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THE GROWTH OF AZOTOBACTER VINELANDII ON p-HYDROXYBENZOIC ACID FROM SOIL MEDIUM

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Fang Jy Wu, B.S.
Denton, Texas
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The purpose of this study was to search for the substrates utilized by *Azotobacter vinelandii* in dialysed soil media. Also, we sought to determine the relationship between these substrates and the growth and morphological variations of *A. vinelandii*.

p-Hydroxybenzoic acid was shown to be used as the carbon and energy source by *A. vinelandii* in dialysed soil medium. The amount of this compound in the soil dialysed soil medium ranged from 14 to 21 micrograms per gram of soil.

In a dialysed soil medium, p-hydroxybenzoic acid induced *A. vinelandii* to form minute bodies, similar to the filtrable forms reported by Gonzalez and Vela, although no growth of minute bodies was detected.

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CHAPTER I

INTRODUCTION

Many bacteria exhibit pleomorphism under certain culture conditions; however, members of the family *Azotobacteraceae* have a tendency toward extreme pleomorphism. Jones (17), Lohnis and Smith (19), Winogradsky (40), Eisenstark et al. (9,10), Bisset and Hale (3,4) and Vela et al. (36) have shown many morphological variations in azotobacter cells. The variations in cell form of this organism include large non-sporeforming rods, coccoid budding cells, dwarf cells, giant cells, branching cells, gram positive and gram negative rods, and fungoid cells; in addition, these organisms form cysts and, some reports say, gonidia (3,4,17, 19,36,40).

Cultures of *Azotobacter vinelandii* in nitrogen-free media (39) contain relatively large cells (2 microns or more in diameter) occurring singly or as diploids. These are the forms commonly seen in all cultures grown in the laboratory and are considered to be typical. They represent the morphology described in *Bergey's Manual of Determinative Bacteriology* and in a major part of the literature on microbiology including all modern textbooks. If the growth medium contains nitrogenous substances, especially organic materials, the morphology of *A. vinelandii* changes dramatically. For example, fungoid

cells can be induced if the organism is grown in 5 per cent Difco peptone (Difco Laboratories, Detroit, Michigan) (36), and giant cells of *A. agile* have been seen after transfering growth from a nitrogen-free medium to a medium containing beef extract or soil extract (9,10). On the other hand, Vela and Cagle (35) were able to see these giant cells in nitrogen-free media at specific times during the cycle of cyst formation and germination in *A. vinelandii*.

Winogradsky and others (1,26,35,39,40) reported that cyst formation might be induced in some strains when organic compound, such as ethanol or butanol, were supplied as the sole carbon and energy source. Socolofsky and Wyss (31,32) reported that the addition of 0.3 per cent n-butanol to Burk's medium would induce the conversion of almost 99 per cent of vegetative cells of *A. vinelandii* into the cyst form.

While the above descriptive morphology has been established in the literature as the result of extensive studies of azotobacter, other parallel studies have claimed that there is a small form of the azotobacter which can pass through filters capable of retaining other bacteria. This morphological variant has been called the filtrable form of the azotobacter (17).

Jones (17) was the first to observe the filtrable azotobacter in 1912, a finding confirmed by Lohnis and Smith in the early 1920's. Many researches doubted their findings, however, since only a very few filtrable forms could be found.

When Gonzalez and Vela (16) were able to devise a medium in which large numbers of the organisms could be found, they were able to suggest that filtrable forms of azotobacter can survive in dry soil for more than fifteen years.

It seems probable that the filtrable forms of _A. vinelandii_ are the forms which naturally occur _in situ_ in the soil. This seems likely since, according to Gonzalez and Vela (16), the filtrable forms have very long survivability, suggesting a relationship between small cell size and long survivability that may be explained by the smaller cells requiring less nutrients and expending less energy.

Although the filtrable form of _A. vinelandii_ has been cultured in the dialysed soil medium repeatedly (16), not much is known regarding their morphological, structural, and functional properties. Rubenchik (28) maintains that morphological variability should be attributed only to environmental influences. Therefore, all questions concerning environmental conditions and the morphological variation of _A. vinelandii_ must, by necessity, be dependent on medium composition since growth temperatures and gaseous environments are generally uniformly constant in the culture of _A. vinelandii_. In the simplest form of azotobacter, then, morphological variations must depend on the nutrients available to the organism. This relationship between cell morphology and nutrients suggests that there are chemical substances in soil of molecular weight less than 20,000 daltons which determine the organism's morpho-

logy. In this case, there must be substances in the soil which induce the formation of the filtrable form (cellulae) of A. vinelandii.

Microorganisms from the following families have been shown to utilize aromatic compounds as the sole source of carbon for growth: Coccaceae, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirillaceae, Bacillaceae and Azotobacteraceae (21,23). However, the abilities of the organisms to degrade phenolic compounds are not easily comparable because of the different enzyme systems involved. On the other hand, it has recently been suggested that two of these, pseudomonas and azotobacter, must share evolutionary histories because they possess similar (almost identical) metabolic pathways for the degradation of benzoic substances (8). It is part of the conventional wisdom of modern microbiology that if an organic substance can be oxidized by a bacterium, then it must be able to grow on that substance using it as a source of energy and material. Therefore, some aromatic substance(s) in the dialysed soil medium of Gonzalez and Vela may prove to be an important source of carbon and energy for A. vinelandii.

According to Stanier (33), Azotobacter cells grow well in soil when they do not need to compete with other aerobic organisms using similar nutrients, that is, in nitrogen-poor environments. They also grow well in the presence of some unusual carbon compounds such as benzoic acid (23). In 1952,

Winogradsky stated that the azotobacter were forced to utilize fermentation or decomposition products of plant residues in soil since, in his view, they could not compete with other soil bacteria for carbohydrates and other easily metabolized substrates.

A variety of phenolic acids have been detected in aquatic extracts of soil (12,24,25,30,31). These are released from lignin decomposition by soil microorganisms or from humic acid degradated by oxidants such as peracetic acid, nitric acid, alkaline cupric sulfate, alkaline nitrobenzene, perhydrodioxide, and sodium hypochlorite solution (5,6,21,29). Therefore, it is assumed that the phenolic acids in the dialysed soil medium of Gonzalez and Vela must play an important role in the growth and morphological variability of A. vinelandii growing in that medium. Further, the successful use of phenolic acids as a carbon and energy source by A. vinelandii in soil is significant since this organism grows better in the dialysed soil medium than in nitrogen-free media containing glucose (16).

The ability of A. vinelandii to utilize aromatic compounds has been long known (13,14,27,41). Biochemical investigations of the oxidative degradation of aromatic compounds by A. vinelandii have revealed that these substrates are metabolized through a pathway involving the diphenols, protocatechuate, or catechol. The phenolic acids are converted to aliphatic compounds by ring cleavage involving direct oxygenation with

molecular oxygen. Two methods of ortho-diphenol cleavage have been demonstrated in azotobacter (7,14,37): (1) ortho cleavage, or oxidative fission of the bond between carbon atoms bearing the hydroxyl groups; and (2) meta cleavage, or rupture of the aromatic structure at the carbon atoms adjacent to the ortho-diphenol group. Completely different metabolic pathways result from these two modes of ring cleavage, but both lead to the final end products, succinic acid and acetyl coenzyme A.

Many reports have appeared in the literature describing the ability of the azotobacters to metabolize phenolic acids when these are added to pure cultures in the laboratory. However, to our knowledge, no one has investigated this phenomenon in media other than those in which the exact chemical composition is known. We have assumed that the azotobacters must utilize benzoic substances in the dialysed soil medium of Gonzalez and Vela and have performed experiments to test this assumption.

The work reported here represents a search for the substrates utilized by *A. vinelandii* in the dialysed soil medium. Also, we sought to determine the relationship between these substrates and the growth and morphological variations of *A. vinelandii*.

CHAPTER II

MATERIALS AND METHODS

Organism

The organism used in this study was *Azotobacter vinelandii* ATCC 12837, obtained from the stock culture collection at North Texas State University. For initial propagation, the organism was streaked on a modified Burk's nitrogen-free agar medium (39) of the following composition in grams per liter of solution: K_2HPO_4, 0.64; KH_2PO_4, 0.16; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.2; $CaSO_4 \cdot 2H_2O$, 0.05; Na_2MoO_4, 0.001; $FeSO_4$, 0.003; agar (Difco Laboratories, Detroit, Michigan), 15; and glucose, 5. The organisms were periodically checked for purity by gram staining and by streaking on Burk's medium and tryptic soy agar medium.

Media and Cultures

A dialysed soil medium was made by placing 10 grams of finely ground garden soil in water-washed dialysis tubing (size #36, VWR Scientific, Inc., San Francisco, California) and placing it in 50 milliliters of distilled water contained in 250-milliliter Erlenmeyer flasks. These were sterilized by autoclaving at 121 C for 15 minutes. All cultures were incubated on a rotary shaker at 28 C and agitated at 90 revolutions per minute. Viable cell counts were obtained

by serial dilution and planting on a Burk's agar medium or on a dialysed soil agar medium by the drop method (22). The latter was prepared by removing the dialysis tubing and soil from the dialysed soil medium after autoclaving, adding 20 grams of pure agar per 1000 milliliters of liquid and autoclaving again before pouring into sterile petri-dishes.

Fractionation of the Dialysed Soil Medium

The dialysed soil medium was frationated as described in Fig. 1. The pH of all final fractions and residues was then adjusted to 7.2 with acid or base by using a pH meter (Horizon Ecology Co., Chicago, Illinois).

Extraction of Phenolic Acids

After the dialysis tubing containing the garden soil was removed from the medium, a measured volume of the dialysed soil medium was heated to 50 C under vacuum to remove the major portion of the water. The volume of the concentrated sample was measured and recorded. Then, the sample was chilled in an ice bath, acidified to a pH between 1 and 2 (indicator paper) by the careful addition of concentrated hydrochloric acid, and saturated with sodium chloride (approximately 26 grams per 100 milliliters). It was then extracted with three successive portions (half volumes) of ethyl acetate. The combined ethyl acetate extracts were washed by shaking with small portions of a 10 per cent (w/v) sodium bicarbonate solution (Fisher Scientific Co., Fair Lawn, New Jersey). The bicar-

bonate extracts were combined, chilled in an ice bath, acidified to pH 1 to 2 by the addition of concentrated hydrochloric acid, saturated with salt, and then extracted with successive small portions of ethyl acetate (20). The ethyl acetate extracts were pooled and stored in the freezer until needed for analysis.

Paper Chromatography

Two-dimensional paper chromatography was used to determine which phenolic acids were present in the dialysed soil medium and which were used by _A. vinelandii_ during the growth of this organism. Dialysed soil medium was inoculated with _A. vinelandii_ and the cultures were incubated at 28 C for five days. They were then centrifuged and passed through 0.2 micron filter membranes in order to remove all cells. Part of this was concentrated at 50 C under vacuum. Phenolic acids were then extracted from this portion of the spent medium and a portion spotted onto Whatman No. 1 filter paper. Two solvent systems (20) were used; isopropanol: 28 per cent aqueous ammonia: water (8:1:1) for the first dimension and propionic acid: benzene: water (2:2:1) for the second. The solvent fronts were allowed to advance 25 to 30 centimeters in each case and all chromatograms were examined under ultraviolet light in order to assay the fluorescent compounds. After this examination, the phenolic acids were visualized by spraying with diazotized p-nitroaniline.

Absorption Spectra

Absorption spectra were determined with a spectronic UV-210 recording spectrophotometer (Shimadzu Ltd., Kyoto, Japan) as previously described (38). The desired zones on two-dimensional paper chromatograms were cut out and extracted with ether. The ether extracts were evaporated to a small volume (approximately 1 milliliter) and again chromatographed in 2 per cent acetic acid by one-dimensional paper chromatography. These chromatograms were dried thoroughly at 100 C in the hot air oven and the spots were cut out and extracted with ethanol. These ethanol extracts were analyzed spectrophotometrically by scanning between 700 and 300 nanometers. Areas of the paper far from the spots selected were extracted with ethanol and used as the "blank" for the spectrophotometric studies.

Ashing of Dialysed Soil Medium

Fifty milliliters of sterile dialysed soil medium were agitated at 28 C for 48 hours. The dialysed soil medium was then condensed by heating at 90 C until dry and then heating at 1000 C for 2 hours. The ash was redisolved in 50 milliliters of water and adjusted to pH 7.2. This solution contains only the mineral substances in dialysed soil medium.

Quantitative Analysis of P-Hydroxybenzoic Acid

The amount of p-hydroxybenzoic acid in the dialysed soil medium was measured by comparison of the authentic compound

and the extracted p-hydroxybenzoic acid on thin layer chromatogram. The stationary phase of the thin layer chromatograms was made by one portion of silica gel and two portions of water. The solvent system (15) was benzene: methanol: acetic acid (45:8:4) and the phenolic acids were visualized by spraying with diazotized p-nitroaniline.

Procedure for Detecting the Effect of p-Hydroxybenzoic Acid on the Growth of *A. Vinelandii*

Azotobacter vinelandii was inoculated into 50 milliliters of dialysed soil medium. The growth was measured using drop method on plates of Burk's medium. The culture was then centrifuged and then filtered through 0.2 micron membranes, and reinoculated with *A. vinelandii*. If there was growth in this "spent" filtrate, the culture was again filtered and reinoculated. Finally, p-hydroxybenzoic acid was added to the last cell-free filtrate and the effect of this compound on the growth of *A. vinelandii* was detected.

CHAPTER III

RESULTS

The dialysed soil medium was separated into various fractions by the procedures summarized in Fig. 1. The growth of *Azotobacter vinelandii* in the various fractions was determined by viable cell count on Burk's agar medium. The results are shown in Fig. 2. No growth and no morphological variation were observed in the ether or pyridine fractions.

In addition, the growth response of *A. vinelandii* to the dialysed soil medium ash residue was investigated. The cells survived in these cultures as indicated by cell counts which showed that the cell number remained constant. Since no growth was obtained in either the ether fraction or the pyridine fraction nor was any seen in the ash residue medium, it can be concluded that the principle substances which support growth of *A. vinelandii* in the dialysed soil medium were non-lipid, non-carbonhydrate, organic materials.

The molecular weights of these substances in dialysed soil medium which support the growth of *A. vinelandii* was determined by using dialysis tubing of different pore size. The range explored was between 6,000 and 20,000 daltons molecular weight. Our results showed that the organism grew equally well in media prepared with dialysis tubing in this entire range. Thus, we could conclude that the molecular

weights of the substances which supported the growth of A. vinelandii in soil were less than 6,000 daltons.

According to the above information, and through previous knowledge of the fact that aromatic acids serve as the sole source of carbon and energy for A. vinelandii, phenolic acids were suspected of being the principle substances used by A. vinelandii for its growth in the dialysed soil medium. As a result, we subsequently investigated the role of phenolic acids in this medium. Figure 3 shows the results obtained by chromatographing the phenolic acids extracted from dialysed soil medium before and after growing A. vinelandii during a period of 120 hours. The phenolic acids numbered 1 to 5 in both dialysed soil media had the same R_F values and gave the same colors when these were developed with diazotized p-nitroaniline. However, the one numbered 6 appeared in the dialysed soil medium only before the growth of A. vinelandii but not after the growth of this organism. This suggests that the phenolic acid numbered 6 was consumed during the growth of A. vinelandii.

The identity of the phenolic acid associated with the growth of A. vinelandii was provided by its R_F value, the color developed with diazotized p-nitroaniline, and its fluorescence under ultraviolet light (Table I). The phenolic acid numbered 6 was red when exposed to p-nitroaniline, and gave a dark blue fluorescence under short wavelength ultraviolet light. Its R_F value in the first solvent system

[isopropanol: 28 per cent aqueous ammonia: water (8:1:1)] was 0.28. In the second solvent system [benzene: propionic acid: water (2:2:1)] it was 0.55. From these observations, it was assumed that the phenolic acid numbered 6 was p-hydroxybenzoic acid.

Positive identification of this phenolic acid was done by infrared and light spectrophotometry. The close correlation between authentic p-hydroxybenzoic acid (Aldrich Co., Milwaukee, Wisconsin) and the phenolic acid numbered 6 is shown in Fig. 4. Therefore, it was concluded that p-hydroxybenzoic acid was associated with the growth and cell morphology of _A. vinelandii_ in the dialysed soil medium.

Quantitative analysis of p-hydroxybenzoic acid in the dialysed soil medium was done by using thin-layer chromatography. Authentic p-hydroxybenzoic acid (0.1 gram/5 milliliter water) and p-hydroxybenzoic acid re-extracted from paper chromatograms were compared on thin-layer chromatograms. Using this method, it was possible to compare the colors developed with diazotized p-nitroaniline as well as the R_F values and spot sizes of authentic p-hydroxybenzoic acid and p-hydroxybenzoic acid obtained from the soil medium. By these criteria, it was shown that the amount of p-hydroxybenzoic acid in the dialysed soil medium ranged in concentration from 14 to 21 micrograms per gram of the soils tested.

In order to prove that p-hydroxybenzoic acid was the substance responsible for the growth of _A. vinelandii_ in soil

medium, the authentic compound was added to a spent, dialysed soil medium in which *A. vinelandii* had grown to stationary phase. It was assumed that the limiting nutrient had been exhausted in this medium. The preparation of this medium was done by repeatedly removing *A. vinelandii* from the dialysed soil medium by centrifugation and filtration and then re-inoculating the filtrate until no growth was possible. Then, the last cell-free filtrate was collected as the test medium. The viable cell concentration in the last cell-free filtrate to which p-hydroxybenzoic acid had been added was much higher than that in the dialysed soil medium (Fig. 5). Also, the *A. vinelandii* culture in the medium with added p-hydroxybenzoic acid turned dark brown and turbid. This result indicated that p-hydroxybenzoic acid was used as the sole carbon and energy source by *A. vinelandii*.

Whether or not p-hydroxybenzoic acid was associated with the morphological variation of *A. vinelandii* was investigated in further experimentation. Authentic p-hydroxybenzoic acid and the phenolic acids extracted from the dialysed soil medium were added to the ash residue medium and to the aqueous residue remaining after the phenolic acids had been removed from dialysed soil medium. Growth and cell morphology was examined in these cultures. No morphological changes were seen. However, when media containing naturally occurring phenolic acids or authentic p-hydroxybenzoic acid were used, the minute bodies were formed which were similar to the filtrable forms

reported by Gonzalez and Vela (14) in every aspect except that no growth of minute bodies was detected in the medium (Table II). On the other hand, no growth and formation of minute bodies was observed in either the ash residue medium or the aqueous residue medium.

Phase contrast microscopy of cells grown in the ash residue medium to which p-hydroxybenzoic acid had been added is shown in Fig. 6. The figure shows the production and release of minute bodies from vegetative cells.

The ash residue medium containing various concentrations of p-hydroxybenzoic acid were prepared as shown in Table III. Growth of _A. vinelandii_ and formation of minute bodies were observed in those media containing 25, 50, and 75 milligrams of p-hydroxybenzoic acid. However, there was no growth and formation of minute bodies in the media containing more than 100 milligrams or less than 10 milligrams of this compound. The results (Table III) indicate that the effect of p-hydroxybenzoic acid on _A. vinelandii_ is critically dependent on the concentration employed.

The phenolic acid numbered 2 shown on paper chromatograms was thought to be ferulic acid. When authentic ferulic acid was added to the ash residue medium in the concentrations indicated in Table IV, no growth and cell morphological variation, nor formation of minute bodies, was observed. Since only the existence of p-hydroxybenzoic acid in the dialysed soil medium supports the growth of azotobacter and induces the

formation of minute bodies, it can be suggested that the presence of p-hydroxybenzoic acid in the medium accounts for the growth and the structural changes in *A. vinelandii*.

To find out whether or not *A. vinelandii* can use other phenolic acids shown on two-dimensional paper chromatograms, we removed this microorganism from the dialysed soil medium when the cells had grown to stationary phase. Then part of the cell-free extract was kept for analyzing phenolic acids on two-dimensional paper chromatography, and the rest of the cell-free extract was reinoculated with *A. vinelandii*. The procedures were repeated twice, and the phenolic acids extracted from each cell-free extract were then compared. Our results showed that other phenolic acids always remained in the cell-free dialysed soil extract even when the p-hydroxybenzoic acid had run out. This suggests that p-hydroxybenzoic acid is the only phenolic acid associated with the growth and structural change of *A. vinelandii* in the dialysed soil medium.

CHAPTER IV

DISCUSSION

The use of soil media in the study of azotobacters is not new (28). It has long been known that *Azotobacter vinelandii* can use some aromatic compounds, such as benzoic acid in soil (28). The results reported here show that p-hydroxybenzoic acid can be used rapidly and efficiently as a sole source of carbon and energy by *A. vinelandii* (Fig. 5). The ash residue from the incinerated dialysed soil medium to which was added either p-hydroxybenzoic acid or naturally occurring phenolic acids supported the growth and formation of the minute bodies (Table II). The results of these tests give proof that p-hydroxybenzoic acid was not only the growth substrate, but was also the minute body-inducing component of phenolic acids. When ferulic acid (one of the phenolic acids shown in Table I) was added to the ash residue medium in the concentration indicated in Table IV, no minute bodies were produced. The effect of p-hydroxybenzoic acid on the formation of minute bodies of *A. vinelandii* is clear (Fig. 6). Hence, the utilization of p-hydroxybenzoic acid in a dialysed soil medium indicates that this compound probably serves as a nutrient and a minute body-inducing component in the natural environment. This conclusion confirms Winogradksy's assumption that *Azotobacter* are forced to utilize the

fermentation or decomposition products of plant residues in soil. The conclusion also favors the assumption that in soil, and under certain conditions, A. vinelandii can compete successfully with other bacteria for various carbon sources.

Since our results showed that A. vinelandii was able to grow on p-hydroxybenzoic acid, using it as a sole source of carbon and energy, the metabolic pathways used by A. vinelandii for the oxidation of p-hydroxybenzoic acid becomes significant. The pathway by which A. vinelandii metabolizes p-hydroxybenaoic acid in modified Burk's medium has been investigated (14). It can be assumed that the organism metabolizes p-hydroxybenzoic acid in the dialysed soil medium similarly, namely by conversion to protocatechuate, through the ortho-cleavage pathway, which is the oxidative fission of the bond between carbon atoms bearing hydroxyl groups (8). The products of this β-ketoadipate pathway are acetyl coenzyme A and succinic acid. Furthermore, Lineweaver (18) found that succinic acid is oxidized entirely (95 to 100 per cent) to carbon dioxide and water by Azotobacter.

According to our data, the amount of p-hydroxybenzoic acid in the dialysed soil medium was very low. The concentration ranged from 14 to 21 micrograms per gram (dry-weight) of soil. However, this concentration in the dialysed soil medium is considered to be high enough to stimulate the formation of the minute bodies of A. vinelandii. On the other hand, when p-hydroxybenzoic acid was added to the ash residue from the incinerated dialysed soil medium, the results (Table III)

showed the need for a higher and more critical concentration than found in the soils of this compound to support the growth and production of the minute bodies. High concentrations of this compound (more than 100 milligrams per 50 milliliters of ash residue medium) would inhibit the growth of *A. vinelandii* and limit the formation of minute bodies, while too low a concentration (1 milligram per 50 milliliters of ash residue medium) would not support detectable growth nor formation of minute bodies. The difference in amount of p-hydroxybenzoic acid required for the formation of minute bodies in the dialysed soil medium and in the ash residue medium may be due to the greater complexity of nutrients in the dialysed soil medium than in the ash residue medium.

Our results, shown by two-dimensional paper chromatography, indicate that *A. vinelandii* can utilize p-hydroxybenzoic acid but not other phenolic acids as growth substrates. Therefore, it is reasonable to think that the growth of *A. vinelandii* with p-hydroxybenzoic acid induces a high rate of oxidation while growth with the other phenolic acids shown on paper chromatograms do not support significant oxidation (14). In addition to this, other possibilities might be that those phenolic acids were cooxidized. As Foster (11) suggests, "the inability to grow at the expense of a particular hydrocarbon is not a consequence only of an organism's inability to attack the substrate; obviously, failure to grow may be due to its inability to assimilate the oxidation products." Furthermore, the

enzymes required for the metabolisms of other phenolic acids may not be induced or may be present at high values in p-hydroxybenzoic acid-grown *A. vinelandii* (14). Therefore, it appears likely that this organism employs only p-hydroxybenzoic acid as a growth substance in the natural environment.

Our experiments showed p-hydroxybenzoic acid to have no effect on the morphological variation of *A. vinelandii*. However, this substance was associated with the formation and release of minute bodies. These minute bodies were similar to the filtrable forms reported by Gonzalez and Vela (16) in every aspect, except that no cellular function (growth) could be detected.

Alexander (2) has suggested that 90 to 99 per cent of the soil bacteria have nutritional patterns appreciably more complex than those known for other organisms. Since *Azotobacter* is a normal constituent of the soil microflora, visible growth may vary according to the nutritional characteristics of the medium employed. Therefore, it is possible that the minute body can be the elusive filtrable form of azotobacters. To confirm this possibility, further investigation of functional and structural properties of minute bodies is required.

TABLE I

MAJOR PHENOLIC ACIDS EXTRACTED FROM DIALYSED SOIL MEDIUM RESOLVED BY TWO DIMENSIONAL PAPER CHROMATOGRAPHY

Solvent systems: INW, isopropyl alcohol: aqueous ammonia: water, 8:1:1; PBW, propionic acid: benzene: water, 2:2:1 (organic phase)
Reagent DzPNA: diazotized p-nitroaniline.
Abbreviations: lt, light; dk, dark; R, red; Bl, blue; P, purple; G, gray; UV, ultraviolet

Compound No.	Identity and Color with DzPNA	R_F Value		Fluorescent Color under UV Light
		INW	PBW	
1	Vanillic acid (Bl)	0.23	0.73	
2	Ferulic acid (Bl-G)	0.25	0.77	Bl
3	p-Hydroxycinnamic acid (lt-Bl-G)	0.31	0.60	lt-Bl
4	m-Hydroxybenzoic acid (R)	0.39	0.53	dk-Bl
5	Dihydroferulic acid (lt-R)	0.44	0.71	lt-Bl
6	p-Hydroxybenzoic acid (R)	0.28	0.55	dk-Bl

TABLE II

THE GROWTH OF _A. VINELANDII_ AND THE FORMATION OF MINUTE BODIES IN DIFFERENT GROWTH MEDIA

Growth Medium	Growth on Agar Medium	Formation of Minute Bodies
DSM[a]	+	+
DSM without phenolic acids[b]	-	-
DMS without phenolic acids + phenolic acids extract[c]	+	+
Ash residue from DSM	-	-
Ash residue from DSM + phenolic acids	+	+
Ash residue from DSM + p-hydroxybenzoic acid	+	+
DSM without phenolic acid + p-hydroxybenzoic acid	+	+

[a]DSM = dialysed soil medium

[b]This is the aqueous fraction remaining after the phenolic acid had been removed from dialysed soil medium.

[c]as in b above plus phenolic acids extracted from dialysed soil medium.

TABLE III

THE EFFECT OF p-HYDROXYBENZOIC ACID ON THE GROWTH AND FORMATION OF MINUTE BODIES

p-Hydroxybenzoic Acid (mg/50 ml of ash residue medium)	Effect
1	No growth detected; no minute bodies observed.
10	No growth detected; few minute bodies observed.
25	Some growth; some minute bodies observed.
50	Growth observed; culture turbid; large amount of minute bodies observed.
75	Same as the effect in 50 mg.
100	Growth was greatly inhibited; some minute bodies observed.
150	Growth was inhibited; culture never turbid; no minute bodies observed.

TABLE IV

THE EFFECT OF FERULIC ACID ON THE GROWTH AND FORMATION OF MINUTE BODIES

Ferulic Acid (mg/50 ml ash residue medium)	Effect
1	No growth detected.
10	No growth detected.
25	No growth detected.
50	No growth detected.
75	Slight turbidity; most vegetative cells had irregular shapes.
100	No growth detected.
150	No growth detected.

FIGURE 1

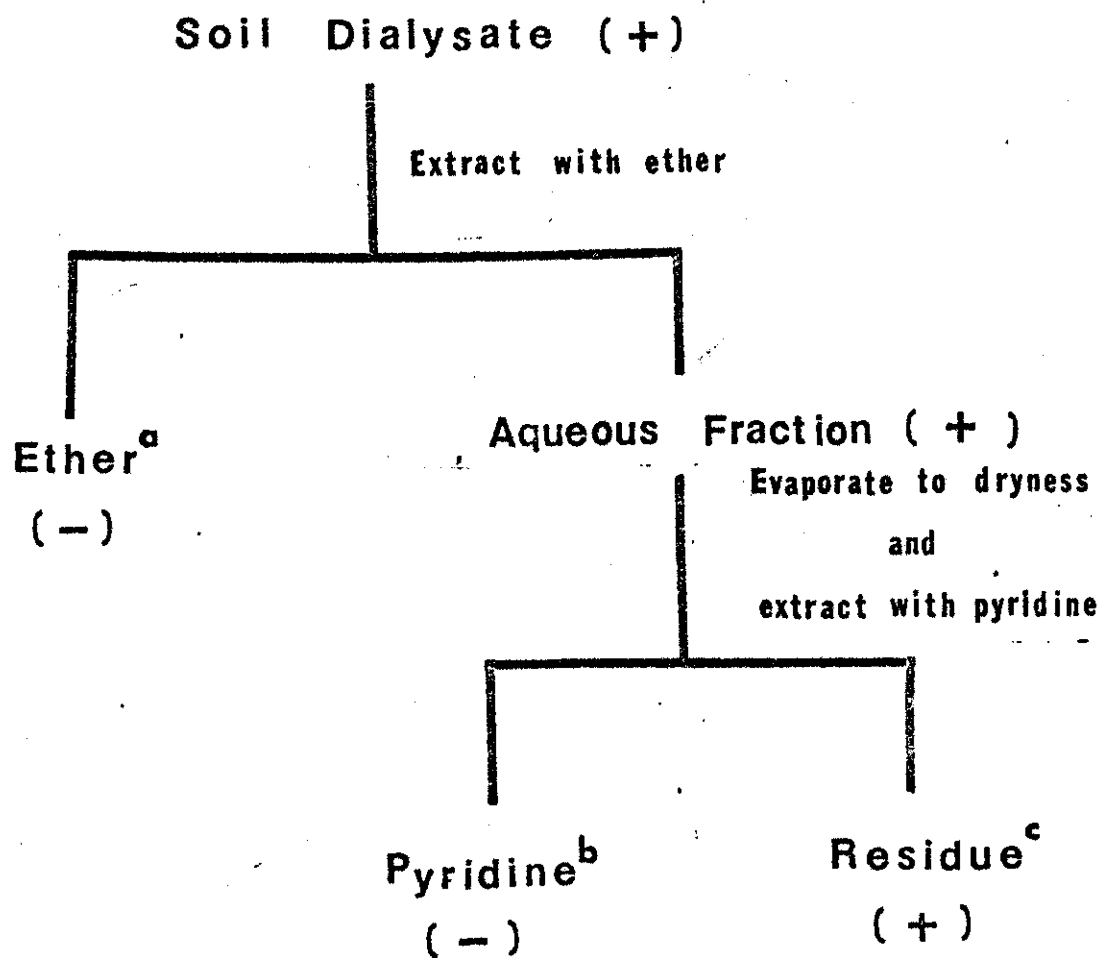
FRACTIONATION OF THE DIALYSED SOIL MEDIUM

Figures in parenthesis indicate whether or not there was growth in the various fractions. Each fraction was inoculated with the same number of cells of *A. vinelandii* and inoculated under the same conditions.

[a]Ether evaporated, water added and evaporated, water added to proportionate volume, pH adjusted and resulting solution tested for ability to support growth.

[b]Pyridine evaporated, water added and evaporated, water added to proportionate volume, pH adjusted and resulting solution tested for ability to support growth.

[c]Liquid evaporated, water added and evaporated, ether added to proportionate volume, pH adjusted and resulting solution tested for ability to support growth.

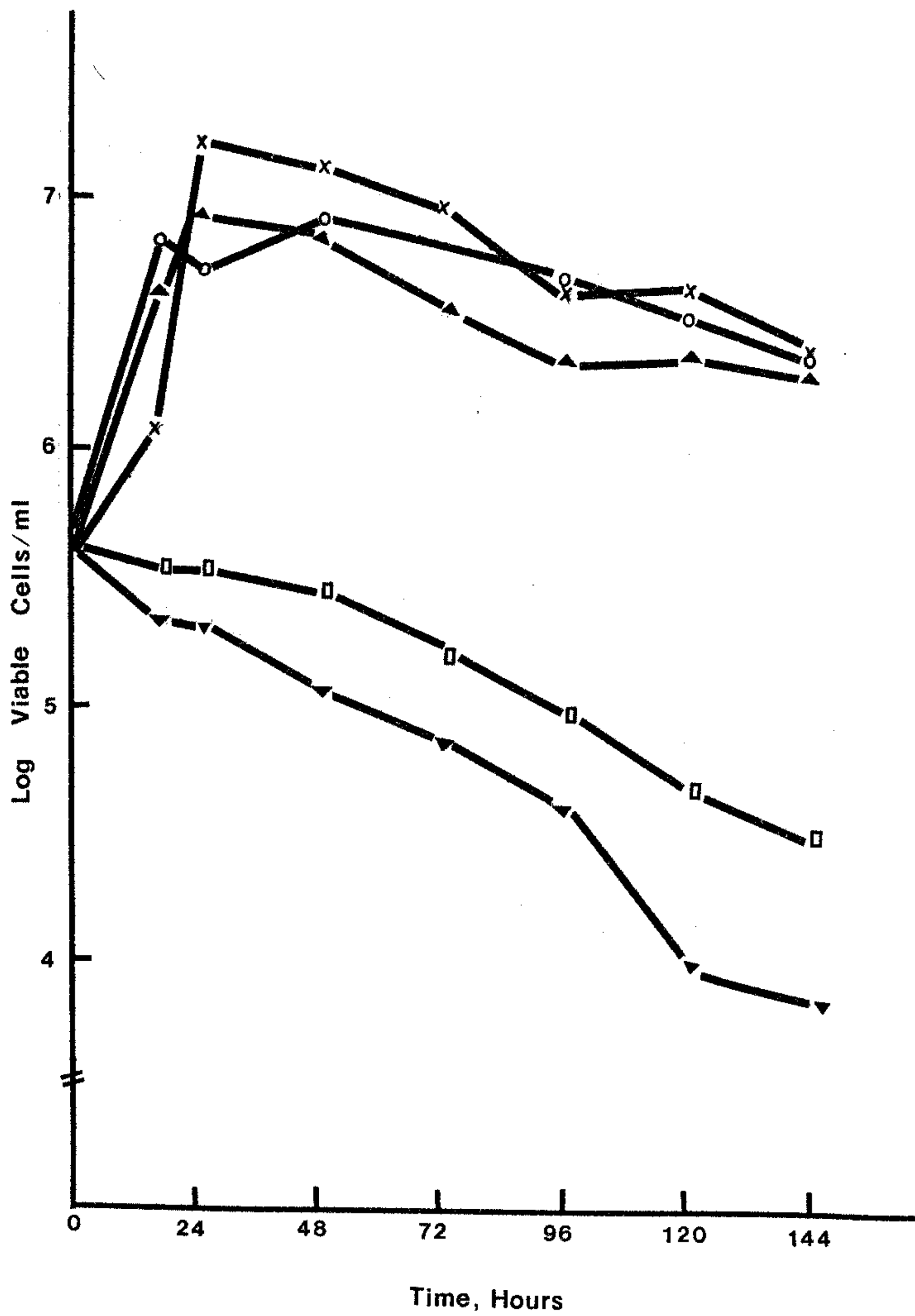


Soil Dialysate (+)
Extract with ether
Ether[a]
(–)
Aqueous Fraction (+)
Evaporate to dryness
and
extract with pyridine
Pyridine[b]
(–)
Residue[c]
(+)

FIGURE 2

EFFECT OF THE VARIOUS FRACTIONS OF DIALYSED SOIL MEDIUM ON THE GROWTH OF _A. VINELANDII_

The numbers of viable cells were determined on Burk's medium. The following symbols indicate the growth in different fractions: x, aqueous fraction remaining after the ether extract had been removed from dialysed soil medium; o, dialysed soil medium (control); ▲, residue after extracting with pyridine; ▯, ether extract; ▼, pyridine extract.

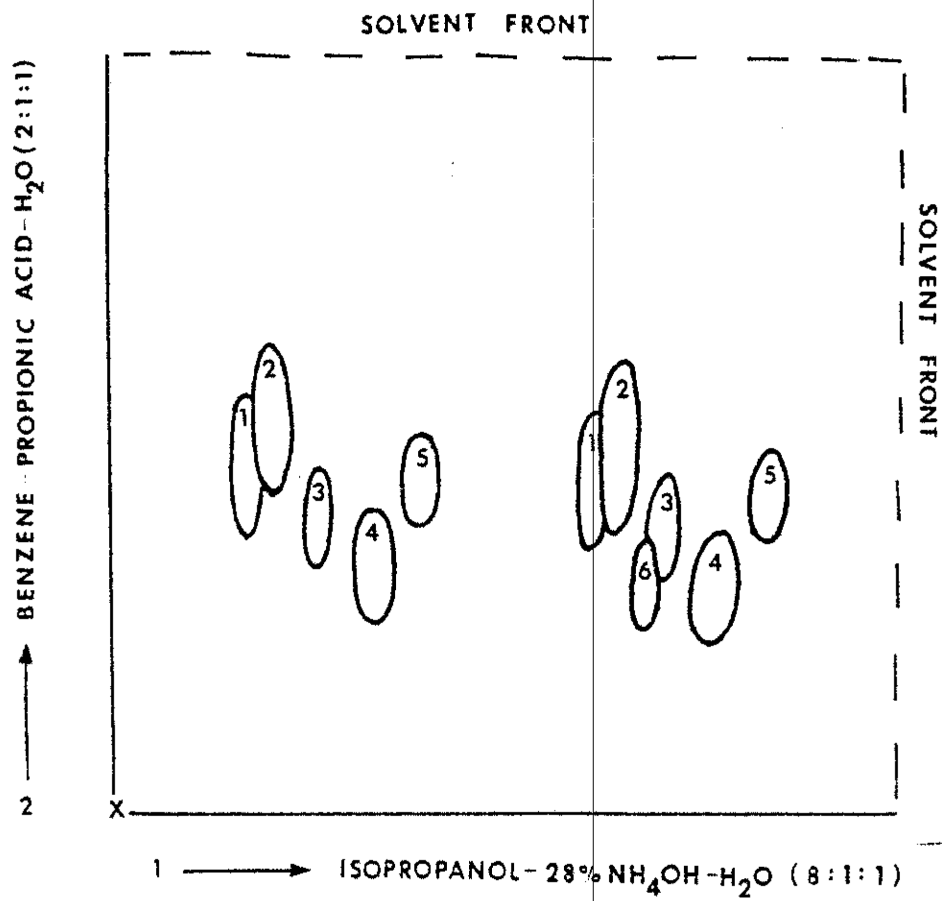


7
6
5
4
Log Viable Cells/ml
0
24
48
72
96
120
144
Time, Hours

FIGURE 3

MAJOR PHENOLIC ACIDS IN THE DIALYSED SOIL MEDIUM BEFORE (RIGHT) AND AFTER (LEFT) GROWTH OF *A. VINELANDII*

The numbers refer to the compounds listed in Table I.



SOLVENT FRONT
SOLVENT FRONT
BENZENE - PROPIONIC ACID - H_2O (2:1:1)
2
X
1
ISOPROPANOL - 28% NH_4OH - H_2O (8:1:1)
1
2
3
4
5
6

FIGURE 4

ABSORPTION SPECTRA

Authentic p-hydroxybenzoic acid (0.1 g/5 ml water), and the No. 6 phenolic acid obtained from paper chromatograms (sample) were chromatographed in 2% acetic acid and extracted with ethanol. These ethanol extracts were analyzed spectrophotometrically.

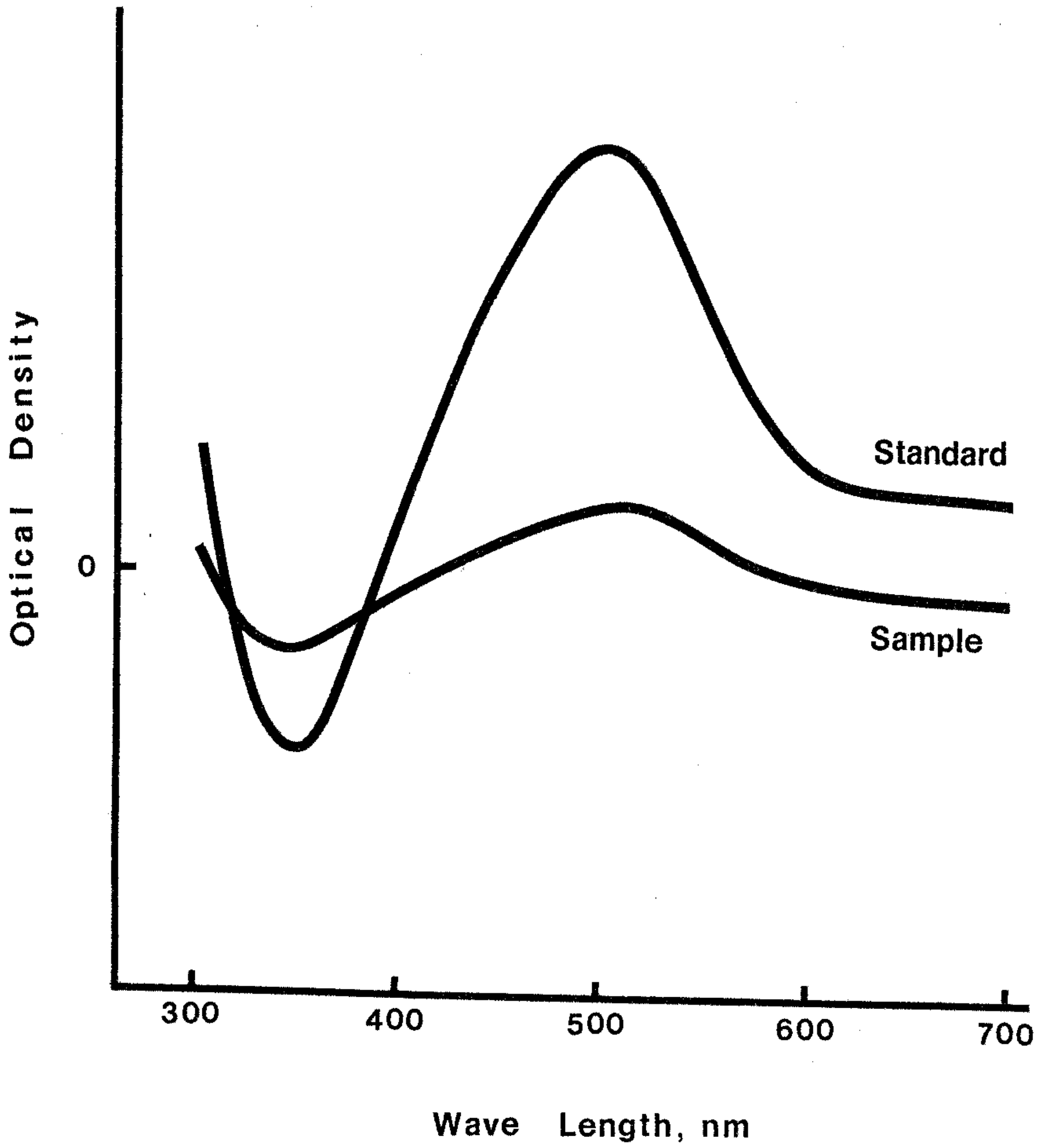

Optical Density
0
Standard
Sample
300
400
500
600
700
Wave Length, nm

FIGURE 5

THE EFFECT OF p-HYDROXYBENZOIC ACID ADDED TO SPENT MEDIUM ON THE GROWTH OF *A. VINELANDII*

The following symbols indicate the growth of *A. vinelandii* in: o, dialysed soil medium; x, first "spent" filtrate; ▫, second "spent" filtrate; ▾, second "spent" filtrate with p-hydroxybenzoic acid added.

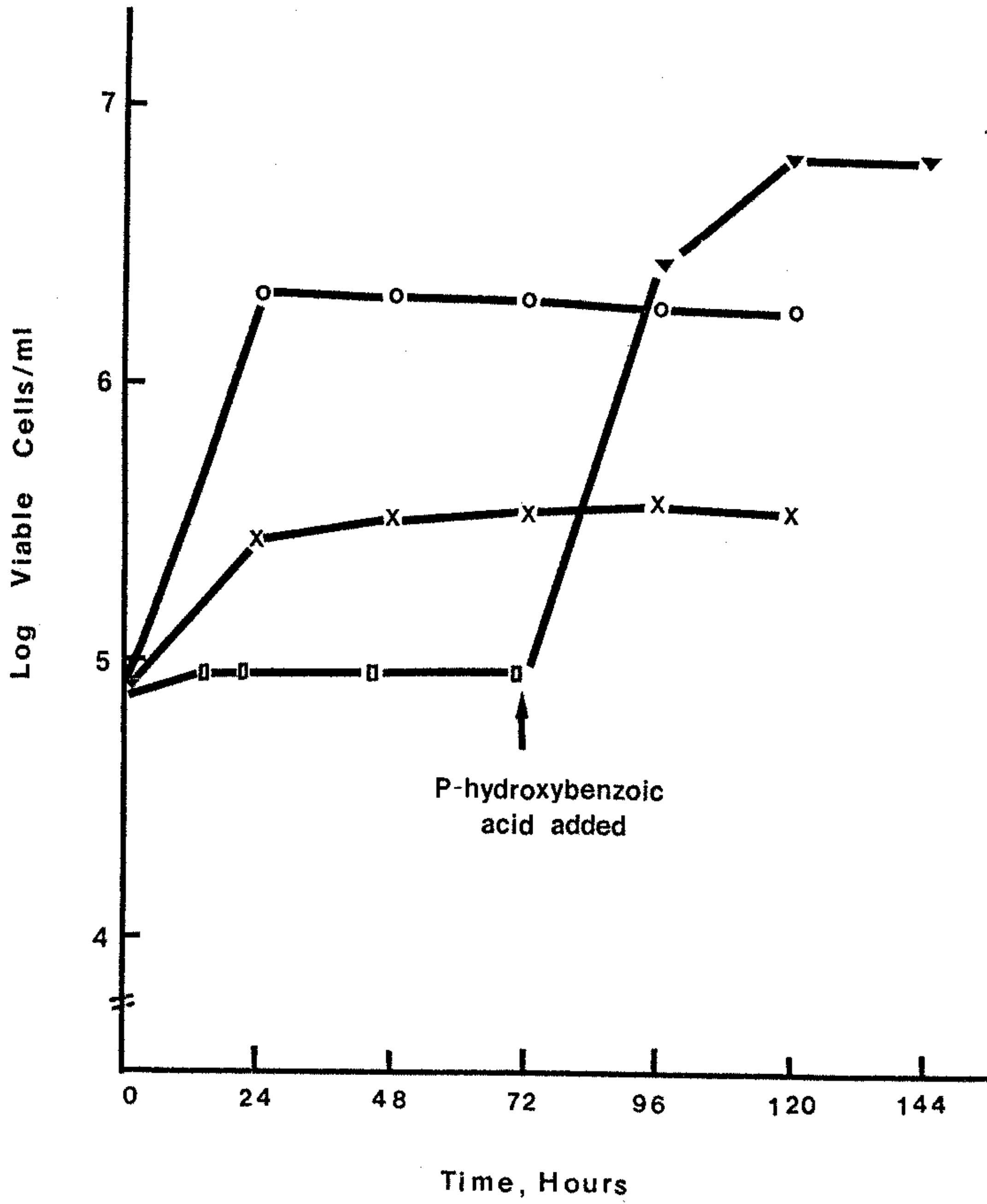
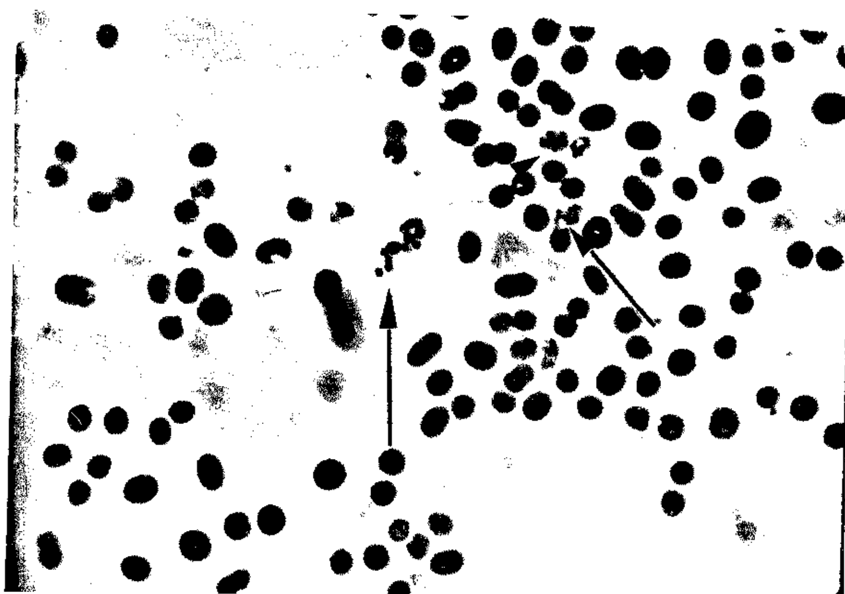

7
6
5
4
Log Viable Cells/ml
P-hydroxybenzoic
acid added
0
24
48
72
96
120
144
Time, Hours

FIGURE 6

FORMATION AND RELEASE OF MINUTE BODIES FROM VEGETATIVE CELLS OF *A. VINELANDII*

These cells were grown in the ash residue medium to which p-hydroxybenzoic acid had been added. The arrows show the minute bodies released from vegetative cells.



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