 6 cm. from the point of application. However, elution of the zone occurred when the strips were placed in a wash solution of 10% acetic acid. Elution also occurred in 65% ethanol.

To avoid elution of sample material, electrophoresis was conducted on Whatman No. 1 filter paper using staining solutions in 95% ethanol. No zones were observed after staining with ninhydrin and aniline blue black. Acridine orange adhered readily to control strips of filter paper producing non-specific fluorescence under ultraviolet light over the entire strip. Retention of the stain occurred after extensive washing in 95% ethanol. Therefore, acridine orange could not be used to stain the strips for detection of zones of nucleic acid.

Since results from the staining solutions were nominal, a more sensitive technique of detection was employed. The electrophoresis strips were sectioned, each section being washed in a small volume of distilled water. The A_{260} of each elutant was then recorded. Control strips possessed no absorbance readings over 0.03.

FIGURE 6 AND 7: The electrophoresis of bacterial cell-free extract (Fig. 6) and cell extract (Fig. 7) on cellulose acetate. Sections of the strip, 0.5 cm. in width, were rinsed with 1 ml. of distilled water. The absorbance of each sample was recorded. The end of the strip which was at the anode is to the right. The arrow marks the region at which the sample was applied.

FIGURE 6

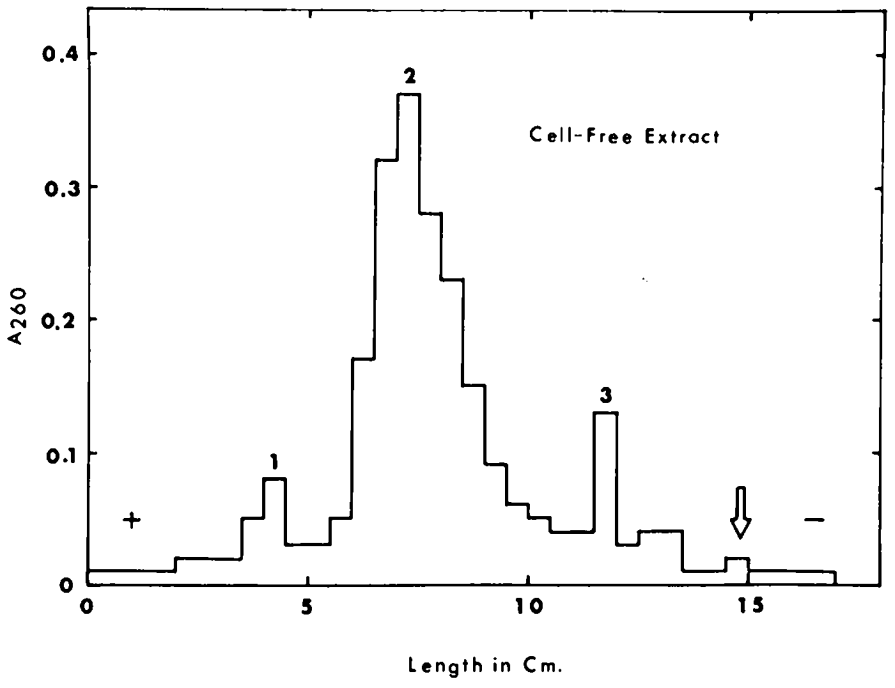


FIGURE 7

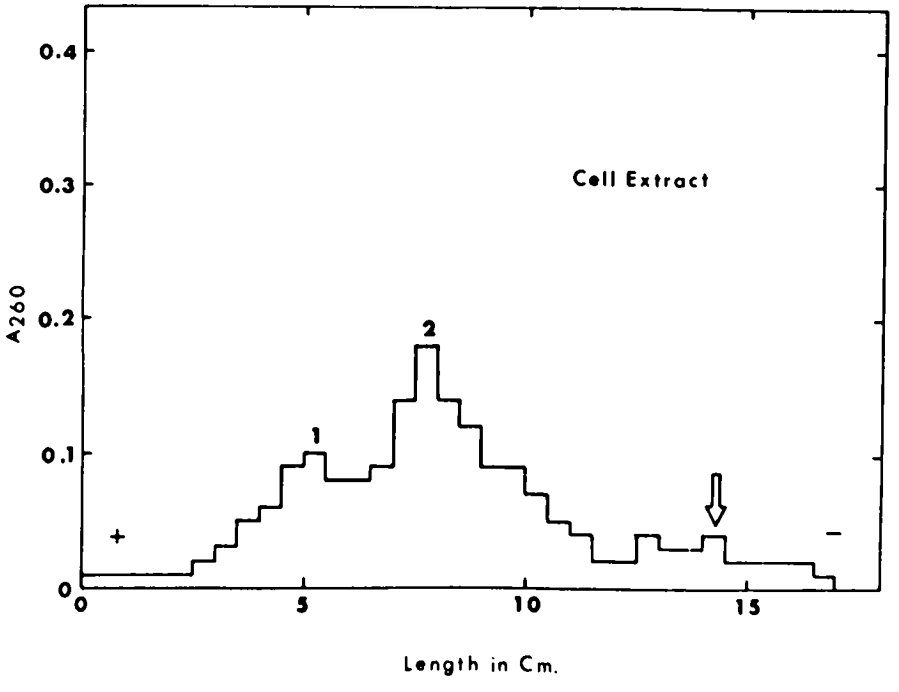
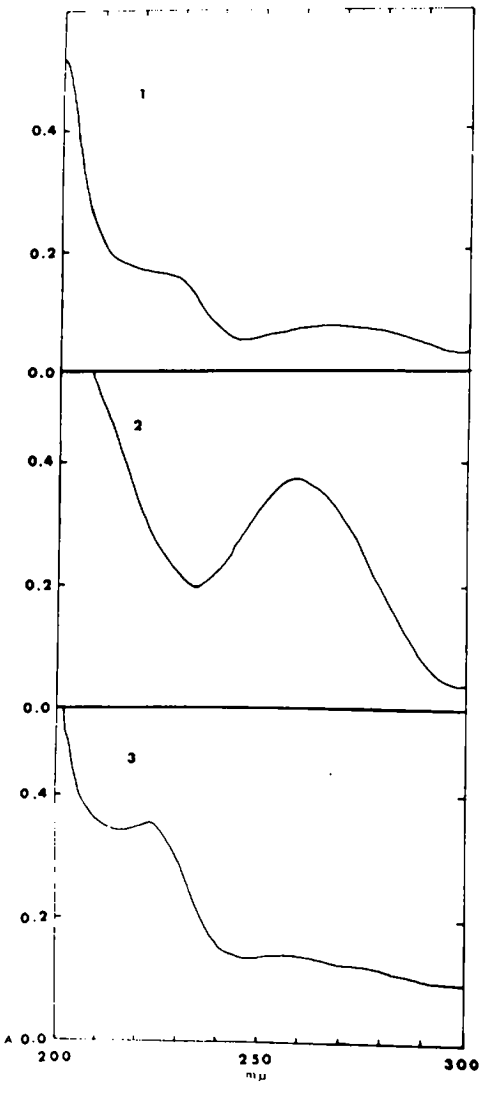


FIGURE 8



Ultraviolet scanning spectra of individual components obtained by electrophoresis of the bacterial cell-free extract on cellulose acetate paper: 1) zone 1 with a 270 mμ maximum and a 245 mμ minimum, 2) zone 2 with a 260 mμ maximum and a 230 mμ minimum, and 3) zone 3 with a maximum at 225 mμ.

Upon electrophoresis of the purified cell-free extract, three distinct compounds were separated on the cellulose acetate strips. The bar graph in Figure 6 illustrates the data obtained. Zone No. 1 had an ultraviolet scanning spectrum with a maximum at 270 mμ and a minimum at 245 mμ (Fig. 8). A comparable zone appeared with the cell extract (Fig. 7) although its scanning spectrum possessed a 260 mμ maximum. Such a result may have been due to the poor separation obtained between the two components of the cell extract. In both extracts, zone No. 2 had a scanning spectrum typical of nucleic acid, and it stained with acridine orange. These two results provided strong evidence that nucleic acid was a constituent of this component. Zone No. 3, present only in the cell-free extract, showed no absorbance peaks at 260 mμ or 280 mμ suggesting that large quantities of nucleic acid or protein were not present. This component crystallized out of the extract solution very readily when stored in the refrigerator. However, at the concentration of 20 mg/ml, the component became soluble after maintaining the cell-free extract solution in a 50°C water bath for twelve hours.

Biological activity - The control broth was biologically inactive until the substance had been dried over P_2O_5 (Table 1). A similar response to drying was observed with cell-free extract but not with the cell extract.

When activity was present, assay plants showed wilting of the primary leaves and partial collapse of tissue in the stem which caused the plant stems to bend while in the short test tubes. Table 1 presents the percentage of stem reduction occurring with treatment of cuttings with the test solutions.

The response induced in the tomato cuttings by the bacterial extracts differed from that of a high molecular weight compound. Plants placed in test solutions of Ficoll (>400,000 M. W.) showed no significant difference in stem strength. However, leaf wilting was observed. This observation agrees with the results obtained by Strobel when plants were treated with a high molecular weight dextran (22).

The first results showed that purified extracts were inactivated by nuclease treatment. However, this conclusion was based only on the visual observation of assay plants. Further studies using crude extracts actually showed an increase in biological activity. Results from the cell-free extract appear in Table 1. Test solutions of nucleases and of nuclease treated r-RNA showed no wilting.

Wilt inducement by nucleic acid - Nucleic acid from Gynura aurantiaca was obtained to determine if nucleic acid from other sources would induce wilting in assay plants. A loss in stem strength in assay plants was measured by the wilt-o-meter after one hour. Concentrations above 0.5 mg/ml. did induce significant differences in stem strength (Table 2).

Double stranded DNA in comparison with single stranded DNA produced more wilting with a greater reduction in stem strength.

The pH of the test solution has an apparent result on the percentage of reduction of stem strength produced by nucleic acid. Solutions of r-RNA induced approximately twice as much activity at a pH of 6.0 in comparison with solutions at pH 7.0 (Table 3). The wilting also proceeded at a slower rate in the dark in relation to wilting in the light; wilting was not inhibited by darkness (Table 3).

TABLE I

WILTING INDUCED BY BACTERIAL AND CONTROL EXTRACTS

Extract (0.5%)	Mean Average Stem Strength (Wilt-o-units)	t value	Statistical Comparison of the Two Means	Percent Reduction of Stem Strength
Cell	2.7	5.7	Non-identical[2]	57%
Control[1]	6.3			
Cell-free	4.8	3.2	Non-identical	37%
Control	7.6			
Cell-free (Nuclease Treated)				
Extract	2.9	4.6	Non-identical	52%
Control	6.1			
Control Broth (non-dry)				
Extract	10.1	0.1	Identical	None
Control	10.0			
Control Broth (dried)				
Extract	4.3	4.6	Non-identical	38%
Control	7.4			
Ficoll (>400,000 M. W.)				
Compound	8.1	1.0	Identical	None
Control	8.3			

[1]Assay cuttings were placed in 0.05 M phosphate buffer, pH 7.0.

[2]As per Steel and Torrie (20) with 95% confidence limit.

TABLE II

WILTING INDUCED BY NUCLEIC ACID FROM GYNURA AURANTIACA

Nucleic Acid	Mean Average Stem Strength (Wilt-o-units)	t value	Statistical Comparison of the Two Means	Percent Reduction of Stem Strength
r-RNA - pH 7.0				
1.00 mg/ml	3.8	8.8	Non-identical[2]	62%
Control[1]	9.9			
0.50 mg/ml	4.8	2.1	Non-identical	26%
Control	6.5			
0.25 mg/ml	6.4	0.6	Identical	None
Control	6.8			
DNA - pH 7.0				
Double stranded				
1.00 mg/ml	1.8	14.4	Non-identical	80%
Control	9.0			
0.50 mg/ml	4.6	6.3	Non-identical	39%
Control	7.6			
0.25 mg/ml	8.9	0.4	Identical	None
Control	8.7			
Single stranded				
0.50 mg/ml	3.8	8.3	Non-identical	65%
Control	10.8			

[1]Assay cuttings were placed in 0.05 M phosphate buffer, pH 7.0.

[2]As per Steel and Torrie (20) with 95% confidence limit.

TABLE III

FACTORS INFLUENCING WILTING INDUCED BY NUCLEIC ACID - r-RNA

	Mean Average Stem Strength (Wilt-o-units)	t value	Statistical Comparison of the Two Means	Percent Reduction of Stem Strength
pH FACTOR				
pH 7.0				
0.5 mg/ml	4.8	2.1	Non-identical[2]	26%
Control[1]	6.5			
pH 6.0				
0.5 mg/ml	3.9	4.9	Non-identical	51%
Control	7.9			
LIGHT FACTOR				
Light				
1.0 mg/ml	2.3	7.3	Non-identical	69%
Control	7.4			
Darkness				
1.0 mg/ml	3.8	5.4	Non-identical	49%
Control	7.4			

[1]Assay cuttings were placed in 0.05 M phosphate buffer, pH 7.0.

[2]As per Steel and Torrie (20) with 95% confidence limit.

DISCUSSION

By sonic oscillation of whole cells of Corynebacterium sepedonicum, a compound which induced wilting in assay plants was isolated. Two similar extracts were obtained from the bacterial culture medium and un-inoculated broth by the procedure of Strobel (21). The three materials apparently possessed identical molecular weights as determined by gel filtration. The extracts contained nucleic acid, but the proportion of nucleic acid in each extract varied considerably.

Each extract had a considerably higher molecular weight in comparison to that previously reported for the glycopeptide phytotoxin (21). Only small quantities of material were detected in the molecular weight region of 18,000. This variation may be due to the method used to detect the product. The ultraviolet absorbance could possibly fail to detect small quantities of polysaccharides: although the presence of large quantities of these substances should be evident. Since our material differed in size from the phytotoxin isolated by Strobel, one cannot conclude that our extracts are identical to the toxin previously reported.

Although the material was thought to be a homogeneous compound, several discrete compounds were present in the purified extracts as determined by electrophoresis.

Component No. 1 may have been nucleoprotein since its scanning spectrum showed a peak maximum at 270 mμ. Component No. 2 appeared to contain nucleic acid although the actual percentage present is not known. Further studies on the chemical nature of these compounds were not made. It is not known which components are required to induce wilting. For this reason, further modification of the purification procedures may be required to eliminate those compounds which do not possess wilting activity.

Evidence suggests that the extracts may be composed of normal cellular constituents, at least constituents which are present in bacterial and fungal cells. A material which induced wilting in assay plants was isolated from un-inoculated culture medium which consisted primarily of yeast extract or the products resulting from the degradation of yeast cells. Preliminary work with Corynebacterium tritici also showed the presence of compounds having an identical molecular weight and scanning spectrum as the compounds isolated from the ring rot pathogen. The wilting ability of this substance is not known.

Physical disruption of the cell may be responsible for the accumulation of normal cellular components in bacterial culture medium. Sufficient quantities of the wilt-inducing materials from the bacteria do not occur in the medium until after six days of incubation (24). At the end of this time,

death with subsequent lysis of the cells could yield an ample amount of cellular constituents in the broth. Our research has shown that these constituents will survive rather harsh degradation conditions without a loss in wilting activity. The products from yeast extract serve to illustrate this point.

Further studies revealed that the wilting response was associated with nucleic acid. The activity was not restricted to a specific type of nucleic acid or to nucleic acid from the bacterial pathogen. Nucleic acid from Gynura aurantiaca induced a reduction of stem strength in assay plants at a concentration as low as 0.5 mg/ml. A reduction was observed with r-RNA as well as with double and single stranded DNA. The wilting in assay plants was identical to that induced by the bacterial extracts, noteably a reduction in stem strength. This response differed from that induced by a high molecular weight compound. Ficoll, with a molecular weight larger than 400,000 caused excessive leaf wilting with no significant difference in stem strength.

The two bacterial extracts were shown to contain nucleic acid by the following criteria: a) a typical ultraviolet scanning spectrum, b) a diphenylamine product with an absorption at 595 mμ, c) a typical color reaction from staining with acridine orange. The extracts could contain as much as 10-30% of this compound; however, this estimation is very

approximate since aromatic amino acids could also contribute to some of the absorbance at 260 mμ. Using this percentage range, the test solutions for the plant assay could contain from 0.5 - 1.5 mg/ml. of nucleic acid.. Comparable concentrations of nucleic acid from Gynura were shown to induce wilting in assay plants.

To determine if nucleic acid was responsible for wilting, extracts were treated with nucleases. The results obtained from these experiments are inconclusive and raise several questions. Initial observations of assay plants showed an entire inactivation with enzyme treatment. However, these results were obtained visually and were not determined with the wilt-o-meter. Using this machine in later studies, crude extracts actually showed an increase in activity after enzyme treatment. Such a response could be similar in nature to that observed with native double stranded DNA. When the DNA had been partially broken into the single stranded form, a sizeable increase in the wilting activity was noted and may have been due to the exposure of more chemical sites of biological activity through a change in structure. In a similar manner, it is possible that nucleases are blocked and do not degrade the intact nucleic acid in the extract material, but instead, they alter the tertiary or secondary structure resulting in an increase in the number of active sites. The molecular structure does not appear to be fragmented since

the molecular weight of the extract apparently remains the same after nuclease treatment. However, a small change in molecular weight might not be detected with gel filtration if fragmentation of the structure did occur. Further evidence that an alteration in structural configuration occurred was that cell-free and control broth extracts were not active unless they were subjected to drying over P_2O_5. After the materials were dried and resuspended in buffer, wilting was induced in assay plants. Dried and non-dried extracts apparently possessed identical molecular weights as determined by gel filtration.

The chemical site of biological activity has not clearly been defined in this text. Although vague in concept, the term is used to denote the specific unit on the nucleic acid which is capable of biological activity. This unit appears to be larger than a nucleotide since nucleic acid which has been degraded by treatment with nucleases does not induce a wilting response. The mode by which this unit induces the permeability alteration in plants is unknown.

The role of the extracts in the plant infected with the pathogen is open to question. It is possible that sufficient quantities of these materials could result from the degradation of cells after prolonged infection. The activity of the extracts after leaving the cells is unknown. Cell extracts obtained by the procedure of sonic oscillation appear to

possess immediate activity. However, the chemical structure of this extract may have been altered by the intense energy released during this procedure. The extracts obtained from the bacterial culture medium did not possess activity until after they had been dried. If compounds similar to those of the cell-free extract are released into the plant, a mechanism of activation of its biological capacities would be required. Our data indicated that nucleases could possibly function to produce this activation if such a mechanism was required. Further research is needed to completely substantiate this idea.

Caution must be used in the assumption that purified extracts function in the production of host wilting. Wilting induced in assay cuttings and that produced by the pathogen in the host may be two distinct cases. One must show that the purified substances occur within the host in ample concentrations to induce wilting. This problem becomes extremely complex when an attempt is made to determine the concentration of material required to induce host wilting in respect to the time required for the formation of symptoms.

SUMMARY

An extract which induced wilting in assay plants was isolated by sonic oscillation of whole cells of Corynebacterium sepedonicum. This material was similar to two extracts obtained from bacterial and un-inoculated culture media. Each extract had an estimated molecular weight of 25,420, and each material consisted of at least 10% nucleic acid. Each extract induced wilting in small tomato cuttings with a reduction in the stem strength of the plant. Nucleic acid from Gynura aurantiaca produced a similar wilting of assay plants; this biological activity was not restricted to a specific type of nucleic acid. The association between the presence of nucleic acid and the wilting of assay plants was strongly established. However, bacterial extracts treated with nucleases showed an increase in biological activity rather than a reduction. For this reason, nucleases may alter the structural configuration without elimination of the nucleic acid constituents from the molecule. A similar structural alteration may occur when extracts are dried over P_2O_5.

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