

THE SECRETION OF ANTIMICROBIAL MATERIAL BY ASPEN TISSUE

(Populus tremuloides Michx.)

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ABSTRACT

The progressive secretion of antimicrobial material by isolated aspen tissue was characterized. The active material diffused at a higher rate in standard culture than alpha-amylase. The pH of the tissue culture medium was not altered by tissue growth and the antimicrobial material proved to be bactericidal. The incorporation of dimethyl sulfoxide did not increase the release of the active material. Catechol was detected in the tissue using enzymatic and chemical analysis but the analysis led to the conclusion that catechol was not the primary material responsible for the observed antimicrobial activity.

Further analysis with electrophoresis and chromatography demonstrated that the inhibitory material may consist of several components.

INTRODUCTION

The occurrence of antimicrobial substances in higher plants has been extensively investigated. Nickell (1960) surveyed the papers dealing with antimicrobial materials found in vascular plants and reported that active substances are found in 157 families. Tissue cultures isolated from higher plants have also demonstrated antimicrobial activity. Carew and Staba (1965) emphasized the importance of plant tissue cultures in the production of materials which may have medicinal importance. Tissue cultures represent a unique experimental approach useful in the study of the physiological and biochemical properties of antibiotic substances. White (1939) and others were the first to develop methods for the growth of callus tissue for an indefinite period. This development provided the impetus for the initiation of a number of research programs dealing with the metabolism of plant tissue cultures. Tissue grown in vitro can be used to provide a constant source of material which is genetically identical to the parent plant. In vitro growth of tissue also provides a system for the control and variation of both nutritional and environmental conditions.

Tulecke et al. (1962) studied the biochemical and physiological relationship between in vitro and in vivo tissue from Ginkgo biloba. Isolated tissue was similar to the parent plant in its ability to produce specific compounds even when the in vivo and in vitro tissues were produced under extremely different environmental conditions. Callus tissue also has the ability to regenerate a whole plant. Such toti-

potency is a common phenomenon among plants.

The release of materials, other than antibiotics, from tissue cultures has also been investigated. Straus and Campbell (1963) have studied the release of enzymes from Nicotiana tabacum tissue cultures and have found that the isolated tissue releases peroxidase, acid phosphatase, and indoleacetic acid oxidase. Similarly, Chang and Bandurski (1964) have shown that the roots of corn release exocellular hydrolytic enzymes which are involved in the breakdown of macromolecules. Inhibitors of bacterial growth have also been detected in the medium used to support the growth of human tissue cultures. Novak and Windsor (1968) investigated the release of such an antibiotic into the growth medium. It inhibited the growth of pathogenic bacteria and has been identified as an alpha-ketoaldehyde attached to a carrier molecule.

The release of antimicrobial materials from plant tissue cultures is a frequent occurrence. Mathes (1963) tested thirty isolated plant tissue cultures and found fifty per cent of these cultures demonstrated antimicrobial activity. Khanna and Staba (1968) performed a similar survey of tissue isolated from twenty-four species and found that eighty per cent of these cultures demonstrated antimicrobial activity. Campbell, Chan, and Barker (1965) extracted the medium which cauliflower (Brassica oleraceae) and lettuce (Lactuca sativa) tissue cultures had grown on with an ethanol-ether solvent. The extraction solvent yielded an alcohol-soluble fraction that was highly inhibitory for Staphylococcus aureus. Maheshwari et al. (1967) detected an inhibitory material in the agar of snapdragon (Antirrhinum majus) and sunflower (Helianthus annuus) tissue cultures. The material was ether and ethanol soluble and inhibited the urediospore germination of rust fungus, Puccinia helianthi. Mathes (1963)

discovered that triploid aspen tissue, Populus tremuloides Michx., demonstrated antimicrobial activity against a variety of microorganisms.

Previous investigators have shown that plants from the genus Populus contain antibiotic materials; Klöpping and van der Kerk (1951) reported fungicidal activity in aqueous extracts from the bark of Populus candicans. Several compounds of low fungistatic activity were also found in the extract. The compounds with low activity were salicin, saligenin, catechol, and salicylic acid derivatives. Hubbes (1962) isolated catechol from the bark of Populus tremuloides Michx. in sufficient quantity to inhibit the growth of the fungus, Hypoxyton pruinaum. Mathes and Kremer (1966) investigated the inhibitory action of catechol against bacteria. Catechol was inhibitory to Bacillus subtilis at a concentration of 1000 ppm. Catechol (ortho-dihydroxybenzene) has been found in other plants and has been shown to be an antibiotic agent. Link and Walker (1933) isolated catechol from the outer scales of pigmented onion and found that catechol at a concentration of 2×10^{-3} molar inhibited the germination of the fungus, Collectrichum circinans. An additional antifungicidal material, protocatechuic acid, was also present in the onion scales.

Harborne and Stimmonds (1964) reported, in a survey of plant phenolic compounds, that catechol was found with certainty in only a few plants. Resorcinol (meta-dihydroxybenzene) has not been reported to occur in a free form in nature. Hydroxyquinone (para-dihydroxybenzene) has been found to be the most widely distributed isomer of these three phenolic compounds. It has been found in the Ericaceae (Vaccinium, Rhododendron), Rosaceae (Pyrus, Docynia), Proteaceae (Protea) and Compositae (Xanthium). Kretovich (1966) stated that the experimental evidence indicates

that the synthesis of aromatic compounds results from the reaction of photosynthetic products. The reaction proceeds from sedoheptulose 1,7-diphosphate by the classic pathway through a series of reactions to phenylpyruvic acid. A cyclic precursor is thus formed for the production of phenolic compounds. Another classic pathway for the production of a benzene ring involves the condensation of three acetic acid residues. The product is quinic acid which is oxidized to hydroquinone.

Nelson and Dawson (1944) performed some of the initial work concerning the enzymatic oxidation of aromatics, especially phenolic compounds. The substrate for this oxidative enzyme is monophenolic compounds such as p-cresol and tyrosine, which are oxidized to the corresponding diphenols. The enzyme is also capable of catalyzing the further oxidation of the ortho-diphenols to the corresponding ortho-quinones. The quinones produced from the oxidative reactions with polyphenoloxidase are capable of further oxidation and polymerization. The color of quinones and their oxidation products depends on the phenol which is used as the substrate. The darkening of plant tissue due to injury or age is generally attributed to these compounds. Treatment of catechol with polyphenoloxidase results in the development of ortho-quinone which is a dark red compound.

Catechol forms a number of specific products which are helpful in its identification (Haas and Hill, 1921): it yields a green color with ferric chloride while hydroquinone does not give a color reaction in the presence of ferric chloride but is oxidized to the quinone; resorcinol gives a violet color with ferric chloride. The dark green color indicating catechol is converted to a violet color in the presence of sodium acetate. The violet color produced by the reaction of ferric chloride

with resorcinol is destroyed by the addition of sodium acetate. Lead acetate specifically precipitates catechol but does not react with resorcinol and hydroxyquinone. All of the dihydroxy-isomers of benzene show a maximum absorption peak between 270 and 280 nanometers, as expected.

The purpose of this paper is to present evidence concerning the antimicrobial material released from triploid aspen tissue grown in vitro and to investigate the possibility that phenolic materials may be involved in the inhibition of microorganisms. Supporting evidence concerning the release and stability of the antimicrobial material is presented.

GENERAL MATERIALS AND METHODS

The callus tissue used in this investigation was aseptically isolated from the cambial region of the stem of Populus tremuloides Michx. in December, 1961 (Mathes, 1964). The isolation procedure for obtaining the tissue consisted of cutting a stem into internode sections and surface sterilizing the sections by immersion for 10 minutes in a 5.25 per cent hypochlorite solution containing a few drops of the cationic detergent Tween 20. The sections were rinsed three times with sterile distilled water and cut into pieces approximately ten millimeters in length. The pieces were transferred aseptically to basal medium containing major elements (White, 1942) and trace elements (Nitsch, 1951). The medium also contained 3.0 ppm glycine, 0.1 ppm thiamine, 2 per cent sucrose and 10 per cent coconut milk. The coconut milk was heated to 60°C and filtered after cooling (Tulecke, 1957). The callus tissue was produced at 24-26°C during a fifteen day period and was then removed from the approximate cambial region.

The tissue used during this investigation was grown on number 23 medium (Mathes, 1964 and Appendix, Table 1) in the dark at a temperature of 24-25°C. The medium contained trace elements, 2 per cent sucrose, 0.8 per cent agar, major elements, 0.5 ppm naphthaleneacetic acid, and 10 per cent coconut milk heated to 60°C and filtered after cooling. The medium was autoclaved for 15 minutes at 15 psi before use. It was poured into petri plates to a volume of approximately forty milliliters. The tissue was removed from the plates after a three week growth period.

The isolated tissue was cut into small pieces, weighing approximately 0.050 grams and placed on fresh medium at the beginning of each experiment. The bacteria used in assaying for antimicrobial activity were either Sarcina lutea or Bacillus subtilis. These bacteria were used because Mathes (1963) found that aspen tissue grown in vitro releases a substance which inhibits their growth. The bacteria were maintained on slants and transferred to a 0.8 per cent nutrient broth for use as an inoculum. The nutrient broth also contained 0.7 per cent sucrose and 0.5 per cent sodium chloride. The inoculum of bacteria was utilized when it reached an optical density between 0.1 and 0.3.

The standard method of assaying for antimicrobial material in medium which had supported tissue growth was as follows. The tissue was removed from the plate and the agar was flooded with a nutrient broth suspension of bacteria. The excess inoculum was removed and the plate incubated at 24-26°C until bacterial growth defined the inhibitory zone. The size of the zone was recorded. The method of assaying a catechol solution, expressed tissue sap, and extractions of the expressed sap and tissue was more complex. The material (0.1 ml) was pipetted on to a sterile antibiotic pad (12.7 millimeters disc of absorbent filter paper) and dried for two hours at 65°C. The pad was placed on a sterile agar plate on a horizontal position for five days in order to allow sufficient time for the diffusion of materials from the pad. It was removed and the agar was flooded in the manner already described.

RESULTS AND DISCUSSION

An initial test was performed to determine whether antimicrobial activity could be demonstrated using the conditions described for tissue growth in the general methods and materials section. The experiment utilized sixty pieces of tissue which were weighed initially and at the conclusion of the 21-day growth period in sterile 10 ml Erlenmeyer flasks. The growth index (final mean fresh weight/initial mean fresh weight) was calculated from this data. The tissue was placed on plates of number 23 medium with three pieces of tissue per plate. The medium was assayed at the end of the growth period with a suspension of either Sarcina lutea or Bacillus subtilis. Control plates which had not supported the growth of tissue were also flooded with Sarcina lutea. Control plates were used in order to determine if materials in the medium in absence of the tissue were inhibitory to the bacteria. Similarly, tissue was grown on a completely defined medium that did not contain coconut milk. The defined medium (Linsmaier and Skoog, 1965 and Appendix, Table 2) was assayed with Bacillus subtilis after it had supported tissue growth for a period of 21 days. The procedure was necessary to eliminate the possibility of compounds in coconut milk, rather than the isolated tissue, inhibiting the growth of bacteria. The results of this experiment indicated that the inhibition of bacterial growth could be obtained using the general methods for tissue growth. A mean inhibitory zone of 3.0 centimeters was obtained using Sarcina lutea while Bacillus subtilis gave a mean inhibitory zone of 3.2 centimeters. The control of

number 23 medium did not inhibit bacterial growth. The defined medium after tissue growth gave bacterial inhibition. The control and defined medium procedure demonstrated that the components of the medium are not required to demonstrate the inhibition of bacterial growth in the presence of isolated tissue. The growth index for the tissue grown on number 23 medium was 14.8. Isolated aspen tissue grew rapidly on this medium and produced satisfactory inhibitory zones; so it was decided to utilize number 23 medium in subsequent experiments.

The next experiment was performed to characterize the growth pattern of the tissue on number 23 medium. The purpose of the experiment was to illustrate that a constant increase in tissue weight occurs during an extended period of tissue growth. Sixty pieces of tissue were weighed in groups of three in sterile, tared 10 ml Erlenmeyer flasks at the beginning of the experiment. Each group of tissue was placed on a plate of medium. The tissue from each plate of medium was removed at four day intervals and weighed aseptically in 10 ml Erlenmeyer flasks. The tissue was aseptically returned to the same plate and position in the agar after weighing. Two groups or six pieces of tissue were placed in a 60°C oven at the beginning and at the termination of the experiment and dried until a constant dry weight was obtained. The initial and final mean per cent dry weight for the tissue was calculated from these data. The mean increase in tissue weight for each time interval was calculated and graphed (Figure 1) for a 28-day period.

The results of the experiment (Figure 1 and Appendix, Table 3) show a relatively constant increase in fresh weight of the tissue during the 28-day growth period. A greater increase in tissue weight was observed during the latter half of the growth period. This observation may be

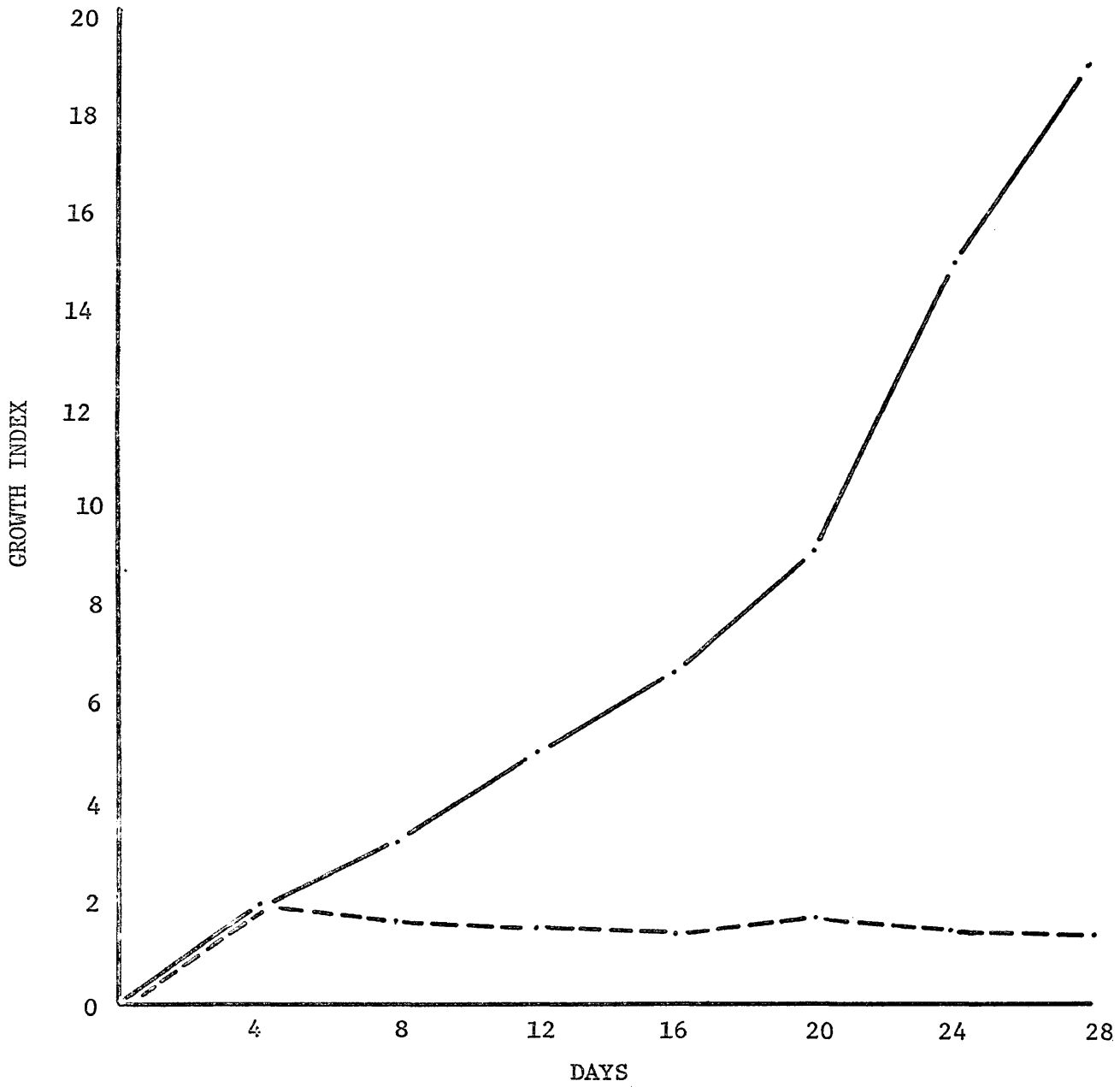


FIGURE I: THE GROWTH OF ISOLATED ASPEN TISSUE
PROGRESSIVE GROWTH INDEX———
GROWTH INDEX / 4-DAY TIME INTERVAL - - - - -

due to the fact that the tissue required an initial short period to adapt to the fresh medium. The rate of tissue growth declined after 20 days, presumably as a result of the exhaustion of nutrients or the accumulation of waste products. The mean per cent dry weight at the beginning and termination of the experiment was 8 per cent and 7 per cent, respectively.

The next area of experimentation was designed to investigate the progressive release of the antimicrobial material from the tissue into the medium. Thirty-six pieces of tissue were weighed individually in sterile, tared 10 ml Erlenmeyer flasks. Each piece of tissue was given a number and its location was marked on the Petri plate. Three pieces of tissue were placed on each plate of medium. Three plates, containing a total of nine pieces of tissue, were removed and the tissue was weighed at time intervals of two, four, eight, and twelve days. The plates were assayed for activity using Sarcina lutea. The mean tissue weight and mean inhibitory zone size was calculated for each time interval. The data are presented in the Appendix, Table 4 and graphed in Figure 2.

There is a progressive release of antimicrobial material as the weight of tissue increases. The slopes of the lines representing the tissue weight and zone size (Figure 2 and Appendix, Table 4) indicate that there is some variability in tissue growth and zone size. The variability in tissue growth and inhibitory zone size can be attributed to differences in the viability of the tissue. It can be concluded from the data that there is a progressive release of inhibitory material with time.

Soluble starch was incorporated into the tissue culture medium in

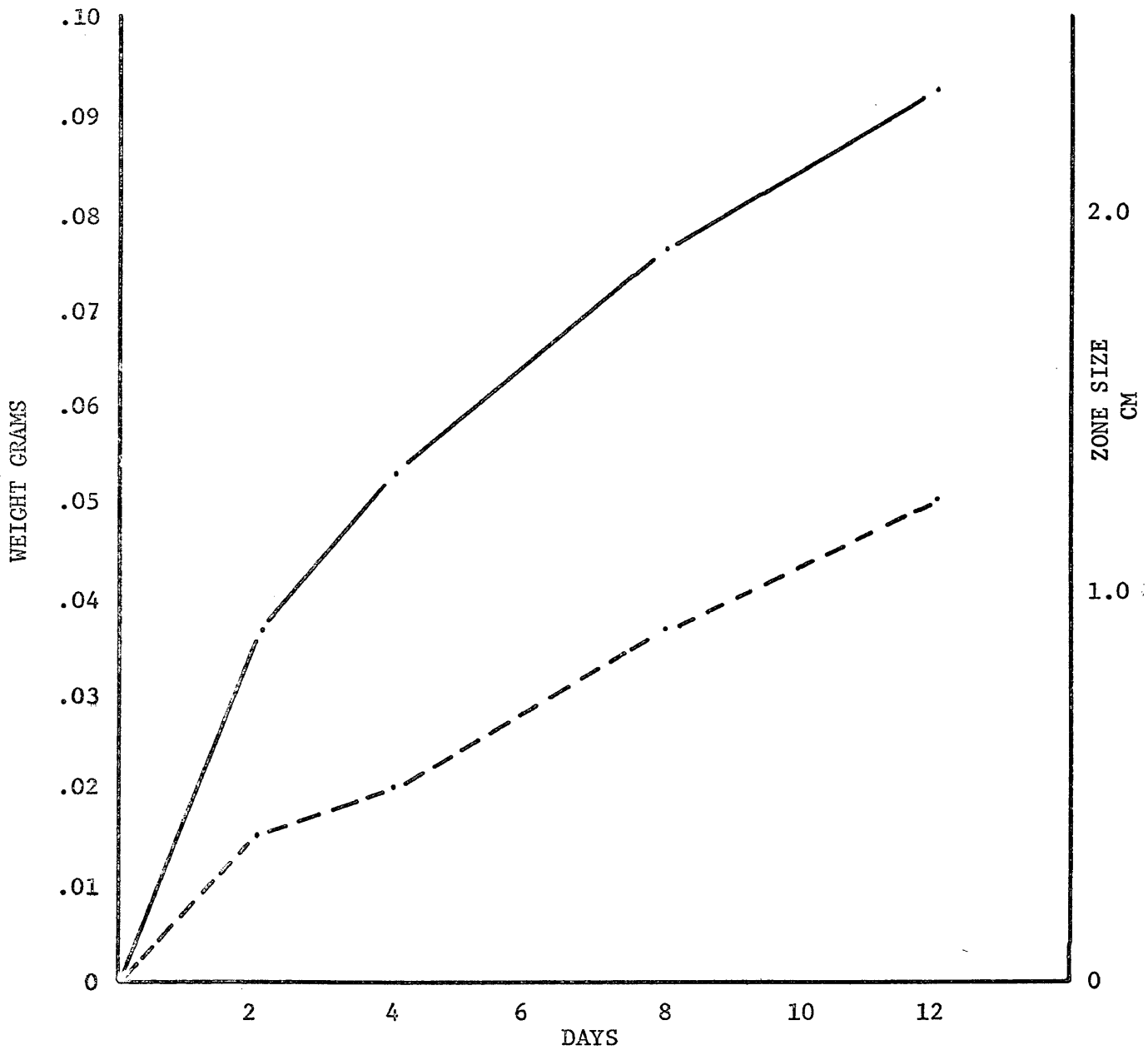


FIGURE 2: THE PROGRESSIVE RELEASE OF INHIBITORY MATERIAL BY ISOLATED ASPEN TISSUE

ZONE SIZE VERSUS TIME —————

TISSUE WEIGHT INCREASE VERSUS TIME - - - - -

order to compare the zone of starch hydrolysis with the size of the inhibitory zone. Nickell and Brakke (1954) found that tissue from common sorrel (Rumex acetosa) grown in vitro, released amylase into the medium. A comparison of the starch hydrolysis zone with the zone of the inhibitory material could possibly give a comparison of the relative molecular size of the enzyme and the antimicrobial compound(s). Three pieces of tissue were placed on each plate of number 23 medium that contained 1 per cent soluble starch and 1 per cent sucrose. The tissue was weighed aseptically at the beginning and again at the termination of the 18-day growth period. The tissue was removed from the plates and the agar was assayed for antimicrobial activity with Sarcina lutea and for starch digestion activity with iodine solution. The bacteria were incubated on the agar until inhibitory zones were visible. The size of the zone was measured and the plates were sprayed with a 0.0025N iodine solution. Colorless zones corresponding to the areas of starch digestion were obtained and measured. These zones were surrounded by areas of intense blue-black coloration indicating the presence of starch. The control was a plate that had supported the growth of tissue for 18 days and was sprayed with the iodine solution after the tissue was removed.

The data (Appendix, Table 5) show that the mean inhibitory zone was 2.5 centimeters and the mean size of the starch digestion was 1.1 centimeters. The tissue used in the experiment showed a growth index of 7.8. The results indicate, based on diffusion rates, that the inhibitory material is probably a smaller molecule than the enzyme which has a molecular weight of 54,180 Daltons. However, this conclusion may not be valid due to the other factors affecting diffusion. In addition to size, the diffusion of a molecule is controlled by its charge and symmetry.

Thus, there are three factors to consider in addition to the possibility that the inhibitor may be secreted in a much greater quantity than the enzyme. The control plate zone for starch hydrolysis was similar to those plates assayed first with bacteria. The bacteria did not appear to influence the diameter of the starch hydrolysis zone of the experimental plates.

The pH of the medium was measured in an attempt to detect changes in the hydrogen ion concentration since the observed inhibition could be the result of a shift in the pH of the medium. The inhibitory zone may be the result of an antimicrobial inhibitor or it may result from a pH change due to materials being released from the tissue. A change in the pH of the medium after supporting tissue growth, especially in the area of inhibition, would reveal that tissue had altered the pH as a result of its metabolism. Isolated tissue was weighed aseptically at the beginning and termination of the experiment. It was removed from the plates after 18 days and the plates were sprayed with universal indicator. The pH of a plate of fresh tissue culture medium was also measured as a control.

Hydrogen ion measurements (Appendix, Table 6) revealed that the area immediately surrounding the position of the tissue (i.e. the inhibitory zone) had a slightly lower pH (5.0-5.5) than the remainder of the medium (5.5-6.0). The area not closely associated with the growth of the tissue had a pH slightly lower than the control (6.0). The results did not reveal a large change in the pH from that of the control. The small change in the pH of the medium used to support the active growth of tissue could be due to nutrient uptake. It was concluded that such small shifts in the pH of the medium could not result in the

observed inhibition.

This conclusion was based on a series of controls which were adjusted to various acidities. Standard medium was adjusted to a pH of 4.5, 5.0 or 5.5 with 1N HCl before autoclaving. The pH of the medium was determined and the plates were flooded with a nutrient broth culture of Sarcina lutea and the excess inoculum was removed. Bacterial growth occurred at each pH.

Plates which had supported tissue growth for an 18-day growth period were used in order to determine whether the inhibitory material was bacteristatic or bactericidal. The plates were flooded with a nutrient broth suspension of Sarcina lutea which had an optical density of 0.15. The excess inoculum was removed from the plate and a wire loop was used to sample the medium from the center of the potential inhibitory zone and from the periphery of the plate. The loop from each location was used in order to inoculate separate flasks which contained 50 ml of nutrient broth. The flasks were placed on a shaker and their optical density was determined at selected intervals for a week. A sample of bacteria was removed from each zone on the plate during a 16 hour period. The data are presented in Table 1.

Inspection of the data revealed that there is a very marked reduction in bacterial growth when samples are removed from the zones of inhibition. The samples removed from the outside of the zone displayed very rapid growth and reach infinite optical density after 76 hours while 160 hours were required for the final (16 hour) sample from the inhibitory zone to reach an infinite optical density. The conclusion drawn from these data was that the antimicrobial material was primarily bactericidal. Eventual bacterial growth in the samples taken from the inhibitory zone was the result of a small number of cells which were capable of surviving

TABLE I

THE BACTERICIDAL PROPERTIES OF THE ANTIMICROBIAL
MATERIAL SECRETED BY ISOLATED ASPEN TISSUE

Time when optical density read hours	Time when sample taken hours	Plate 1		Plate 2	
		Inhibitory Zone	Control	Inhibitory Zone	Control
76	0	0.83	.85	0.85	1.20
	1	0.82		1.40	
	2	0.05	1.40	0.10	1.50
	3	0.25		0.17	
	4	0.04	1.60	0.00	1.80
	5	0.01		0.01	
	6	0.01	1.60	0.00	1.90
	7	0.00		0.02	
	8	0.07	1.90	0.00	1.60
	10	0.00		0.00	
	12	0.00	2.00	0.00	2.00
	16	0.00	2.00	0.00	2.00
	107 (a)	16	0.02		0.02
118	16	0.06		0.06	
132	16	0.21		0.28	
160	16	2.00		2.00	

(a) All optical density readings were greater than 2.00 except the 16 hours samples

the antibiotic or were not subjected to it long enough for complete lethality.

The influence of dimethyl sulfoxide (DMSO) on the release of antimicrobial material from isolated tissue was investigated. Kligman (1965) demonstrated that the penetration of pharmaceutical substances through the membranes of tissue is dramatically improved when used in conjunction with DMSO. Experiments were performed in order to determine the influence of DMSO on the permeability of isolated tissue. An increase in the release of the inhibitory material might be obtained with an increase in the permeability of the tissue in the presence of DMSO. Tissue culture medium was autoclaved for 15 minutes and the appropriate concentration of DMSO (b.p. 189°C) was added to the medium after it had cooled to 50°C. The medium was poured into Petri plates. Each experiment utilized five plates of medium with three pieces of tissue per plate at each concentration of DMSO (12.5, 25, 50, 1000, 1600, and 2000 ppm). The tissue used for each concentration was weighed aseptically in tared 10 ml Erlenmeyer flasks at the beginning and termination of the 12-day period of growth. The plates of medium were assayed for antimicrobial activity using Sarcina lutea.

Inspection of the data (Appendix, Table 7) indicates that the addition of dimethyl sulfoxide to tissue culture medium does not appreciably influence the size of the inhibitory zone under the conditions employed. The concentration of DMSO utilized may not be adequate to illicit a greater release of the antimicrobial material.

Mathes and Kremer (1966) have suggested the possibility of catechol being the inhibitory material. Catechol should be found in the tissue if it is solely or partially responsible for the antibiotic activity of isolated aspen tissue. Thus, it was necessary to analyze for its

presence in the tissue and an aqueous extract of aspen tissue was used initially in the chemical analysis for catechol. White actively growing tissue (12.0489 grams) was frozen and homogenized in a blender with 100 ml of double distilled water. The homogenate was heated to 100°C in a water bath and filtered through Whatman No. 1 paper. The filtrate (10 ml) was filter-sterilized using a 0.22 micron Millipore filter. The extract was assayed by pipetting 0.1 ml on an antibiotic pad and placing the pad in a vertical position in the medium of a Petri plate which was previously flooded with a nutrient broth suspension of Sarcina lutea. The excess inoculum was removed from the plate. The plate was incubated at 24-25°C until bacterial growth covered the agar. The control was an antibiotic pad with 0.1 ml of sterile double distilled water. The assay for activity demonstrated that the aqueous extract was inhibitory to Sarcina lutea. The control did not give antimicrobial activity.

Catechol was also assayed for antimicrobial activity in order to establish its antibiotic properties. The method of assay included two different techniques. The first technique consisted of pipetting 0.1 ml of catechol on an antibiotic pad and placing the pad in a vertical position in the medium of a Petri plate which was previously flooded with a nutrient broth suspension of Sarcina lutea. The excess inoculum was removed from the plate. The plate was incubated at 24-25°C until bacterial growth covered the agar. The second technique (described in general materials and methods) involved drying the pads at 65°C. When additional amounts of catechol were added, the pad was dried between each successive addition of 0.1 ml. Catechol, at concentrations of 350, 650, and 1300 ppm was assayed using the first method and was assayed

using previously dried pads at concentrations of 100, 500, 1000, 2000, and 3000 ppm. These concentrations were assayed with either 0.1 ml or 0.2 ml on an antibiotic pad.

The results of the assay (Appendix, Table 8) revealed that catechol in the concentrations used and the conditions employed did not inhibit the growth of Sarcina lutea. The agar which catechol had diffused into from the antibiotic pad turned a reddish color when the bacteria began to grow on the plate. Since catechol is oxidized to the red colored ortho-quinone by the appropriate enzyme (tyrosinase), the red color probably indicates that the bacteria were capable of oxidizing catechol. The red color in the agar disappeared after four or five days and this may indicate that the bacteria have the ability to metabolize and breakdown catechol.

Three ml of either the filter-sterilized extract or the catechol standards (1000, 1500, 2000, and 3000 ppm) were each tested with 0.1 ml of 1 per cent ferric chloride. The extract and standards gave a positive green ferric chloride test for catechol. The optical density of each ferric chloride tested solution was measured at 560 nanometers using a Spectronic 20. Comparison of the optical density of the extract with those of the standards revealed that the aqueous extract had an absorption equal to a catechol concentration of approximately 220 ppm.

The ferric chloride treated aqueous extract and catechol standards were next tested with a drop of 1 per cent sodium acetate. The green ferric chloride-catechol color complex in the extract and standards turned a violet color when the sodium acetate was added. Three ml of each standard and the aqueous extract were tested with a drop of 1 per cent lead acetate in the absence of ferric chloride. A pinkish white

precipitate of catechol and lead acetate occurred in each fraction. The absorption spectrum between 340 and 220 nanometers was recorded using the aqueous extract, using a DK-2A Recording Ratio Spectrophotometer. The extract gave a peak of maximum absorption at 287 nanometers. The aqueous catechol solution gave a peak of maximum absorption at 283 nanometers. The spectra are presented in Figure 3 and the data are presented in the Appendix, Table 9.

The nonsterile aqueous extract was washed five times with a 1:1 volume of ethyl acetate. Catechol is soluble in ethyl acetate and the solvent is insoluble with water. The ethyl acetate wash was dried with anhydrous calcium chloride. A spectrum of the aqueous extract, the washed aqueous extract and the ethyl acetate wash was recorded between 340 and 220 nanometers. These spectra are presented in Figure 4. Each fraction gave a peak of maximum absorption at 287 nanometers. The aqueous extract displayed a reduction in its peak after the ethyl acetate extraction. Two ml of the ethyl acetate wash was tested with a small amount of ferric chloride (1 mg). A positive green color test was obtained. The remaining ethyl acetate wash was divided into two equal fractions. One fraction was washed four times with a 1:1 volume of double distilled water. Two ml of the aqueous wash were tested for the presence of the substrate (catechol) by the addition of 0.1 ml of tyrosinase (polyphenoloxidase). The enzyme had a concentration of 54 units of activity/0.1 ml. Two ml of the ethyl acetate were also tested with the addition of 0.1 ml of tyrosinase. A number of controls were included for the purpose of excluding the possibility that the enzyme is not reactive in ethyl acetate. They included: (1) 2 ml of ethyl acetate wash

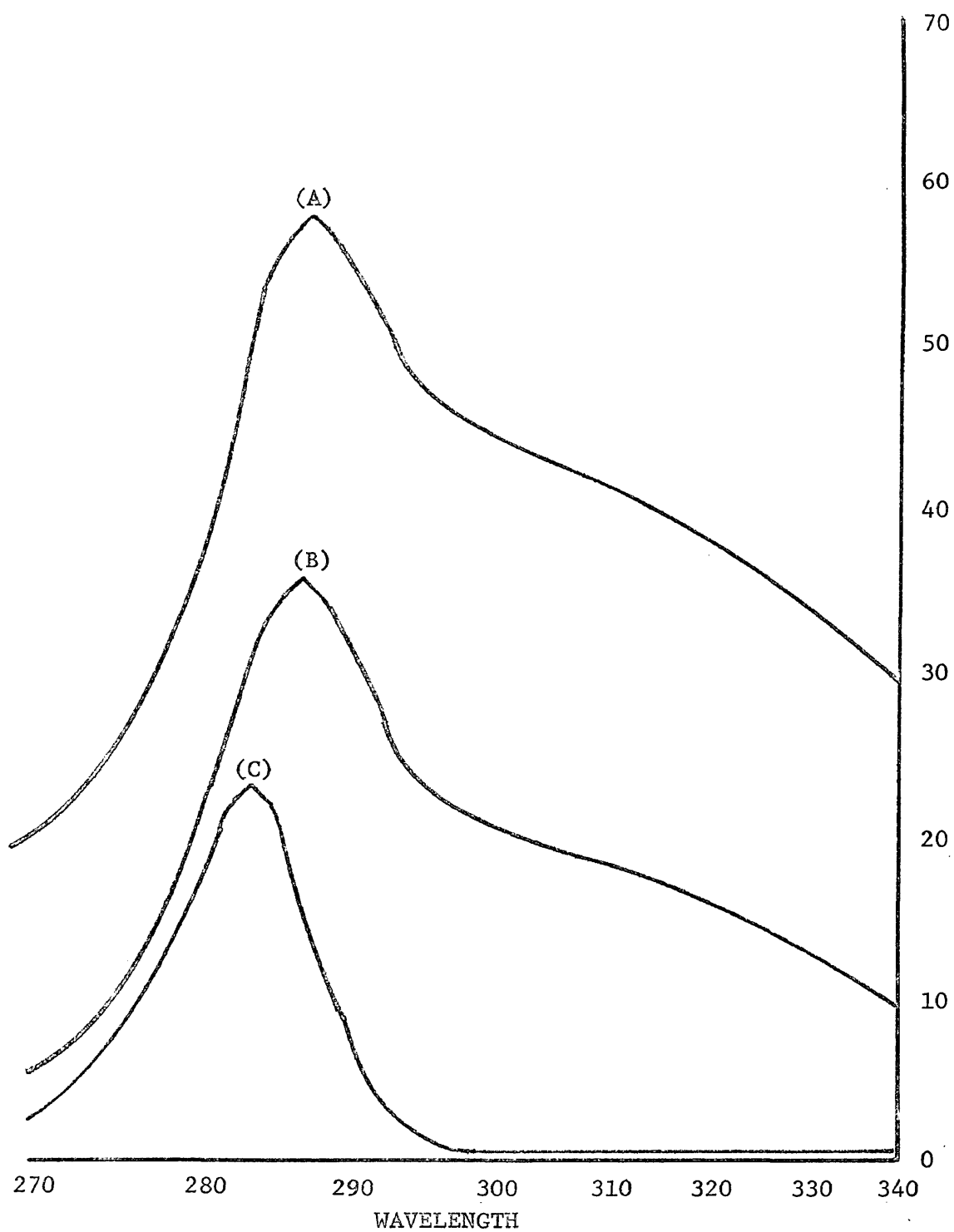


FIGURE 3: THE SPECTRA OF THE AQUEOUS EXTRACT OF ASPEN TISSUE
(A) AQUEOUS EXTRACT OF ASPEN TISSUE
(B) FILTER STERILIZED AQUEOUS EXTRACT
(C) AQUEOUS CATECHOL

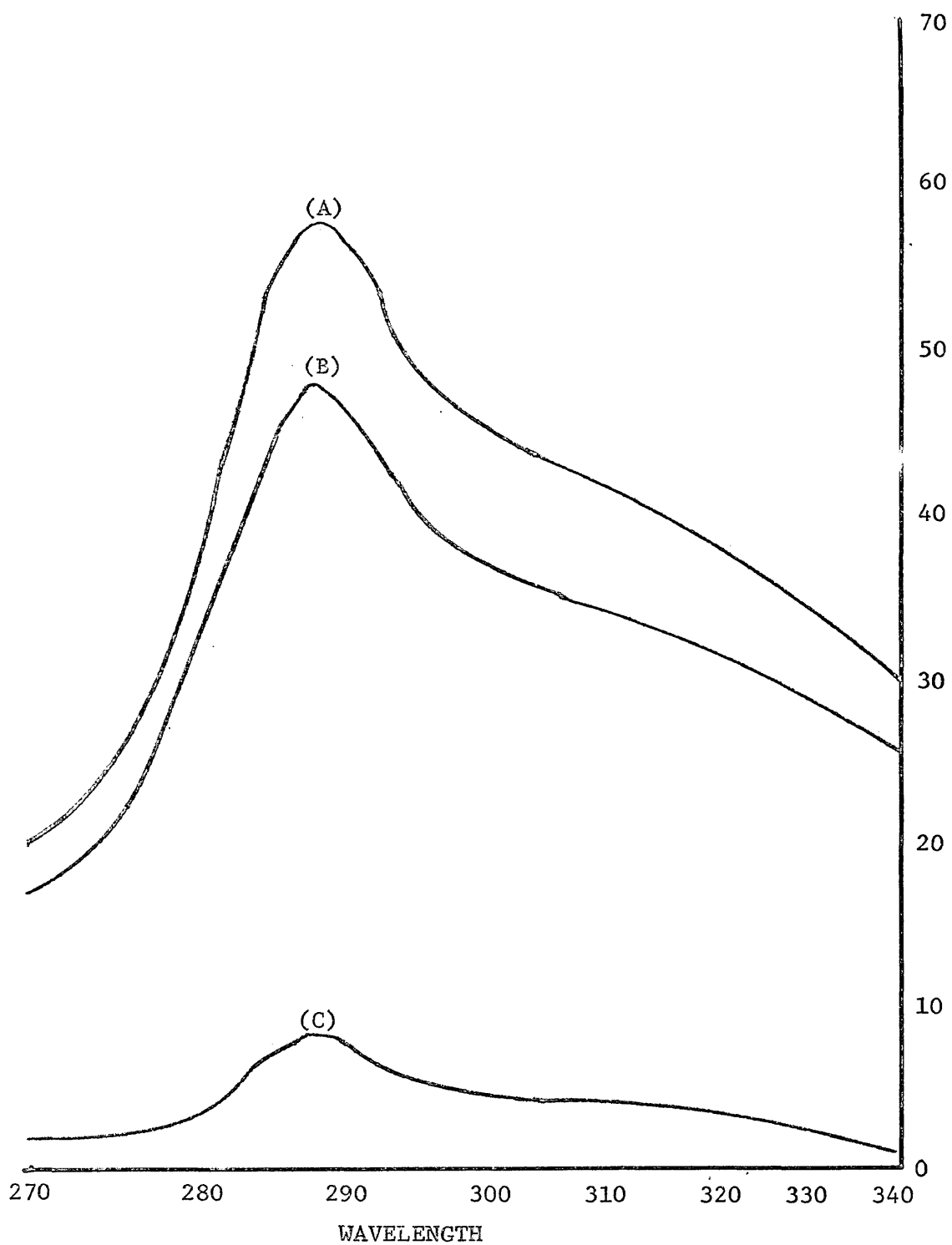


FIGURE 4: THE SPECTRA OF THE ETHYL ACETATE WASH
(A) AQUEOUS EXTRACT OF ASPEN TISSUE
(B) AQUEOUS EXTRACT AFTER ETHYL ACETATE WASH
(C) ETHYL ACETATE EXTRACT

+ 0.1 ml of double distilled water, (2) 2 ml of ethyl acetate + 0.1 ml of double distilled water, (3) 2 ml of ethyl acetate + 0.1 ml of tyrosinase, (4) 2 ml of catechol in ethyl acetate solution + 0.1 ml of enzyme. The enzymatic treatment of the ethyl acetate wash gave a dark red color reaction which is characteristic of the oxidation products of an ethyl acetate-catechol solution treated with tyrosinase. The enzymatic treatment of the water wash gave a dark red color reaction which is characteristic of the oxidation product of aqueous catechol plus tyrosinase. The controls revealed that polyphenoloxidase was active in ethyl acetate. The data for the above experiment are presented in the Appendix, Table 10.

Standard tissue culture medium which had supported the growth of isolated aspen tissue for a period of 18-days was tested for the presence of catechol. The medium was liquified and washed with ethyl acetate. The ethyl acetate was tested with ferric chloride and polyphenoloxidase. Neither a green ferric chloride test nor a red color reaction product from the enzymatic oxidation of catechol was obtained. Catechol does not appear to be in the medium in a detectable quantity.

The possibility that catechol is the primary inhibitory material was further investigated utilizing the enzymatic and chemical analysis of expressed cellular sap. Actively growing white aspen tissue was placed in a sterile plastic tube and crushed under aseptic conditions in a vise. The cellular sap was assayed for activity using Sarcina lutea. The sap inhibited bacterial growth. The expressed sap was also filter-sterilized. Three ml of both the filter-sterilized and catechol standards (1000, 1500, 2000 and 3000 ppm) were tested with 0.1 ml of 1 per cent ferric chloride solution. A positive green ferric chloride test was obtained for each fraction. The optical density of each

fraction was measured using a Spectronic 20. The colorimetric technique revealed an absorption equal to a catechol concentration of approximately 1000 ppm in the filter-sterilized expressed sap. One drop of 1 per cent sodium acetate was added to each ferric chloride tested fraction and a violet color appeared. A drop of 1 per cent lead acetate was added to 1.0 ml portions of the sterile expressed sap and the catechol standards. A pinkish white precipitate was obtained for all solutions tested. The data are presented in the Appendix, Table 9.

The filter-sterilized sap was treated with tyrosinase in order to oxidize the catechol present to the ortho-quinone. Loss of inhibition due to tyrosinase oxidation would implicate a phenolic compound as the inhibitor. One ml of the sap was oxidized with 0.1 ml of tyrosinase (105 units of activity/0.1 ml). The control was 0.1 ml of denatured enzyme added to 1.0 ml of sap. The enzyme was denatured by autoclaving it at 121°C and 15 psi for 15 minutes. Another control for enzyme activity was 1.0 ml portions of 3000 ppm aqueous catechol oxidized with 0.1 ml of active or inactive enzyme. Each reaction was allowed to proceed for 24 hours at room temperature before assaying the reaction mixture for antimicrobial activity with Sarcina lutea. A spectrum was recorded using aqueous catechol, enzyme oxidized catechol, filtered expressed sap and the enzyme oxidized sap between 340 and 220 millimicrons (see Figure 5). The spectra did not reveal any significant change in absorption due to the enzymatic oxidation of the active expressed sap or catechol.

Enzymatic oxidation of the expressed sap caused the reaction mixture to darken. However, the assay for activity showed the enzyme oxidized sap to still retain its antimicrobial activity. The control sap

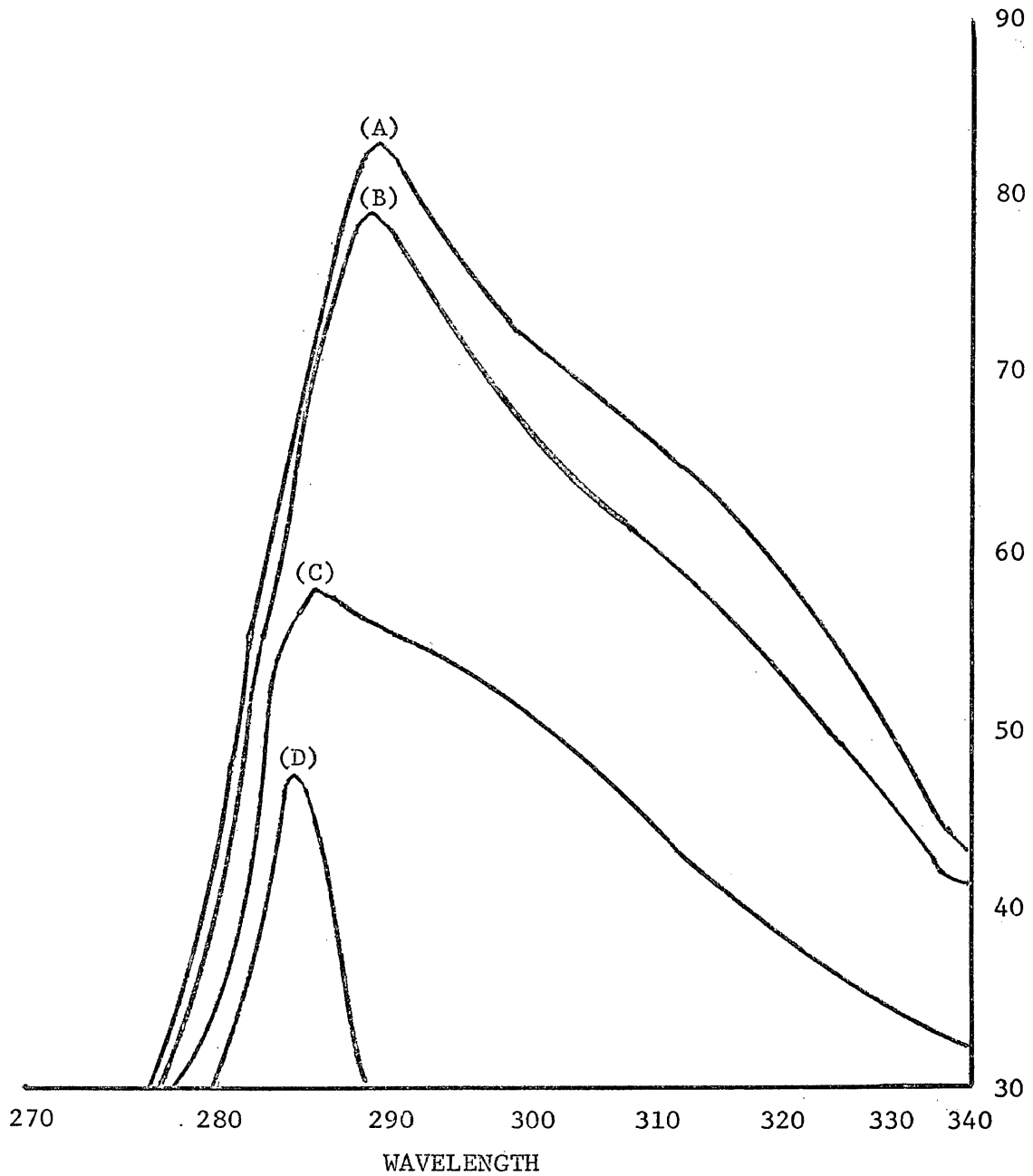


FIGURE 5: THE SPECTRA OF THE EXPRESSED CELLULAR SAP
(A) FERTILE-STERILIZED EXPRESSED SAP
(B) FERTILE-STERILIZED EXPRESSED SAP + TYROSINASE
(C) AQUEOUS CATECHOL + TYROSINASE
(D) AQUEOUS CATECHOL

containing the denatured enzyme did not give a color reaction but did give inhibition of bacterial growth. The catechol controls demonstrated the tyrosinase to be chemically active. The enzyme oxidized catechol gave a dark red solution. Thus, these data (Appendix, Table 11) support the hypothesis that catechol is not responsible for the antibiotic activity since enzyme oxidation of the phenolic compounds in the expressed sap did not destroy activity.

Electrophoresis was considered as a possible tool for the isolation of the antimicrobial material. It was necessary to initially determine the magnitude and type of charge on the inhibitory molecule(s) so that the material could be placed under the proper electrophoretic conditions. The electrode chambers used in the procedure were filled with a solution containing mineral nutrients in the same concentration as found in the standard medium. Sodium chloride was also added to the solution in order to maintain a greater resistance across the medium. The charge on the molecule was investigated by electrophoresing a plate which had supported tissue growth for 18 days. The plate was placed above and between the electrode chambers and cheesecloth salt bridges, soaked in the salt solution, were placed in contact with the salt solution in the electrode chamber and the opposite edges of the agar. The current was set at 15 milliamperes and the potential was maintained at 200 volts/1.0 centimeters. The agar medium was electrophoresed under these conditions for 15 minutes. The plate was immediately assayed for antimicrobial activity with Sarcina lutea. The electrophoresis was repeated using antibiotic pads which were soaked in a solution containing 2000 ppm catechol and placed vertically in a plate of fresh medium. This procedure introduced catechol into the agar for electro-

phoresis. The plate was electrophoresed for five minutes as above and flooded with a 1 per cent ferric chloride solution after the electrophoresis.

The results from this experiment revealed that the antimicrobial material had no net charge at the pH range of the standard medium (5.5-6.0). This conclusion was made because the zone of inhibition remained circular and evenly distributed around the indentation in the medium where the tissue had grown. The catechol placed in the medium at a pH of 5.5-6.0 migrated to the cathode. An affirmative ferric chloride test (green color) identified the position of the catechol in the agar. The results of this experiment eliminated the use of electrophoresis as a technique for the separation of the antibiotic material since the material did not migrate under the conditions employed.

Liquid standard tissue culture medium was used for the analysis of antimicrobial material because agar was not present to interfere with the extraction of the inhibitory material. The use of liquid medium would also provide a method for the concentration of the inhibitory materials. Tissue was grown in liquid standard medium for a period of four weeks. Five pieces of tissue were placed in 250 ml of liquid medium and incubated in the dark on a shaker at 200 rpm at 26-27°C in a controlled environmental New Brunswick incubator-shaker. The tissue was removed and the medium was stored at -18°C until used. One liter of the liquid medium was evaporated to dryness at 60°C in vacuo in a flash evaporator. The residue in the two liter round bottom flask was extracted with 50 ml of chloroform and the residue was extracted with 50 ml of ethyl acetate. The two solvents were evaporated at room temperature to an approximate volume of 5 ml. They were both assayed for activity using Sarcina lutea.

Pure solvents were placed on the antibiotic pads and used as controls. The 5 ml extracts of chloroform and ethyl acetate were evaporated to complete dryness at room temperature in separate watch glasses. Each watch glass was washed with 2 ml of sterile double distilled water and each of these fractions were assayed for antimicrobial activity. Double distilled water was assayed as a control.

The results of the extraction procedure (Appendix, Table 12) were negative. The results are not conclusive in view of the fact that only two solvents were used for extraction. Further work with liquid defined medium may represent the best method for isolating the antimicrobial material(s).

Paper and column chromatography were used in an attempt to isolate the antimicrobial material from expressed cellular sap. Expressed cellular sap was chromatographed on Whatman No. 1 paper using distilled water or butanol, acetic acid, and water at a ratio of 12:3:5, respectively. The chromatographs were loaded with 0.1 ml of the expressed sap on each spot and the solvent front was allowed to ascend approximately 15 cm. The chromatograph was loaded again with 0.1 ml of the expressed sap in an area above the solvent front. This spot served as a control. The chromatographs were dried at room temperature and placed on the surface of a 9 X 12 plate containing 250 ml of 10 per cent agar. The chromatograph was removed from the plate after 3 days and the plate was flooded with a nutrient broth suspension of Sarcina lutea. The excess inoculum was removed from the plate by aspiration. Chromatographs were also placed on the surface of 10 per cent agar which had been inoculated with a suspension of Sarcina lutea. The bacteria were added to the sterile agar at 45°C and the mixture was poured into

the 9 X 12 plate to solidify. The chromatographs did not result in antimicrobial activity using either solvent system or method of development. The control spots did give antimicrobial activity. The lack of activity in the expressed sap may be attributed to several factors. The antimicrobial activity may be due to a concerted effect of several compounds and the separation by the chromatography would inactivate the system. The dilution effect of chromatography might also serve to eliminate active inhibition. Although contamination was not observed, it also could serve as a factor to eliminate activity.

Bio-Gel P-10, a polyacrylamide gel, was used in the column chromatography procedure. The gel has a molecular weight exclusion limit of 12,000 Daltons. It was hydrated for 24 hours in 0.1M NaH_2PO_4 at a pH of 5.7 before use. Three ml of filter-sterilized expressed juice were placed on a 5.0 ml column and a series of seven 1.0 ml fractions were collected. The void volume of the column was approximately 2.0 ml since it usually consists of 38 to 42 per cent of the column's volume. Each 1.0 ml fraction was assayed using Sarcina lutea. The control was the assaying of 0.1M NaH_2PO_4 . The fractions assayed did not result in the inhibition of microbial growth. The conclusion made from these results was that either the antimicrobial material(s) was caught in the mesh of the gel or that the inhibitory material(s) consists of two or more components necessary for activity. If the former conclusion is correct, this would indicate that the material(s) has a molecular weight smaller than 12,000 Daltons. The latter conclusion would mean that the inhibitor consists of two or more components which the column was able to separate.

CONCLUSION

Isolated aspen tissue demonstrated a constant rate of growth which was correlated with a progressive release of the inhibitory material. The diffusion rate of the inhibitory molecule(s), when released progressively, was more rapid than that of the starch hydrolysis enzyme, alpha-amylase. Although other factors affecting diffusion could not be controlled entirely, it was concluded that the antibiotic molecule is smaller than the alpha-amylase molecule.

The pH of the medium, after it had supported the growth of tissue, revealed no significant change that might be responsible for the observed inhibition. Further investigation revealed the inhibitory molecule(s) to be bactericidal. The bacteria, after removal from agar which contained the antimicrobial material, demonstrated a marked reduction in growth when placed in nutrient broth. The inclusion of dimethyl sulfoxide, which enhances the permeability of membranes, did not increase the release of the inhibitory material.

An aqueous extract of the tissue was utilized to inhibit bacterial growth. Chemical and enzymatic analysis of the extract revealed that it contained catechol which many workers have suggested as the major antimicrobial agent in the genus Populus. However, the assay of an aqueous catechol solution for antimicrobial material proved to be negative using the procedures outlined. The possibility that catechol is a constituent of the inhibitory material was further investigated using expressed cellular sap. It was concluded that catechol was at least not exclu-

sively responsible for the antimicrobial activity of the tissue.

Several techniques were applied to the problem of isolating the inhibitory material from the tissue. Electrophoresis did not prove to be an applicable tool for isolating the inhibitor because the material is apparently not charged, as is catechol. Liquid tissue culture medium, a desirable bulk source, did not yield an active fraction after extraction procedures but this technique may prove to be fruitful with further work. Chromatographic separation of the expressed sap was not successful. However, the absence of antimicrobial activity, after chromatographic development, may indicate that it was separated into two or more inactive components. Column chromatography supported this hypothesis because none of the fractions collected from the gel were active. The gel had a molecular weight exclusion limit of 12,000 Daltons. The column could have either trapped an inhibitory molecule smaller than 12,000 Daltons or removed one of the smaller components of an inhibitory complex.

APPENDIX

TABLE I

THE COMPOSITION OF THE STANDARD MEDIUM (NUMBER 23)

chemical	concentration--mg/liter
$\text{CaNO}_3 \cdot 4\text{H}_2\text{O}$	200
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	360
Na_2SO_4	200
KNO_3	80
KCl	66
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	16
Ferric citrate	10
MnSO_4	3
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$	0.025
H_2SO_4 (sp. gr. 1.84)	0.0005 ml/liter
H_3BO_3	0.05
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.05
Agar	8000
Sucrose	20000
Naphthalene Acetic Acid	0.50
Coconut milk	100 ml

TABLE 2

THE COMPOSITION OF THE DEFINED MEDIUM (LINSMAIER AND SKOOG)

chemical	concentration--mg/liter
NH_4NO_3	1650
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
KH_2PO_4	170
Na_2EDTA	37.3
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
KNO_3	1900
H_3BO_3	6.2
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	17.1
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10.7
KI	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
Kinetin	0.2
Indoleacetic Acid	2.0
Agar	8000
Sucrose	30000
Thiamine HCl	0.40
Inositol	100
Adjust pH to 5.6 with .06 ml of 1N NaOH/liter	

TABLE 3

THE GROWTH RATE OF ISOLATED ASPEN TISSUE

Time-days	Mean tissue weight (gms) (a)	Standard deviation of mean weight (gms)	Progressive growth index (b)	Growth index/4-day time interval (c)
0	0.089	0.006		
4	0.172	0.021	1.933	1.933
8	0.290	0.036	3.258	1.686
12	0.454	0.059	5.101	1.566
16	0.584	0.081	6.561	1.286
20	0.977	0.097	10.977	1.673
24	1.356	0.137	15.236	1.388
28	1.692	0.054	19.011	1.248

(a) All values reported as mean fresh weight

(b) Progressive growth index denotes the mean tissue weight of each time increment/initial mean weight

(c) Growth index/4-day increment indicates the mean tissue weight/mean tissue weight of previous 4-day time interval

TABLE 4

THE PROGRESSIVE RELEASE OF INHIBITORY MATERIAL BY ISOLATED ASPEN TISSUE

Time-days	Mean initial weight (gms)	Mean final weight (gms)	Mean increase weight (gms)	Mean zone size (cm)
2	0.024	0.039	0.015	0.9
4	0.027	0.047	0.020	1.3
8	0.029	0.065	0.036	1.9
12	0.024	0.074	0.050	2.1

TABLE 5

THE DIAMETER OF BACTERIAL INHIBITION AND STARCH HYDROLYSIS ZONES

Tissue number ^(a)	Zone size for bacterial inhibition	Zone size for starch hydrolysis
1	2.7	1.5
2	2.5	1.0
3	2.5	1.5
4	2.1	1.0
5	2.5	1.5
6	2.2	1.0
7	2.5	0.5
8	2.5	0.5
9	2.9	1.0
	mean 2.5	mean 1.1
Control	---	2.0, 2.0, 1.9
 (a) Mean initial tissue weight per piece 0.0403 Mean final tissue weight per piece 0.3150		

Table 6

THE HYDROGEN ION CONCENTRATIONS OF TISSUE CULTURE MEDIUM

Plate number(a)	pH of plate	pH of area where tissue had grown
1	5.5-6.0	5-5.5
2	5.5-6.0	5-5.5
3	5.5-6.0	5-5.5
4	5.5-6.0	5-5.5
Control	6.0	6.0

Bacterial growth on standard medium which had an adjusted pH served as a control. Bacteria grew on medium which had a pH of 4.5, 5.0, and 5.5 as well as the normal medium.

(a) Initial average tissue weight per piece 0.0403 gms
 Final average tissue weight per piece 0.3150 gms
 Average tissue weight increase per piece 0.2647 gms

TABLE 7

THE INFLUENCE OF DIMETHYLSULFOXIDE ON THE RELEASE
OF ANTIMICROBIAL MATERIAL BY ISOLATED TISSUE

Conc. of DMSO (ppm)	Mean initial weight (gms)	Mean final weight (gms)	Mean increase in tissue weight (gms)	Mean zone size (cm)
Experiment I				
2000	0.217	0.1251	0.0934	3.0
1600	0.0289	0.1163	0.0974	2.7
1000	0.0199	0.1540	0.1341	2.5
500	0.0251	0.0926	0.0675	3.3
50	0.0201	0.1394	0.1193	4.2
Control	0.0186	0.0578	0.0392	2.6
Experiment II				
1600	0.0237	0.1087	0.0850	2.4
1000	0.0230	0.1176	0.0946	3.5
500	0.0176	0.0851	0.0665	2.4
50	0.0229	0.0976	0.0747	3.3
Control	0.0236	0.0556	0.0333	1.7
Experiment III				
50	0.0591	0.2278	0.1687	3.7
25	0.0551	0.1720	0.1149	3.9
12.5	0.0586	0.2096	0.1510	3.8
Control	0.0588	0.2125	0.1537	4.4

TABLE 7 (CONTINUED)

Conc. of DMSO (ppm)	Mean initial weight (gms)	Mean final weight (gms)	Mean increase in tissue weight (gms)	Mean zone size (cm)
Experiment IV				
1000	0.0364	0.0849	0.0585	0.7
500	0.0296	0.0728	0.0432	0.9
50	0.0341	0.0717	0.0376	1.1
Control	0.0368	0.0824	0.0556	1.0

TABLE 8

THE ASSAY OF CATECHOL FOR ANTIMICROBIAL ACTIVITY

Concentration of catechol--ppm	Inhibition (+ or -)
Method--0.1 ml on antibiotic pad--placed on flooded plate	
Control	-
1300	-
650	-
350	-
Method--0.1 ml on antibiotic pad--dried at 65°C for two hours	
Control	-
1000	-
500	-
100	-
Method--0.1 ml on antibiotic pad--dried at 65°C for two hours	
Control	-
3000	-
2000	-
1500	-
1000	-
500	-
100	-

TABLE 9

THE QUALITATIVE AND QUANTITATIVE ANALYSIS OF CATECHOL
IN THE EXPRESSED SAP AND THE AQUEOUS EXTRACT

Solution	Ferric chloric test(+ or -)	Sodium acetate (+ or -)	Lead acetate ppt.(+ or -)	Optical density at 570 mu.
aqueous extract (filter-sterilized)	+	+	+	0.06
expressed sap (filter-sterilized)	+	+	+	0.27
3000 ppm catechol	+	+	+	0.27
2000 ppm catechol	+	+	+	0.28
1500 ppm catechol	+	+	+	0.29
1000 ppm catechol	+	+	+	0.32
ethyl acetate wash	+	(a)	(a)	(a)

(a) Test not completed

TABLE 10

THE ENZYMATIC OXIDATION OF THE AQUEOUS EXTRACT

Fraction Oxidized	Color Reaction
aqueous catechol + enzyme	dark red
water wash + enzyme	dark red
ethyl acetate wash + enzyme	dark red
ethyl acetate + water	no reaction
catechol ethyl acetate + enzyme	dark red
ethyl acetate + water	no reaction
ethyl acetate wash + water	no reaction

TABLE 11

THE ENZYMATIC ASSAY FOR ANTIMICROBIAL ACTIVITY

Solution	Inhibition (+ or -)	Zone size (cm)
expressed cellular sap	+	1.0
expressed cellular sap + active enzyme	+	1.0
expressed cellular sap + inactive enzyme	+	1.0
3000 ppm catechol + active enzyme	-	---
3000 ppm catechol + inactive enzyme	-	---

TABLE 12

EXTRACTION OF LIQUID TISSUE CULTURE MEDIUM

Extract assayed	Inhibition (+ or -)
chloroform extract	negative
chloroform control	negative
ethyl acetate extract	negative
ethyl acetate control	negative
water wash of chloroform extract	negative
water wash of ethyl acetate acetate	negative

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