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Pseudomonas as Causative Agents of Fluorescence in Anguispira kochi (Pfeiffer) Mucus

John Michael Baum
Eastern Illinois University

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Pseudomonas as Causative Agents of Fluorescence in *Anguispira kochi* (Pfeiffer) Mucus

(TITLE)

BY

John Michael Baum

THESIS

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in *Anguispira kochi* (Pfeiffer) Mucus

Presented by

John Michael Baum

a candidate for the degree of Master of Science

and hereby certify that in their opinion it is acceptable.

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I dedicate this thesis to my wife, Roberta Sue, whose understanding and unselfishness has made the completion of my graduate work possible; and to my parents, Mr. and Mrs. John A. Baum, for giving me what they never had — an opportunity to obtain a college education. I hope this thesis can in some way repay these people for their sacrifices made on my behalf.

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ABSTRACT

The mucus from land snail specimens of *Anguispira kochi* collected from Coles and Edgar counties, Illinois, emits a blue fluorescence when exposed to ultra-violet light. *Pseudomonas*, a genus of bacteria which produces fluorescent pigments, has been isolated from the mucus of these snails. The object of the study was to compare the fluorescence of the mucus from individuals of *A. kochi* with the fluorescence of the pigments produced on a synthetic medium by pseudomonads isolated from the mucus of these snails. An effort was made to establish that *Pseudomonas* is the causative agent of fluorescence in the mucus of *A. kochi* by comparing the physical character and the spectrophotometric records of the two substances. The pigment produced by these bacteria and the fluorescent substance in the mucus were identical in regard to dialysis, withstanding high temperatures, and solubility in the 18 solvents used. Differences in fluorescent color were explained experimentally. The absorption curve of the bacterial pigment (maxima at about 265 and 400 nm) was similar to that of the *A. kochi* mucus (maxima at about 265 and 385 nm). The results from the study indicate that *Pseudomonas* is the causative agent of the fluorescence in the mucus of *A. kochi*. It is plausible that the mucus of these snails contains the appropriate nutritional requirements which allow the bacterial to produce diffusible pigments that fluoresce under ultra-violet light.

INTRODUCTION

The mucus from living specimens of three endodontid species and the alcohol used to preserve specimens of these and other species of the family has been found to emit a blue fluorescence when exposed to ultra-violet light (Rawls and Yates, in press). A survey of the literature has revealed no prior work directly related to investigating the mechanism responsible for fluorescence of this nature in gastropods. Investigation has shown Pseudomonas, a genus of bacteria capable of producing fluorescent pigments (Breed, Murray, and Smith, 1957), to be present in the mucus of Anguispira alternata, Anguispira kochi, and a non-fluorescent species of the Polygyridae, Mesodon thyroidus. Ward et al. (1967) reported the use of ultra-violet light for the detection of fluorescent pigment producing pseudomonads in burn wound infections of man, thus illustrating that fluorescent pigment production can occur in living hosts. Therefore, it is plausible that the mucus of certain snails contains the appropriate nutritional requirements which allow these bacteria to produce diffusible pigments that fluoresce under ultra-violet light.

The object of this study was to compare the fluorescence of the mucus from individuals of A. kochi with the fluorescence of the pigments produced on a synthetic medium by pseudomonads isolated from the mucus of these snails. An effort was made to establish that Pseudomonas is indeed the

causative agent of fluorescence in the mucus of A. kochi and perhaps in the mucus of other snails exhibiting the same phenomenon. This was achieved by comparing the physical character and spectrophotometric records of the two substances.

Michejda (1958), Michejda and Urbanski (1958), and Wright (1959, 1964) described chromatographic studies using the ninhydrin and ultra-violet patterns of snail mucus for taxonomic purposes. Michejda (1958) found the ninhydrin and ultra-violet patterns of the mucus similar to those of the foot. Wright (1959) discovered that the substances producing the fluorescent pattern are not derived from the snail tissues proper but from body surface secretions; and that, before running chromatograms of three species of Limnaea, two mucus spots fluoresced blue and one fluoresced yellow, the concentration of the mucus being responsible for the difference. McGee (1964) extracted from the eggs of Tegula funebralis a crude green pigment which partitioned into zeaxanthin, lutein, alpha-carotine, and an unknown green pigment with an attached protein. The last pigment had absorption maxima at 640 and 273 nanometers in the oxidized state and 273 nm in the reduced state, with 273 nm being near the peak of coenzyme Q, a quinone found in aerobic bacteria and invertebrates.

From the foot of Monodonta turbinata, Bannister, Bannister, and Micallef (1968) isolated a green pigment which had absorption maxima at 680 and 370 nm, and inflections at 440, 330, and 270 nm in methanolic hydrochloric acid. Rawls and Yates (in press) reported that when the mucus

of A. alternata, A. kochi, and Discus patulus is exposed to ultra-violet light, a blue fouorescence is emitted. They hypothesized that this characteristic may be common to the entire Endodontidae. Rawls and Baum (in press) also reported a blue-green fluorescence in live and preserved specimens of Mesodon clausus, the only species of the Polygyridae known to exhibit fluorescence.

Wright (1959) reported that the biochemistry of mucoid substances in snails has not been studied to any great extent; his own work indicated the presence of an amino sugar in the mucus of Limnaea palustris. He stated that other workers have extracted glucosamine, mannose, and galacturonic acid from the mucin of snail mucus and galactogen from the albumen glands and egg capsules of Helix pomatia. Using the mucus of the giant slug, Ariolimax columbianus, Taylor (1963) found protein, polysaccharide and inorganic ions, with glucosamine and fucose making 40 per cent of the macromolecular substance.

Pigment production of Pseudomonas species has been investigated. Breed et al. (1957) described the bacteria as straight, soil or water inhabiting rods which may produce diffusible pigments which vary from fluorescent green through blue, violet, lilac, rose, and yellow. Elliot (1958) demonstrated that riboflavin was absent as a free component of the bacterial pigment complex. Chodat and Gouda (1961) found several genetically linked pigmented substances possessing a common chemical skeleton in culture

filtrates of Pseudomonas fluorescens. De Ley (1964) reported that Pseudomonas produces phenazine pigments including pyocyanin, chlororaphin and oxychlororaphin, phenazine alpha-carboxylic acid, iodinin, indigoidine, brown pigments, and fluorescent pigments whose biological function is not clearly known but appear to play a role in electron transfer. In regard to fluorescent pigments, he reported that some workers believed a pterine and a flavine were responsible for a blue and yellow fluorescence respectively, while another worker suggested phenazine-1-ol as the fluorescent compound. Newkirk and Hulcher (1969) made an amino acid analysis which showed serine, threonine, glutamic acid, and lysine to comprise 26 per cent of the fluorescent molecular mass of the pigment.

The fluorescent water-soluble pigment produced by Pseudomonas has been called a variety of names by different authors. Its physical and chemical nature as well as its fractions have only recently begun to be elucidated. Jordan (1899) and Sullivan (1905) referred to the substance simply as "the fluorescent pigment." Burton, Campbell, and Eagles (1948), King, Campbell, and Eagles (1948), Totter and Moseley (1953), King, Ward, and Raney (1954), and Lenhoff (1963) all used the term "fluorescin." Elliot (1958) felt "pyoverdine" should be used because it specifically identified the bacterial pigment complex. Osawa et al. (1963) identified two substances, "Fluorescence I" and "Fluorescence II," from the bacterial pigment. Fluorescence I was the main constituent of the

fluorescent pigment produced by the strains studied (Osawa et al., 1964). Later, Osawa et al. (1965) crystallized a non-fluorescent "C-substance" from Fluorescence II. Newkirk and Hulcher (1969) isolated "Compound I" from the pigment produced by P. mildenbergii.

There is a variance of opinion among workers concerning the substances necessary for fluorescent pigment production by Pseudomonas species. Using strains of P. fluorescens, Jordan (1899) devised a medium containing asparagine with sulfur and phosphorus of which the bases were considered unimportant. Sullivan (1905) found that P. aeruginosa, grown on a medium of asparagine, magnesium sulfate, dipotassium hydrogen phosphate, and glycerine proved to produce pyocyanin and a blue-green fluorescent pigment. He discovered that the carbon contained in sugars and glycerine added bacterial pigment formation and that sulfates, a high phosphate content, and alkalinity were necessary for the development of blue-green fluorescent pigments. Georgia and Poe (1931) used P. fluorescens and found that magnesium, sulfate, and phosphates are necessary inorganic constituents for fluorescent pigment production. Georgia and Poe (1932), comparing peptones as suitable agents of support for the production of fluorescent pigments, found considerable variation in composition and discovered that some peptones lacked the necessary constituents required for pigment production. Burton et al. (1948) noted that at high concentrations of phosphate, there was a decrease in the formation of pyocyanin and a proportional increase of pyoverdine. Using P. aeruginosa, King et al. (1948)

concluded that the magnesium ion was essential for pyoverdine production; that the formation of pyoverdine depended upon the concentrations of dipotassium hydrogen phosphate and iron; that although the sulfate ion was not essential for growth, its presence was required for pyoverdine production; and that the production of pyoverdine could be maintained at a high level over a wide range of magnesium, phosphate, and sulfate concentrations. In their development of Medium A for pyocyanin enhancement and Medium B for pyoverdine enhancement, King et al. (1954) found asparagine to be unnecessary and a greater production of pyoverdine to occur when the phosphate concentration was increased. Pigment production was increased by the addition of glucose to the medium and influenced by the type of peptone used (Kluyver, 1956). A glutamic acid medium composed of sodium glutamate, potassium dihydrogen phosphate, magnesium sulfate, and sodium chloride was developed by Osawa's (1963) co-workers who felt this medium gave more distinct color tones than did the A and B media developed by King et al. (1954).

Pigment production appears to be influenced by the concentration of the medium. Simpler modifications of media promoted pigment production (Jordan, 1899), and even if the necessary constituents are present, fluorescent pigment production will not occur if the organisms are grown on a highly concentrated medium (Georgia and Poe, 1932).

The presence of acid appeared to inhibit the production of pigment by affecting the metabolic activities of the bacteria rather than interfering

with the pigment proper, for the pigment is not destroyed by the acid once it is formed (Jordan, 1899). Slight alkaline or acidic changes of the media had little effect on pigment production but great variations in either direction resulted in a colorless growth (Sullivan, 1905). The optimum pH for fluorescent pigment production appeared to be in the neutral range. Georgia and Poe (1931) used a pH range of 6.8 to 7.2 for the media in their research. In another study, Georgia and Poe (1932) found a pH between 6.9 and 7.1 gave optimum fluorescent pigment production. King et al. (1954), found a final pH of 7.0 and 8.0 to be satisfactory for the determination of pyoverdine on Medium B substantiating Osawa et al. (1963) who used a pH of 7.2 in their study.

Iron appears to be of great importance in the formation of pyoverdine. Burton et al. (1948) noted maximum bacterial growth and an appreciable production of pyoverdine in media free of ferrous sulfate. King et al. (1948) stressed that the amount of iron added to media must be minimal. Iron and pyoverdine combine to form part of a respiratory cytochrome; in the absence of iron, the pigment is useless and is excreted into the medium (Knight, 1951). Totter and Moseley (1953) found an inverse relationship to exist between available iron and fluorescent pigment production. They thought pyoverdine to be a substitute for iron-containing cytochromes when a forerunner of the cytochromes is absent. Garibaldi and Neilands (1956) found that Pseudomonas has given no visual evidences of the formation of iron-binding agents. Lenhoff (1963) observed that when P. fluorescens was grown at high oxygen

tension (constant aeration) and in the absence of iron, large amounts of pyoverdine were produced, while less than 0.1 per cent protein was attributed to cytochrome c; at low oxygen tension (no aeration) with iron, cytochrome c composed 3.5 per cent of soluble cellular protein with minimal pyoverdine production. Kraft and Ayres (1964) counted over one million cells per milliliter before the pigment became apparent and concluded that all of the iron in the medium had to be utilized by the organisms before pyoverdine would be produced. Wasserman (1964) found the ferrous ion to be utilized in pigment formation and believed that the bacteria reduce the iron from the ferric state to the ferrous state when it serves as the chromogen for compounds formed in metabolism. Garibaldi (1967) discovered that a totally iron-free medium will not support pseudomonad growth, for it is an absolute requirement of enzyme synthesis; and that conalbumen, an iron-binding egg white protein, was responsible for an increase in pigment production.

The fluorescent color of pyoverdine appears to vary slightly with the species of Pseudomonas producing it and the conditions under which the organisms are grown. When P. ovalis was grown aerobically in asparagine broth and examined under a black light, Elliot (1958) saw the top few milliliters become yellow and later diffuse throughout the entire medium; but when grown anaerobically, small quantities of blue fluorescing pyoverdine were produced. He discovered that the yellow-green fluorescence changed to blue when the pigment was diluted. Paton (1959)

found that the presence of the fluorescent pigment was not dependent upon being visible to the naked eye. Kraft and Ayres (1961) found P. aeruginosa to fluoresce a bright blue-green when grown in asparagine broth. Fluorescence I, isolated by Osawa et al. (1963), appeared as a brilliant bluish-white under ultra-violet light and a yellow-green under ordinary light. Two parts of Fluorescence I, fluorescent blue and yellow-green substances separated by gel filtration, were shown to be the same compound (Osawa et al., 1964). The Fluorescence II fraction was yellow-brown in visible light and a fluorescent yolk-yellow under ultra-violet light (Osawa et al., 1965). Compound I, elucidated by Newkirk and Hulcher (1969) emitted an intense blue-green fluorescence when exposed to ultra-violet light.

Color changes of pyoverdine due to the alteration of pH have been found to be reversible (Jordan, 1899; Sullivan, 1905; Elliot, 1958; Wasserman, 1965; Newkirk and Hulcher, 1969). The fluorescent pigment, in a study by Young (1947), fluoresced an orange when acidified and a blue-green when neutralized. Elliot (1958) made a study of visible and fluorescent color changes when the pH of the pigment was altered. Changes in the tone of Fluorescence I as well as the ultra-violet absorption, fluorescent character, and the electrophoretic pattern due to pH alterations were noted by Osawa et al. (1964), who also observed that the fluorescent yellow-green portion of Fluorescence I was very bright at a pH of 8.6 and changed to a blue fluorescence at a pH of 7.5. Wasserman (1965) noted

that of a pH of 9.8, the fluorescent color was yellow-green or blue-green and when acidified, ranged from blue at a neutral pH to blue-white, white, whitish-orange, or orange at a pH of 2.0. Newkirk and Hulcher (1969) noted that the blue-green fluorescence from P. mildenbergii was most strongly colored at a pH from 7.0 to 9.0.

Hugo and Turner (1957) isolated a soil pseudomonad, the green fluorescent pigment of which had absorption maxima (405-414 nm in an alkaline solution, shifting to 360-380 nm when acidified) that were identical with P. aeruginosa, P. eisenbergii, and P. fluorescens and corresponded to those of phenazine-1-ol, Crude pyoverdine, dissolved in water, gave absorption maxima at 400 nm and 275 nm (Elliot, 1958). The absorption maxima of pigments produced by P. aeruginosa grown in asparagine broth gave peaks at 410 and 270 nm, and at 405 nm for P. fragi (Kraft and Ayres, 1961). The absorption maxima for Fluorescence II was at 360 nm (Osawa et al., 1963). Both the blue and yellow-green portions of Fluorescence I had absorption maxima at 400, 260, and 230 nm at a pH of 7.0 (Osawa et al., 1964). The ultra-violet absorption maxima of the C-substance were found to be at 346, 256, and 210 nm (Osawa et al., 1965). Wasserman (1965) found that the absence of an absorption maximum at 405 nm was related to the disappearance of visible color and attributed the absorption peak at 260 nm to nucleic acids liberated into the culture media by the degradation of non-viable cells. Compound I, isolated by Newkirk and Hulcher (1969), had absorption maxima at 402 and 278 nm when

the pH was 7.0; in 0.1 M sodium hydroxide, maxima of 401, 311-312, and 278 nm were obtained; and in 0.1 M hydrochloric acid, the maxima shifted to 370-376 and 277 nm.

The maximal wavelength of Fluorescence I was shown to be 460 nm in two strains of P. aeruginosa and 470 nm in another (Osawa et al., 1964). A spectrophoto-fluorometer study of the C-substance gave a fluorescent maximum at 490 nm with an activation wavelength of 360 nm (Osawa et al., 1965). The wavelength of emission for Compound I was at 462 nm with an excitation wavelength of 402 nm (Newkirk and Hulcher, 1969).

Discoveries concerning the relationships between other bacteria and the pigment produced by Pseudomonas have been reported. Bacterial inhibiting substances have been noted in the fluorescent pigment of Pseudomonas (Young, 1947). Chmura and Pelczar (1959) observed that when P. aeruginosa was grown in conjunction with Seratia marcescens, enhancement of the pseudomonad pigment occurred.

Species of Pseudomonas, some of them pigment producing, appear to be established inhabitants of a variety of marine molluscs. Colwell and Liston (1960) found that the Pseudomonas/Vibrio group was the largest single bacterial group inhabiting Pacific oysters, Crassostrea gigas. Pigment producing strains of P. fluorescens have been isolated from Cyclostoma elegans and C. sulcatum by Mahdihassan (1961) who found

that the bacterial growth of these strains was kept under control in the molluscan hosts. Colwell and Liston (1962), in an extensive bacterial study of marine vertebrates and invertebrates, discovered that 48 per cent of the total bacteria found in the invertebrate group were Pseudomonas; a bacterial flora consisting of Pseudomonas and Achromobacter was found in the snail, Lambis lambis, and the squid, Loligo opalescens, of which 89 per cent of the Pseudomonas species from the squid produced a green fluorescent pigment in culture; and in C. gigas, Pseudomonas was one of the predominant genera isolated from the body tissue and fluids. Surianinova (1962) isolated Pseudomonas from the surface and various body parts of Mytilus galloprovincialis. Beeson and Johnson (1967) found that clams in the genus Donax contained Pseudomonas and Vibrio as their bacterial symbionts. Although Pseudomonas appears to be one of the predominant groups of bacteria found in marine molluscs, no discoveries regarding fluorescence in these molluscs was reported by any of the preceeding authors.

MATERIALS AND METHODS

Specimens of A. alternata, A. kochi, and M. thyroidus were collected from the south-eastern and south-western borders of Foley's Woods, near Paris, Illinois, and from the north-eastern and south-eastern borders of Fox Ridge State Park, eight miles south of Charleston, Illinois. Mucus extractions were taken with a bacterial transfer loop from two individuals of each species collected at the four locations. This method of mucus extraction was utilized throughout the study. A loopful of mucus was streaked for obtaining isolated colonies (Pelczar and Reid, 1965) on Petri plates containing Medium B (King et al., 1954). This procedure was repeated the following day using the same snails, yielding 48 inoculated plates, two per snail. Medium B was made in the following manner: Proteose Peptone #3 (Difco), 20 gm.; Glycerine, 10 ml.; K_2HPO_4, 1.5 gm.; $MgSO_4$, 1.5 gm.; Agar, 15 gm.; Distilled water, 1,000 ml.; and pH adjusted to 7.0-7.2. For liquid media, the agar was omitted.

After 48 hours, the colonies that fluoresced under a UVSL 13 combination ultra-violet light (Ultra-violet Products Inc.) were isolated in pure culture by re-streaking isolated colonies at least three to four consecutive times during which no foreign bacterial growth was detected. A gram stain of each pure culture was made as a primary test for purity and a means for gross identification. Bacto-Differentiation Disks, Oxidase (Difco) (Difco Supplementary Literature, 1966) were also employed as a

means for determining the identification of the bacteria. The isolated bacteria were identified as members of the genus, Pseudomonas. The pure isolates of Pseudomonas were streaked on nutrient agar (Difco) slants and refrigerated for storage.

All of the cultures used in the investigation were grown at room temperature without constant mechanical aeration. Capall or Kaput (Bellco) caps enclosed the 16 mm. culture tubes. Before inoculating any media for experimental purposes, each of the Pseudomonas isolates used in the investigation were transferred from the slants and grown in 3 ml. of nutrient broth (Difco) for 48 hours. To obtain a simultaneous growth of all the cultures isolated from a particular snail species, a loopful from each of the 48 hour pseudomonad suspensions was taken and inoculated into a common culture tube containing 3 ml. of nutrient broth. The bacteria in this suspension were then immediately inoculated on the experimental medium. Jordan (1899) and Sullivan (1905) found that daylight will cause the decomposition of pyoverdine. Therefore, all fluorescent pigments produced by the Pseudomonas isolates were stored in a dark place. Those cultures isolated from specimens of A. kochi were the only ones used except where otherwise indicated.

To compare solubility properties between the bacterial pigment and the fluorescent pigment in the mucus of A. kochi, Pseudomonas cultures were grown simultaneously for several days in culture tubes containing 5 ml.

of liquid Medium B. Pigment production in these tubes was checked both visually and under ultra-violet light. Mucus from several specimens of A. kochi was dissolved in 10 ml. of distilled water and fluorescence checked under ultra-violet light. Both the bacterial suspension and the mucus solution were centrifuged in a Sorvall superspeed centrifuge at 13, 500 RPM for 30 minutes. Several drops of each fluorescent supernatant were added to test tubes containing a number of solvents. The tubes were gently shaken and then examined under the ultra-violet light to determine whether the fluorescent substances were soluble in the given solvents.

As a second experimental method, mucus was quantitatively extracted from snails using a medicine dropper calibrated with one ml. of water. This served to quantify the volume of mucus extracted from the snails. A total of one ml. of mucus was taken from seven specimens of A. kochi and dissovled in 5 ml. of 0. 01 M phosphate buffer, pH 7. 0. The isolates were grown in 3 ml. of Medium B for several days and then added to 2 ml. of the buffer. Both solutions were centrifuged at 13, 500 RPM for 30 minutes. More buffer was added to each supernatant, bringing the total volume of each to 10 ml. The fluorescent solutions were then put into dialysis bags and suspended in 40 ml. of the phosphate buffer contained in a sealed 250 ml. flask. The dialytic property of the solutions was observed under ultra-violet light.

Bacteria isolated from A. kochi were all inoculated in a common

tube of nutrient broth and then streaked on three plates of Medium B to obtain solid masses of growth. After 72 hours, the bacteria were scraped from the plates with a clean glass slide and placed in a tube containing 10 ml. of distilled water. The tube was covered with Parafilm (American Can Co.) and aggitated until a homogenous suspension was obtained. The suspension was centrifuged and 0.1 N HCl and 0.1 N NaOH were added to 3 ml. portions of the supernatant to obtain pH's of 4.0, 7.0, 10.0 on a Beckman Zeromatic II pH meter. The same procedure was followed for a 1:10 dilution of the supernatant and for one ml. of mucus obtained from a total of six specimens of A. kochi dissolved in 10 ml. of distilled water. This solution was also centrifuged. The pH of the mucus was recorded before the pH changes were affected by the HCl and NaOH. Observations were made of the fluorescent color changes due to alterations of pH and dilution.

Pseudomonad isolates obtained from A. kochi were grown simultaneously in culture tubes of Medium B for several days until pigment production was evident. Additional mucus samples obtained from several specimens of A. kochi were dissolved in water and poured into a culture tube. Each of the culture tubes was autoclaved at 250°C. at 15 lbs. for 15 minutes. The fluorescent substances were then observed under ultra-violet light.

In an effort to determine any visible difference in pigment production

between those bacteria isolated from A. kochi and from M. thyroidus, cultures isolated from the two snail species were grown independently on plates of Plate Count Agar (Difco), 1% Tryptone (Difco) Agar, and 0.5% Peptone (Difco) Agar. After 48 hours, the cultures were examined visually and under ultra-violet light to determine whether any extraordinary pigment production had occurred.

For the spectrophotometric study, pigments produced by the Pseudomonas isolates and the mucus solutions from A. kochi, M. thyroidus, and specimens of Liguus from Florida were compared. Mucus solutions from M. thyroidus and Liguus were studied spectrophotometrically to provide records for comparison of non-fluorescent mucus to those of A. kochi and the bacterial pigment. A total of one ml. of mucus was extracted from six specimens of A. kochi and diluted in 10 ml. of the 0.01 M phosphate buffer. Also, several loopsful of mucus from other individuals of A. kochi were diluted in 4 ml. quantities of standard buffer solutions (Sargent-Welch) at a pH of 4.0 and 10.0. A total of one ml. of mucus from seven specimens of M. thyroidus and a total of one ml. from two specimens of Liguus were each added to 10 ml. of the phosphate buffer. Individual Petri plates of Medium B were inoculated to obtain a solid mass of growth with each isolate. A suspension of nutrient broth containing all of the 48 hour isolates was also inoculated in the same manner to obtain a simultaneous growth of the organisms. After 72 hours, clean

glass slides were used to scrape the bacteria from the plates and to place the scrapings in tubes containing 10 ml. of the three buffers. The tubes were covered with Parafilm and shaken vigorously to obtain homogenous suspensions. All of the mucus and bacterial suspensions were centrifuged at 13,500 RPM for 30 minutes. In most cases, repeated centrifugation was necessary. Phillips (1964) stated that aliphatic ethers are transparent except for some possible end absorption. Therefore, ether was added to prevent bacterial growth in those solutions containing the phosphate buffer. In solutions containing the other two buffers, the addition of ether was not necessary because spectrophotometric recordings were made immediately following centrifugation. In addition to these solutions, the supernatant containing the phosphate buffer with pigments produced by all of the Pseudomonas isolates from A. kochi was added to a M. thyroidus mucus solution in proportions of 1:3, 1:1, and 3:1. Absorption spectra were recorded on a Beckman DB-G spectrophotometer. The phosphate buffer with ether was used as a reference in recording all absorption spectra at a pH of 7.0, and the two other buffers were used when spectra were run at their corresponding pH.

Haynes (1951) stated most fluorescent pseudomonads will not grow at 37°C. and Schneierson, Amsterdam, and Perlman (1960) used varied amounts of chloramphenicol to prevent pigment production by P. aeruginosa in culture. Therefore, efforts were made to prevent bacterial growth

and/or pigment production in live specimens of A. kochi by temperature control and by the use of chloromycetin (chloramphenicol). Mucus was extracted prior to each experiment to diminish the amounts of bacteria and pigment already present. Mucus was extracted from three specimens of A. kochi before the snails were placed in an oven at 37°C. In a second effort, mucus was extracted from two specimens and solutions of 30 mg. chloromycetin per one ml. of water and 7.5 mg. per one-half ml. of water were administered into the apertures of the snails with a pipette. These two snails were turned to their apertures faced upward to prevent the chloromycetin solution from draining out of their aperatures before they were observed 18 hours later.

RESULTS

The mucus from A. alternata and A. kochi was viscous and contained a visible amber pigment. A brilliant blue fluorescence was emitted when mucus from specimens collected during the summer months was exposed to ultra-violet light. Recent investigation has shown the mucus from specimens of A. alternata collected at Fox Ridge during Spring to fluoresce blue-green. When most of the mucus had been extracted from any particular individual of these two species, the amber color disappeared and the mucus became less viscous. The blue fluorescence did not appear to be affected by the disappearance of the amber color or the viscosity change of the mucus. The mucus from M. thyroidus was not viscous nor was it pigmented, and no fluorescence could be detected when the mucus was exposed to ultra-violet light.

Bacteria which produce fluorescing pigments were isolated from the fluorescent mucus of A. alternata and A. kochi as well as from the non-fluorescent mucus of M. thyroidus. All of the bacteria that produced fluorescent pigments on Medium B were gram negative rods and oxidase positive. These characteristics are indicative of the genus, Pseudomonas. A total of 63 fluorescent isolates were obtained from the mucus of the three snail species. Twenty isolates were obtained from A. alternata, 22 from A. kochi, and 21 from M. thyroidus. Differences in habitat did not appear to affect the fluorescence in the snails or the snail-bacteria

relationship.

When grown on solid or in liquid Medium B, most of the isolates produced a blue-green fluorescent pigment, but some colonies produced fluorescent blue or yellow-green pigments when grown on agar plates of Medium B. It was impossible to group the colonies by their fluorescent pigment because distinct color demarcations could not be made. The pigment produced by all of the isolates was yellow-green in daylight and diffused evenly throughout the agar plates of Medium B. In culture tubes of Medium B, the pigment had the same visible color but was formed near the surface of the medium.

Both the fluorescent pigment in the mucus of A. kochi and the pigment generated by the bacteria isolated from this species appear to have the same solubility properties. These properties and the solvents used in the study are shown in Table 1.

The fluorescent pigment from the mucus of A. kochi and the pigment produced by the Pseudomonas isolates both exhibited the same dialytic property. After 24 hours, fluorescence was apparent on both sides of the dialyzing membrane.

Autoclaving at 250°C. for 15 minutes at 15 p. s. i. did not appear to affect either of the fluorescent pigments.

The pigment produced by the bacteria fluoresced blue-green in liquid Medium B. At a pH of 7. 0, the pigment fluoresced blue much

like that of A. kochi but was not as brilliant, and changed to fluorescent whitish-blue at a pH of 4.0 and to blue-green at a pH of 10.0. All color changes were reversible. When the original solution was diluted 1:10 with water, the blue-green fluorescence changed to a blue fluorescence of the same hue as found in the mucus of A. kochi. The diluted pigment did not show any color changes due to pH alterations. The pH of the mucus from A. kochi was found to be 7.0, and the blue fluorescent color did not change when the pH was altered to acidic and basic ranges. Mucus from the recently collected specimens of A. alternata emitted a blue-green fluorescence, but when the mucus was diluted with water, a blue fluorescence was produced.

There did not appear to be any differences in pigment production between those pseudomonads isolated from A. kochi and those isolated from M. thyroidus. Each culture isolated from the two snail species produced fluorescent pigments that diffused evenly throughout the Medium B Agar but did not diffuse throughout the Plate Count Agar, Tryptone Agar, and Peptone Agar. Fluorescence, when present, was restricted to the colonies proper. Pigment production by the cultures isolated from both snail species was variable on Plate Count Agar and Peptone Agar. Approximately 50 per cent of these isolates produced fluorescent pigments in the two media while the others did not. The Tryptone Agar supported small white colonies when streaked with the isolates from A. kochi and

M. thyroidus, but fluorescence was difficult to determine because of the reflection from the colonies.

The absorption peaks of those pigments formed by each culture isolated from A. kochi ranged from 258 to 270 nm and from 315 to 410 nm. Two peaks, one at 265 nm and another at 400 nm were found to be characteristic of the isolates. The spectra of the mucus solutions from the non-fluorescent snails, M. thyroidus and Liguus, were similar, each with a peak at about 270 nm (Fig., 1-A). When the supernatant of the fluorescent pigments produced by the simultaneous growth of the A. kochi isolates was added to a mucus solution from M. thyroidus, absorption peaks similar to those of the supernatant alone were obtained (Figs., 1-A and 1-B). The absorption spectra of the supernatant-M. thyroidus mucus solution were the same for the 1:3, 1:1, and 3:1 proportions. Figure 1-B depicts the absorption spectra of the bacterial pigment compared to the mucus solution of A. kochi. The spectra are somewhat similar with the peaks of the bacterial pigment at about 265 and 400 nm, and those of the mucus solution at about 265 and 385 nm. Changes in pH to 4.0 and 10.0 appeared to have some similar effects on the absorption spectra of both the bacterial pigment and the pigment from the mucus of A. kochi (Figs., 2-A and 2-B). At a pH of 4.0, the bacterial pigment had a peak at about 320 nm with an inflection at about 405 nm. The mucus solution showed absorption peaks at about 310, 370, and 450 nm. At a pH of 10.0, the

bacterial pigment showed peaks at about 235, 270, and 400 nm. Absorption peaks at about 225, 290, and 365 nm appeared when the mucus solution was altered to the same pH. Oscillations occurred in all spectra recorded in the acidic and basic ranges. When the pH was 4.0, oscillation began at about 305 nm for both the bacterial pigment and the mucus solution. At a pH of 10.0, the recordings of both the bacterial pigment and the mucus solution showed oscillations beginning at 215 nm.

The efforts to prevent fluorescence in living specimens of A. kochi were a failure. The snails died within 18 hours after the temperature and chloramphenicol experiments were started.

DISCUSSION

King et al. (1954) stated that if only one medium could be used for general use, Medium B would be suitable for detecting Pseudomonas. They observed that a slight yellowing of either Medium A or B was the most frequently observed reaction with bacteria other than Pseudomonas and upon examination with ultra-violet light, the foreign substance would not fluoresce. According to the Difco Supplementary Literature (1966), oxidase differentiation discs may be used to differentiate strains of Pseudomonas from other organisms, a positive reaction indicative of the genus. Osawa et al. (1963) used the production of fluorescent pigments and the oxidase reaction to confirm their Pseudomonas identifications. Since the colonies used in this study produced fluorescent pigments on Medium B and were oxidase positive, gram negative rods, their identification to the genus Pseudomonas appears to be justified. Colwell and Liston (1961) discussed the difficulty they encountered using Bergey's Manual (Breed et al., 1957) to identify their strains of Pseudomonas to species. For this reason, no attempt was made to identify any of the 63 isolates beyond the genus level.

The literature indicates that Pseudomonas is a predominant genus of bacteria found in many marine molluscs. The results from this study indicate that Pseudomonas is present in the mucus of A. alternata, A. kochi, and M. thyroidus. It is likely that these bacteria may also be found in the mucus of other species of land snails. Since Pseudomonas has been isolated

from fluorescent and non-fluorescent mucus alike, a presence-absence aspect of these bacteria does not appear to be responsible for the fluorescence in some snail species. However, their presence establishes a potential for the production of diffusible fluorescent pigments in the mucus of certain land snails. If Pseudomonas is responsible for this phenomenon, the physiology of the fluorescent snails may be responsible for the production of substances like or unlike those found in Medium B which enable the bacteria to produce the fluorescent pigment. Conversely, there may be substances, such as iron, in the non-fluorescent mucus that inhibits the liberation of the pigment. In describing the media for the support of pigment production, King et al. (1954) stated that the usefulness of a culture medium is determined not only by the presence of certain necessary constituents, but also by the absence, or minimal concentrations of substances which may have adverse effects. This principle is applicable to the constituents of snail mucus in regard to its role as a medium for the enhancement of fluorescent pigments produced by species of Pseudomonas. The results from previous work in developing ideal media for fluorescent pigment production are useful for speculating the nature of mucus which would permit the production of fluorescent pigments. It would appear that sulfates (Sullivan, 1905, Georgia and Poe, 1931, King et al., 1948), phosphates (Sullivan, 1905, Georgia and Poe, 1931, Burton et al., 1948, King et al., 1948, 1954), and magnesium (Georgia and

Poe, 1931; King et al., 1948) are necessary inorganic constituents for the production of such pigments. Burton et al. (1948), King et al. (1948), Knight (1951), Totter and Moseley (1953), and Kraft and Ayres (1964) all indicated that the amount of free iron in such media should be minimal. Therefore, it is possible that the mucus from non-fluorescent snails may lack sulfates, phosphates, and magnesium; or have a high concentration of iron; or both. Conversely, the mucus from A. kochi may contain sulfates, phosphates, and magnesium; have a low content of free iron; or both. The media used by Georgia and Poe (1931, 1932), King et al. (1954), and Osawa et al. (1963) all had their pH near or at neutrality. The mucus from A. kochi was found to have a pH of 7.0, indicating another factor which may be of importance.

Paton (1959), Osawa et al. (1963), and Wasserman (1965) found that the presence of fluorescence was not dependent upon the bacterial pigment being visible to the naked eye. The mucus from A. kochi was similar in that fluorescence was still present when the amber pigment was not visible. Both fluorescent substances dialyzed, suggesting a small molecular size, and withstood autoclaving, an indication that the fluorescing substances are not made of protein. These results and the solubility properties indicate that the fluorescent substance in the mucus and the fluorescent substance produced by the bacteria are similar.

It was found that the blue-green fluorescence liberated by the Pseudomonas isolates changed to blue when the pH was 7.0. Also, when

the original pigment was diluted, a blue fluorescence was emitted which did not change color when the pH was altered. Although the mucus from A. kochi fluoresced blue and not the blue-green shown by the bacterial pigment, the pH of the mucus was found to be 7.0 indicating that if the bacterial pigment is present in the mucus, it would produce a blue fluorescence. The blue fluorescence of the mucus did not change color when the pH was altered. The diluted bacterial pigment reacted in the same manner, suggesting that if the bacterial pigment is in the mucus, its quantity is minute. The pH of the mucus and the small quantities of the bacterial pigment may both be responsible for the fluorescent color of the mucus. These results show an even greater likeness between the fluorescent substances in the mucus and those produced by Pseudomonas. It is reasonable to assume that a low oxygen tension exists in the mucus of land snails such as A. kochi. Lenhoff (1963) found that when P. fluorescens was grown at a low oxygen tension (non-aerated) containing less than $8X10^{-6}$ M ferric chloride, a blue fluorescence was produced with only a trace of pyoverdine being excreted into the medium. This further substantiates the blue fluorescence in the mucus with respect to small quantities of bacterial pigment. It also suggests that oxygen tension and iron concentration may affect the fluorescent color of the pigment.

Although this study was primarily concerned with A. kochi, the

blue fluorescence in the mucus of A. alternata appears to be identical to that of A. kochi. Having isolated Pseudomonas from members of both of these congeneric species, it is possible that whatever is causing fluorescence in A. kochi may also be responsible for the fluorescence in A. alternata. The blue-green fluorescent pigment in recently collected specimens of A. alternata resembles the bacterial pigment both in original color and in the fact when the mucus was diluted with water, the fluorescence changed to blue. The presence of both the blue and blue-green fluorescence in the mucus of A. alternata is compatible with the hypothesis that pigment production by Pseudomonas may be a function of the physiology of the snail.

When grown on Medium B, Plate Count Agar, Tryptone Agar, and Peptone Agar, there did not appear to be any significant differences in pigment production among the cultures isolated from A. kochi and those isolated from M. thyroidus. Therefore, it is not apparent that A. kochi has selected for strains of Pseudomonas whose nutritional requirements are more easily satisfied for pigment production than are those found in M. thyroidus. Once again, it appears that the fluorescence in the mucus is dependent upon the physiology of the snails rather than the metabolism of the bacteria.

The mucus from A. kochi, M. thyroidus, and Liguus and the bacterial pigment all had an absorption peak at about 265 nm (pH 7.0). Only the mucus from A. kochi and the bacterial pigment had a second

absorption maximum at about 385 and 400 nm respectively. Consequently, it appears that these second peaks are related to the fluorescent nature of the substances. Wasserman (1965) suggested that an absorption peak at 260 nm may be due to nucleic acids liberated into the medium by the degradation of non-visible cells by other bacteria. The present study has established that Pseudomonas is present in the mucus of A. kochi and M. thyroidus and it very likely that they are also present in the mucus of Liguus. Therefore, nucleic acids may be liberated into their mucus, thus causing absorption peaks at about 265 nm for the mucus of these snails.

The absorption maxima at about 265 and 400 nm appear to be indicative of the fluorescent pigments produced by the Pseudomonas isolates. Variable results obtained by other workers (Hugo and Turner, 1957, Elliot, 1958, Kraft and Ayres, 1961, Osawa et al., 1964, Wasserman, 1965, Newkirk and Hulcher, 1969) regarding absorption maxima of Pseudomonas pigments is in accordance with the variation in maxima among the pigments produced by the isolates in this study. However, maxima near 265 and 400 nm for the pigments in this study are also characteristic for the absorption maxima recorded by these workers.

The absorption curves of A. kochi mucus and the bacterial pigment are similar except the peak at about 400 nm for the bacterial pigment does not perfectly match the peak at about 385 nm for the mucus. It must be remembered that neither of the fluorescing substances in this study have

been isolated and purified. It is possible that during the production of the bacterial pigment in the snail, something in the mucus may be metabolized by the bacteria that is a part of the fluorescent compound which shifts the peak to a lower wavelength. This is substantiated by the fact that the absorption maxima of the bacterial pigment with the addition of M. thyroidus mucus gave peaks resembling those up the bacterial pigment alone. This indicates the peak at about 385 nm may not be merely a function of the physical mixing of the mucus and the bacterial pigment but a function of microbial metabolism.

The effects of pH on the absorption spectra of the two pigments do not corrolate with the spectra of Pseudomonas pigments reported by Osawa et al. (1964) and Newkirk and Hulcher (1969). The oscillations and absorption maxima found in this study are unique. At first, the oscillations appeared to be artifacts; however, they disappeared when water blanks were recorded and then reappeared when the pigments were recorded for a second time. Therefore, they appear to be characteristic of the pigments and not artifacts. Although the corresponding buffer was used as a reference at each pH, it is possible that the buffer may have reacted with the pigments, thus altering the absorption curves. If this is true, the oscillations show that the buffer probably reacted with both pigments in a similar manner, for the oscillations begin at the same wavelength for each pigment at each pH. The two substances also have several absorption peaks

in common. At a pH of 4.0, the bacterial pigment had a peak at about 320 nm which is near that of about 310 nm for the mucus. At the pH of 10.0, the bacterial pigment had peaks at about 235 and 270 nm which are near the peaks at about 225 and 290 nm for the mucus. Osawa et al. (1964) believed that a change occurs in the structure of the bacterial pigment between a pH of 4.0 and 5.0. Such a change may account for the change in absorption spectra recorded in the acidic range. Perhaps a similar change occurs when the substances are made alkaline. Emission values of the mucus and bacterial pigment could not be obtained because there was no access to a spectrophoto-fluorometer.

It has been well established that pyocyanin is soluble in chloroform (Sullivan, 1905, Veron, 1961, Azuma and Witter, 1964) while the fluorescent pigments produced by Pseudomonas are not (Sullivan, 1905, Veron, 1961, Wasserman, 1964). Since Medium B is selective for the production of pyoverdine and the pigment produced on this medium was insoluble in chloroform, it is likely that the pigment is pyoverdine, especially since the fluorescent color characteristics and the absorption spectra at pH 7.0 are similar to those described in the literature for this pigment.

CONCLUSIONS

Pseudomonas which produce fluorescent pigments are present in the mucus of Anguispira alternata, Anguispira kochi, and Mesodon thyroidus. The fluorescent pigment in the mucus of A. kochi has physical characteristics and absorption spectra very much like the bacterial pigment produced by Pseudomonas isolated from the mucus of these snails. Although the results are not totally conclusive, they do show beyond a reasonable doubt that Pseudomonas is the causative agent of fluorescence in the mucus of A. kochi. The isolation and purification of each fluorescent substance as well as a chemical analysis of mucus from fluorescent and non-fluorescent snails would be advantageous in further studies.

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TABLE 1. Solubility properties of the fluorescent bacterial and mucus pigments[a]

Solvent	Bacterial pigment	Mucus pigment
Water	+	+
Methyl alcohol (absolute)	+	+
Ethyl alcohol (85%)	+	+
Isopropyl alcohol (absolute)	+	+
Formaline (10%)	+	+
Glacial acetic acid (conc.)	+	+
Hydrochloric acid (conc.)	+	+
Glycerine	+	+
Acetone	+	+
Dioxane	+	+
Chloroform	-	-
Ether	-	-
Amyl acetate	-	-
Aniline	-	-
Benzene	-	-
Essence of Euparal	-	-
Wintergreen oil	-	-
Xylol	-	-

[a] + = soluble
- = insoluble

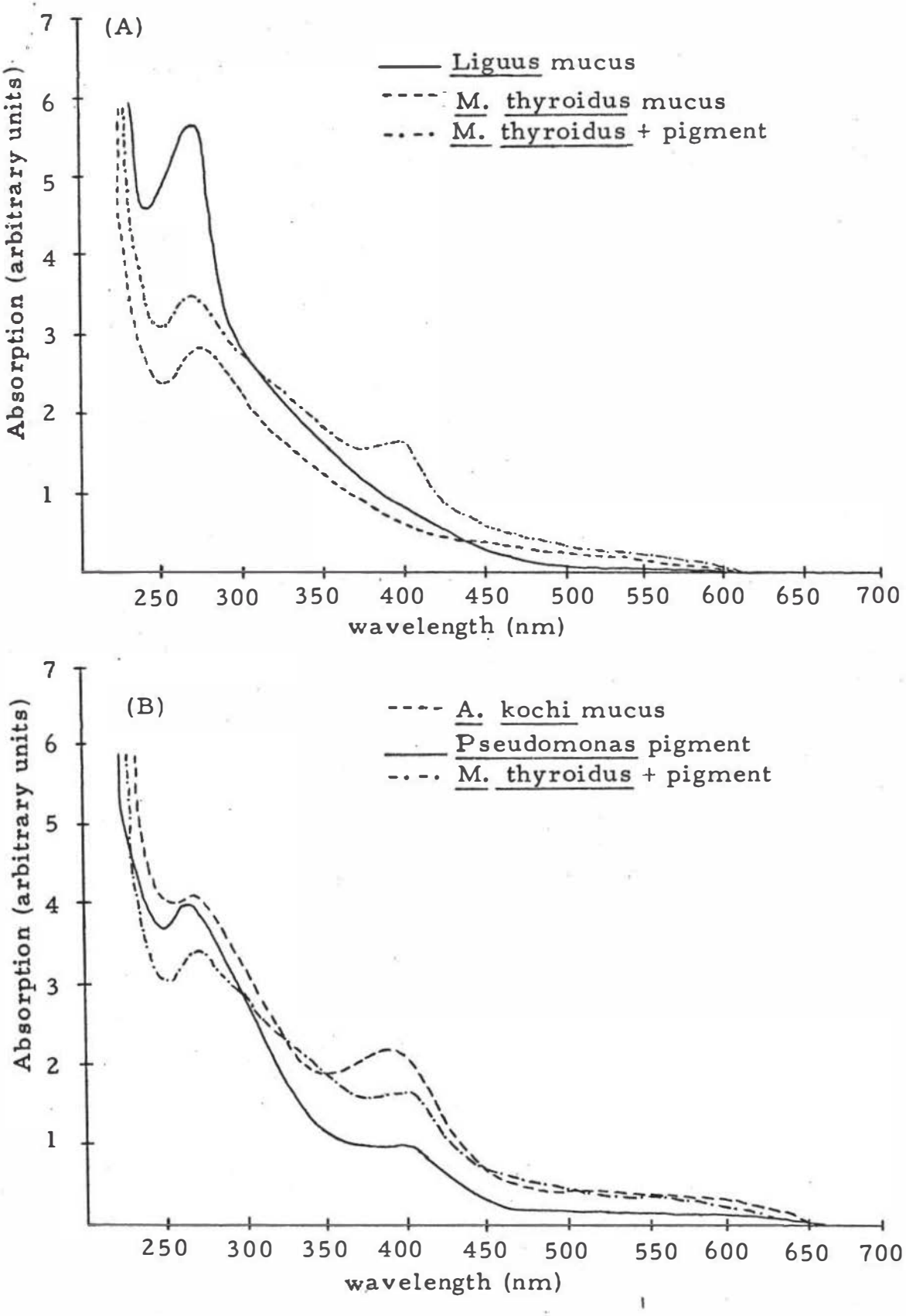

FIG. 1. Absorption spectra (pH 7.0) of snail mucus solutions, mucus solutions with bacterial pigment, and bacterial pigment.

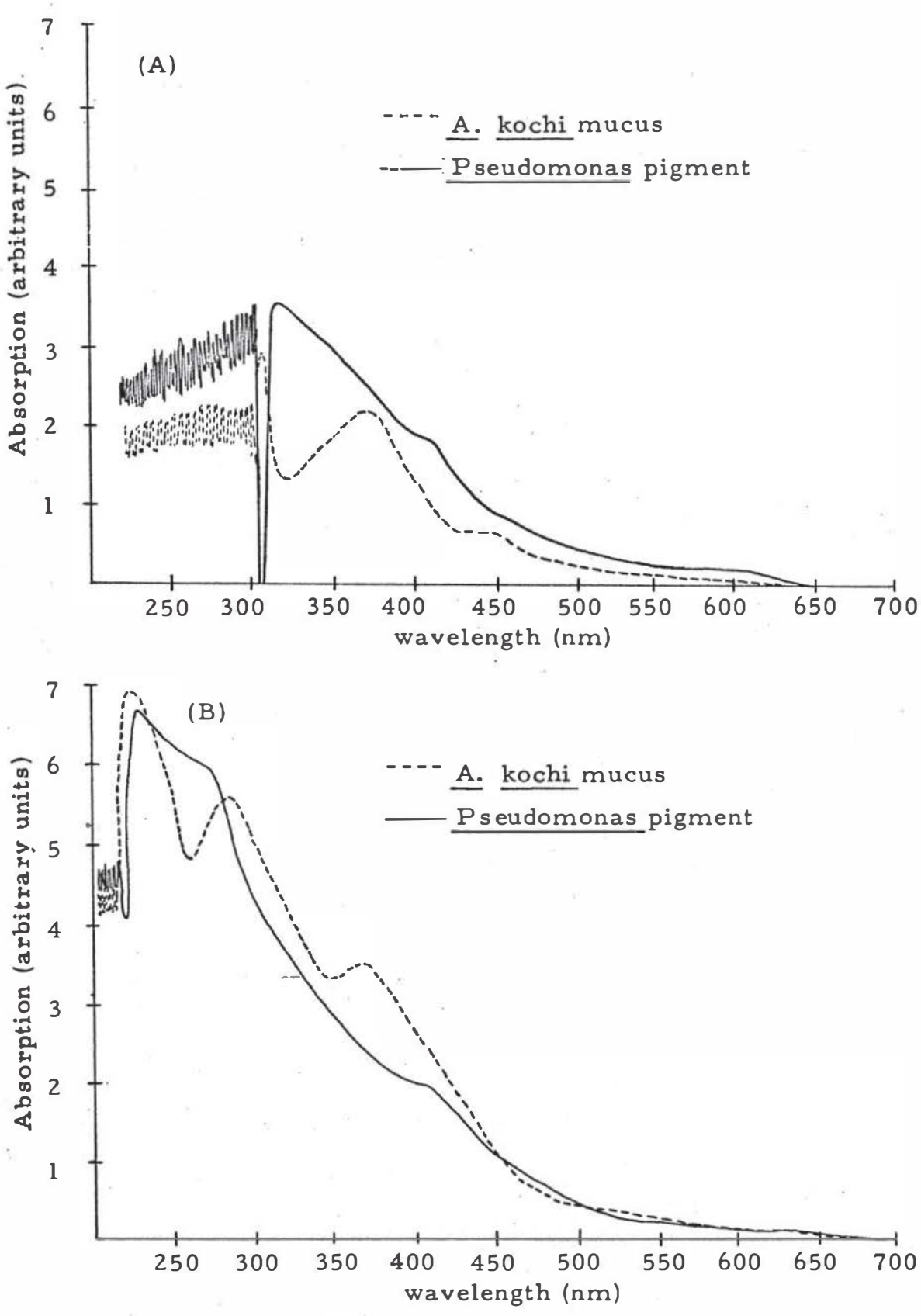

FIG. 2. Absorption spectra of A. kochi mucus solution and bacterial pigment at (A) pH 4.0 and (B) pH 10.0.