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Distribution of Pseudomonad Fluorescence in the Body of Anguispira kochi (Pfeiffer)

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Distribution of Pseudomonad Fluorescence in the Body of Anguispira kochi (Pfeiffer)

(TITLE)

BY

Roger Lowell Yates

THESIS

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Presented by

Roger Lowell Yates

a candidate for the degree of Master of Science

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I would like to dedicate this thesis to my wife, Suzi, whose quiet diligence and understanding has allowed for the completion of my graduate training. Also to my parents who instilled in me the desire to obtain an education.

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INTRODUCTION

Fluorescence is a phenomenon observable in many substances which, when exposed to ultraviolet radiation, absorb energy and emit light at longer wave lengths. This response to ultraviolet light is not uncommon in biological compounds, but it usually is associated with a particular type of tissue or secretion and not found over large areas of an animal body.

During a study of chromatograms of tissues of various species of land snails, certain chromatograms were observed to exhibit a vivid blue fluorescence when subjected to ultraviolet illumination. Further investigation indicated that this fluorescence was characteristic of two endodontid species, Anguispira alternata and A. kochi, and suggested the possibility that it would be found in all members of the family. The fluorescence of the chromatograms led to the discovery that the bodies of these snails fluoresced brilliantly when exposed to ultraviolet light (Rawls and Yates, 1971). Subsequently, Rawls and Baum (in press) reported fluorescence in Mesodon clausus, the only member of the family Polygyridae known thus far to exhibit the phenomenon.

A related study by Baum (1971) established the fact that

bacteria of the genus Pseudomonas are the causative agents of fluorescence in the mucus of Anguispira kochi. Baum also found Pseudomonas in the mucus of non-fluorescing snails, and suggested the existence of some specific physiological mechanism, as yet undetermined, which permits bacterial pigment production in fluorescent snails or inhibits it in those snails which do not so respond to ultraviolet light.

The role of pseudomonads in fluorescence of Anguispira kochi having been established, further investigation was indicated when a next step was suggested by the fact that this phenomenon was not observed to be uniform over the entire body of the snail. The purpose of this thesis is to demonstrate the anatomical distribution of fluorescence in the body of Anguispira kochi, and attempt to establish a basis for understanding why fluorescence occurs in certain body parts and not in others.

LITERATURE REVIEW

The first investigations of animal fluorescence were concerned with construction of catalogues listing the colors of fluorescence observed in different animals (Stübel, 1911; De kowalski, 1911; Arloing et al. 1925). Later work included study of fluorescence of tissues of the human female genitalia (Hauser, 1929), various organs of the human body (Bommer, 1929, 1933), the brain (Exner, 1932, 1933, 1934), the cerebrospinal fluid (Exner and Klemperer, 1930), the argentaffin cells of the intestines (Erös, 1932), the eye (Böck, 1934), animal tissues (Von Querner, 1933; Hamperl, 1934; Sutro, 1936) and the shells of some gastropods (Furreg and Querner, 1929). Most of these researchers were content with descriptions of observed colors, but Prowazek (1914) was the first to discuss the possible cause of their formation, believing physico-chemical conditions to be responsible. Bommer (1929) was the first to attempt extraction and identification of a fluorescent compound from prepared sections with little success.

Ellinger (1940) wrote a comprehensive paper listing all the authors and their works through 1939 and went in great detail describing the development of fluorescence microscopy. The use of fluorescence and fluorescence microscopy has led to many important discoveries

in the field of applied biology. Sutro and Buman (1933) described the advantages of using ultraviolet radiation in microscopic examination of living and dead tissue, and later Sutro (1936) published a paper relating to techniques of fluorescence microscopy. The importance of the fluorescence microscope and the use of fluorochromes to induce fluorescence was discussed by Metcalf and Patton (1944). Since that time, techniques have been developed which permit diagnosis of various types of cancer by the use of ultraviolet light (Bertalanffy et al. 1958), the use of fluorescence microscopy in the detection and diagnosis of disease, and the presence of certain bacteria (Darken, 1961).

The most current knowledge concerning fluorescence was summarized by Passwater (1970), who published a bibliography of all fluorescence research done between 1967 and 1970 in the areas of biology, chemistry and physics.

Baum (1971), demonstrated that pigments produced by bacteria of the genus Pseudomonas fluoresced in the mucus of specimens of Anguispira kochi exposed to ultraviolet radiation. Related research (Baum, private communication) showed that Pseudomonas was also present in the mucus of other land snails, such as Mesodon thyroidus, Mesodon inflectus and Triodopsis albolabris, but failed to fluoresce except in culture. He hypothesized that the mucus of Anguispira kochi contained the appropriate nutritional requirements which allowed the

bacteria to produce the diffusible pigments.

Pseudomonads of various species have been isolated from several organisms. Meyer (1925) published an exhaustive thesis on the isolation of bacteria from the nephridia of some land molluscs. The object of the original research was to determine if the bacteria ultimately found in the nephridia of some molluscs were transmitted from egg to egg or were regularly infected from outside; invariably, pseudomonads were isolated. The results fully harmonized with later work by Mahdihassen (1960) who stated that symbiotic bacteria did not grow at random within the host and were kept within control. Conclusions of the research included the fact that Cyclostoma elegans showed infections with Pseudomonas fluorescens and that infection took place after the organism emerged from the egg.

Several other researchers have isolated Pseudomonas and fluorescent pigments from both invertebrates and vertebrates, among them Colwell and Liston (1961), McGee (1964), Beeson and Johnson (1967), and Bannister, Bannister and Micallef (1968). The presence of Pseudomonas in marine vertebrates was noted in the study by Colwell and Liston (1961). In an attempt to compile a list of taxonomic relationships among the pseudomonads, key characters used were included, such as the requirements needed which might be supplied by the environment including the host. McGee (1964) reported green and

yellow pigments extracted from eggs of the trochacean species Tegula funebralis and Tegula brunnea. In the oxidized form, these pigments formed a green fluorescence. The absorption spectra showed a maximum absorption at 273 millimicrons and indicated a marked resemblance to compounds which act as respiratory pigments. Beeson and Johnson (1967) performed studies of bacterial isolates from the digestive gland and part of the gut of the bean clam, Donax gouldi, and showed Pseudomonas to be present. Later, Bannister, Bannister and Micallef (1968) referred to the presence of a green fluorescent pigment in the foot of trochids of the genus Monodonta, namely M. lineata, M. turbinata and M. articulata. The pigment was isolated by homogenation of the foot. Newkirk and Hulcher (1969), using thin layer chromatography and spectrofluorimetry, isolated and noted the properties of the pigments produced by Pseudomonas mildenbergii. The spectrofluorimeter revealed an emission maximum at 462 millimicrons, and absorption maxima at 402 and 278 millimicrons. The latter findings agree with those of Baum (1971). Osawa (1964) reported the production of a brilliant yellow-green fluorescent pigment in a broth culture of Pseudomonas aeruginosa.

The anatomical distribution of fluorescence has long been a center of interest. One of the first to isolate fluorescent material from animals was Von Querner (1932) who described fluorescent

droplets in liver cells.

Later, Popper (1941) discovered this compound in the liver cells to be vitamin A. and he described the distribution of the vitamin in various organs of the rat and man. However, he found that. because of the fat solubility of this compound, morphologic demonstration was only possible in aqueous fixatives.

Although fluorescence in land snails, per se, was not reported until Rawls and Yates (1971), fluorescent materials have been reported by several authors, with some attempt at localizing or finding the tissue sources of the fluorescence. Bowden (1950) observed a luminous freshwater limpet, Latia neritoides, from New Zealand. Attempts were made, unsuccessfully, to prepare the organism for histological study, by fixation and embedding in paraffin. Instead, the author was forced to determine the regions which contained the luminous material by stimulation and observations of secretions. Specific regions were cited for maximum luminous responses. The regions included the free surface of the mantle, anterior tentacles, lateral surfaces of the foot and the surface of the head. Specifically, on the surface of the head, the author stated that the luminous source appeared to be in the intermingled mucus cells, just beneath the simple cuboidal surface epithelium. Also. the luminous products were seen in the subepithelial connective tissue interspersed between muscle fibers.

The histological study was performed by using common microtomist techniques, but these procedures were not explained nor was any mention made of problems encountered by the luminous material being washed out during fixation.

Pigment present in the foot of trochids of Monodonta was found by Bannister, Bannister and Micallef (1968) to be present in the integument and probably in the haemolymph. Their hypotheses were based on the fact that pigmentation of the sole of the foot varied with relaxation and contraction of the pedal musculature. Other researchers. including Beeson and Johnson (1967) and Colwell and Liston (1961) isolated fluorescent pigment producing bacteria from different parts of aquatic organisms. Colwell and Liston (1961) demonstrated that Pseudomonas was present in the skin of Sebastodes melanops, Ophiodon elongatus, Pleuronichthyes sp., Merluccius productus and Acantharus sp. The gills of certain other species served as sources, and isolates were made from the foot of Lambis lambis. In each case the pseudomonads were confined to these areas, showing a tissue specificity. No mention was made of fluorescence or the use of ultraviolet light, but Beeson and Johnson (1967) isolated Pseudomonas from the digestive gland and part of the gut of Donax gouldi.

Chromatography has been used in attempts at identifying fluorescent compounds and studying the correlation between using

different body tissues or inserting variables such as change in diet. Among the first workers to make use of fluorescent compounds in tissues were Hadorn and Mitchell (1951), who found that the intestinal contents did not influence chromatography patterns given by Drosophila. Later, Buzzati-Traverso and Rechnitzer (1953) used paper chromatography for the first time on untreated squashes of muscle tissue of fishes. Both fluorescent and ninhydrin-positive patterns were used in this work, and the authors suggested that geographical races of some species of marine fishes could be distinguished by this method. Subsequent study by Buzzati-Traverso (1953) revealed that in snails, contamination of a tissue crush by gut content or part of the digestive gland usually produced additional bands on the fluorescent chromatograms.

Methods of identifying snails by the use of paper chromatography were first described by Kirk, Main and Beyer (1954). Tissue samples were cut from the posterior and lateral edges of the foot, and crushed onto the paper; the chromatograms were developed in butanol-acetic acid-water and visualized with a Wood's glass mercury vapor lamp. One phase of their work utilized specimens of Theba pisana which were reared in the laboratory. No significant differences between young, intermediate or mature animals could be detected in the characteristic fluorescent patterns, nor differences between mature specimens from widely separated localities. They concluded that

fluorescent patterns were uninfluenced by variation in age or environmental conditions. Similar results were recorded by Varty (1956) in solving taxonomic problems in insects of the genus Adelges. Constant fluorescent patterns were obtained from eggs, larvae and adults, with no apparent variation among adults even though host plants were varied.

The source of the fluorescent substances exhibited by chromatograms of animal tissues has been investigated by several authors. Wright (1959) used the posterior tip of the snail's foot for the tissue-crush, but subsequent work by Wright seemed to show that the substances yielding the fluorescent patterns were not derived from the tissues directly, but from the mucus secretion of the body surface. He reported that the slime-trail mucus of the pedal gland in Helix aspersa gave no fluorescent substances, but the pattern obtained from the general body surface secretion showed all the bands which were present in chromatograms of tissue crushes and a clearer separation was obtained in the absence of tissue. Of eight species of Lymnea studied, he found only four that exhibited even a faint fluorescence.

Homogenates of the foot, hepatopancreas, mucus and the whole organism were made from specimens of several species of freshwater snails by Michejda (1958). The chromatograms using these

substances revealed that the patterns for the hepatopancreas were extremely variable, but Michejda noted that the fluorescent pattern produced by snail mucus was similar to that obtained from tissue preparations of the foot, which implied that all the fluorescent compounds in tissue-crush preparations were present in the body surface mucus. Further work by Michejda and Urbanski (1958), used ultraviolet analysis of chromatograms and compared the foot and the whole organism of representatives of several families, including one species of Endodontidae, Discus rotundatus, the ultraviolet patterns of which did not resemble those of any of the other families discussed. Subsequent work was not done in the Endodontidae but the authors did not say how typical the patterns were of the family. Later, Michejda and Turbanska (1958) qualitatively compared the ultraviolet patterns of various tissues and concluded that ultraviolet patterns of homogenates of foot muscle were much less fluorescent than those of skin and suggested a lack of mucus-secreting glands in the muscular tissue. The ultraviolet patterns of the visceral sac and hepatopancreas differed markedly from those of the skin and foot parts, indicating significantly different chemical composition.

Experiments performed thus far have not explained differences in the fluorescence among individuals. Wright (1959) claimed that the most reasonable explanations for variation in chromatograms seemed

to lie in seasonal metabolic changes in the snails; preparing for hibernation in the autumn and sexual activity in the spring. Colwell and Liston (1962) stated that variations in particular genera and the relative number of bacteria found in particular organisms are due to differences in physico-chemical conditions of the animals and to differences in the external environment. Beeson and Johnson (1967) concluded that extrinsic environmental factors play a role in the presence of absence of certain bacteria, but that intrinsic factors are more vital in certain portions of the body of the infected organism. These results reflected results of a paper by Liston (1956) which demonstrated that types of bacteria in the gut of flatfish from the North Sea were quite distinct from those in the skin and gill.

MATERIALS AND METHODS

Specimens of Anguispira kochi were collected during 1970 and 1971 from several locations in the southwest corner of Foley's Woods near Paris, Illinois. The snails were identified according to Pilsbry (1940) and Burch (1962). Also collected were specimens of Mesodon thyroidus, Mesodon elevatus, Triodopsis albolabris, and Limax sp. for comparative studies in the latter phases of the experimental work.

All the snails were placed in a terrarium partially filled with humus, sand and leaf litter taken from the collection site to ensure that a change in environment could not cause alterations in available food, etc. The snails collected in the fall were retained in the terrarium until early February, when experimental techniques were refined. Work was carried out on those collected in early spring and mid-summer immediately following collection to ensure that no changes could take place due to a time lapse.

To become familiar with the histological structure of Anguispira kochi and to determine sites of fluorescence a number of microscope slides were prepared. Normal histological techniques of fixation and embedding were used and cross-sections of the entire organism were made. The sections were placed serially on slides

and stained with Harris haematoxylin and eosin. Cover slips were placed on the sections to make permanent slides to be used for reference.

Attempts at gross dissection and histological fixation for microscopic study were abandoned because of the solubility properties of the mucus and fluorescent compounds. An alternate method using a CO_2 freezing microtome (American Optical Co., model 880) was then adopted. A Dynazoom stereoscope (Bausch and Lomb Co.) with its base removed was positioned over the stage of the microtome to observe the snail histologically. An ultraviolet filter was attached below the nose of the microscope to absorb most stray ultraviolet light and keep it from entering the microscope. The filter absorbed 89% of the ultraviolet rays from 200-320 millimicrons and 84% in the range of 321-378 millimicrons as determined with a manual spectrophotometer (Hitachi Perkin-Elmer). Nearly all wavelengths above 378 millimicrons passed through the filter uninhibited.

Illumination for the tissue block was provided by a UVSL-13 hand-held Mineralight (Ultraviolet Products, Inc.), which emitted mixed long and short wavelengths. For observation of tissues not under ultraviolet radiation, an incandescent illuminator (Bausch and Lomb) was fixed in the mount provided for it in the dissecting microscope.

Preparation of the snails for sectioning was accomplished by using two methods, the first of which utilized no tissue fixation. The specimen was placed in a tightly sealed container of water to drown the snail and relax its body. After 24 hours it was removed from the container and the shell was carefully broken away from the body. The body of the snail was placed in a perforated plastic refrigerator container and allowed to remain in a stream of tap water for 20-30 minutes to gradually rinse away surface mucus from the tissues. Rinsing completed the preparation and the snail was thus ready for immediate sectioning.

The second method of tissue preparation was a variation of the technique described by Miller (1967) for preparation for dissection. The snail was drowned by immersion in water for about 24 hours to ensure relaxation and expansion, and then heated gradually (approximately 1^{o} per minute) to a temperature of 56^{o} C., the temperature of denaturation of collagen, at which time the dead animal could be pulled easily from the shell. Care was taken not to allow the animal to remain too long at this temperature or at higher temperatures, because the apical viscera would tend to harden and break off in the pulling process. The animal then was placed in the water rinse and the procedure from this point was identical to the first method. Both of these techniques were used interchangeably since neither proved superior. After the surface

mucus was removed from the body, the snail was ready for sectioning and was placed directly on the freezing head of the microtome. Embedding media of the commercial varieties and other common embedding compounds (e.g. glycerin) exhibited fluorescence under ultraviolet light; therefore, distilled water was used. The snail was positioned on its left side with the foot and mantle lying flat. Several drops of water were placed around the body of the snail to form a base. Upon freezing, a few more drops were added to form a block.

The angle of the blade was not critical, but was maintained at about 20-30°. To obtain the best possible sections without smearing the tissues or the fluorescent compounds, the knife blade was cooled with ice at all times during use. After the block was frozen and initial cuts were made, the surface of the ice block and exposed tissue were kept at the freezing point to prevent the fluorescent compounds from becoming intermingled in adjacent tissues. However, to avoid chatter and improper sectioning, the temperature was maintained in a range to avoid freezing the tissues to an extreme where they would become brittle.

Lateral sections were made at 50 microns since thickness in the sections made no difference, because tissues removed by sectioning were not saved. Incident light was used for observation of exposed tissues directly on the frozen block, since trying to use the

excised sections was not feasible.

Fluorescence in tissues and the extent of its occurrence throughout the entire body was noted. Certain histological features had to be determined to recognize the actual position of the fluorescence, but generally fluorescence was described on the tissue level rather than on the cellular level, except in a few areas (e.g. foot), because of the low magnification at which observations were made. Comparisons among individuals and variations of occurrence of fluorescence in various tissues were noted, and as an adjunct to explanation several sketches were made at various levels throughout sectioning as various organ systems were exposed.

Three series of experiments utilizing specimens of Anguispira kochi were performed; a fourth utilized other non-fluorescent species. The first, arbitrarily designated series A, was concerned with those specimens taken from Foley's Woods in the fall of 1970; this series was not examined until February of 1971. Series B utilized a collection of 25 specimens of A. kochi taken early in April when the activity of the snails was first becoming evident; the animals were sectioned in the first three weeks after collection. Series C also utilized A. kochi, but these had been collected in mid-summer; sectioning was done within the first 6 days following collection. The fourth and final group examined was composed of snails known not to

exhibit fluorescence, including specimens of Mesodon thyroidus, M. elevatus, Triodopsis albolabris and Limax sp. These organisms were used because Baum (1971) determined that the mucus of all but Limax, which he did not include in his study, contained bacteria of the genus Pseudomonas. Therefore, in an effort to hypothesize why certain snails fail to fluoresce even in the presence of these bacteria. these snails were examined for any internal fluorescence.

The histological structure and distribution of fluorescence was carried out in great detail in the foot region. Since the previous sectioning technique allowed for viewing the foot only laterally, a second experimental procedure was performed. The foot of a specimen of Anguispira kochi was amputated immediately below the mantle and cut transversely into three pieces. Each piece was then sectioned transversely to give a cross-sectional view, and the location of fluorescence in tissue was noted.

Previous study showed that the fluorescence of a solution of mucus of Anguispira kochi could be quenched by lowering the pH significantly. This phenomenon could be detected visually as well as by using a scanning spectrophotometer. As one method of confirming that the fluorescence seen in the tissues of the snail was indeed the compound being investigated, 0.1 N HCl was added dropwise directly onto the body of a snail as tissues were exposed via the microtome.

One of the features of the fluorescent compound in Anguispira kochi is the shade of blue it fluoresces. The wavelengths of fluorescence were determined by spectrofluorimetry to permit a filter system to be used which would eliminate all but the wavelength of light desired in an attempt to further ensure that the right compound was being observed.

To record fluorescence emission values, a standard mucus solution was used; the standard contained one ml. of mucus diluted to 10 ml. with 0.1 Molar KH_2PO_4 buffer solution, pH 6.9. The mucus solution was then centrifuged at 13,500 rpm for 30 minutes to remove any particulate matter which might reflect the excitation beam. An Aminco-Bowman spectrofluorimeter was utilized to obtain emission spectra. Four separate scans were made at excitation wavelengths of 265, 300, 350 and 400 millimicrons and emissions were read through the range of 200-700 millimicrons.

Adipose tissue fluoresces blue according to Popper (1941). In an attempt to delineate any fatty tissues, the blue fluorescence of which might be confused with that of the compounds under observation, a method suggested by Haitinger (1938), who used an alcoholic solution of chlorophyll, was adopted. The chlorophyll solution, when applied to the tissues of the snail being sectioned, yielded a reaction with fatty tissue to form a red compound which could easily be recognized.

RESULTS

The anatomical features of Anguispira kochi are presented in Figure 1. Examination of a fresh specimen of A. kochi under ultraviolet light reveals fluorescence over all of the surface of the foot, with the exception of the sole. The fluorescence is most concentrated around the genital aperture and anal opening and absent over the antennae and most of the surface of the head. Gross dissection of A. kochi reveals little concerning the distribution of fluorescence which is distributed throughout the foot, with all organs and systems being partially contaminated by the fluorescent pigment in mucoid suspension.

Cross-sections of the foot and head stained with haematoxylin and eosin were studied before the sectioning of frozen tissues was undertaken. The edge of the foot is covered with a layer of epithelial cells of the columnar type, the rest of the body being covered with a layer of stratified squamous epithelial cells. The epithelium of the foot is interrupted at intervals by intercellular spaces and the necks of the gland cells. Inward from the epithelial layer, most of the foot is composed of a layer of connective tissue cells and fibers forming a reticulum in which are situated a number of mucus cells and

connective tissue fibers and small amoeboid cells. The mucus cells were found as individuals in the connective tissue above the sole of the foot, but in aggregations in the foot fringes; some of these cells were seen to have necks which were quite long. Internal to the connective tissue layer is a thick layer of non-striated muscle which, in turn, is covered on its inner surface by a simple squamous epithelial layer which encloses a hemocoelic cavity which extends through much of the length of the foot.

Some tissue and organs contain naturally fluorescent pigments (Metcalf and Patton, 1944). In Anguispira kochi, for instance, the digestive gland emits a faint blue-green fluorescence when excited by ultraviolet light; portions of the reproductive tract fluoresce green; and the pericardial tissues at times emit an orange fluorescence. Such fluorescence is not the result of excitation of Pseudomonas pigment by ultraviolet light because acidification of the tissues of the snail with 0.1 N HCl quenches the blue fluorescence emitted by bacterial pigment (Baum, 1971). Fluorescence of other than bacterial origin is not affected by lowering of the pH nor do such fluorescent materials exhibit solubilities characteristic of pigments produced by pseudomonads (Baum, 1971). The fluorescence of pseudomonad pigment is quite characteristic also. Spectrofluorimetry revealed that the compounds isolated from Anguispira kochi emitted a single band of fluorescence

at 462 millimicrons when the excitation wavelengths were at 265, 300, 350 and 400 millimicrons.

Frozen sections of the foot viewed under ultraviolet light revealed fluorescence in mucus glands, deep beneath the epithelium; the epithelial cells themselves did not fluoresce. No fluorescence was observed in the connective tissue proper, with the exception of that seen in the necks of gland cells which opened to the surface of both the sole and sides of the foot through the epithelial layer. The muscular layer beneath the connective tissue failed to exhibit the phenomenon, as did the simple squamous epithelium next inward. The hemocoelic space enclosed by the epithelium fluoresced brilliantly.

Sectioning the viscera of Anguispira kochi revealed little fluorescence. The largest organ of the visceral mass, the digestive gland, failed to fluoresce, as did the nephridium and pericardial region. The antennae and the area immediately adjacent were also devoid of the fluorescence; the epithelium of this region was sclerotized and lacked the mucus glands so abundant in the foot. Observable fluorescence in the reproductive system varied with the dates of collection. Specimens collected in the fall and sectioned in the winter exhibited fluorescence in major portions of the reproductive system; in the penis, vagina, ovotestis, seminal vesicles and the genital aperture. Spring and summer collections revealed fluorescence only

in the genital aperture. Other than the reproductive system, only one area in the visceral mass, the hemocoelic space, showed any variation in fluorescence during the year. No fluorescence was found in this area in the fall-winter group, but a vivid fluorescence was seen in specimens collected in the spring and summer. Brilliant fluorescence was observed in the subepithelial connective tissue layer of the edge of the mantle, and in the mantle cavity. Less vivid fluorescence could be seen throughout the rest of the organ, but bright spots occurred in scattered locations; on the right, along the posterior arc of the edge of the mantle, immediately above the anal opening, the greatest concentration of fluorescence was observed. In several cases, the area where the mantle overlapped the foot contained a substantial amount of the fluorescent material, but it was extracellular and looked like mucoid material which had not been affected by the rinsing during preparation. Lastly, fluorescence was observed in the cavity of the anterior portions of the gastro-intestinal tract, but never any deeper than the pharyngeal bulb. The wall of the gut was devoid of fluorescence.

DISCUSSION

Fluorescence observed in Anguispira kochi is produced by pseudomonads, which are motile bacteria commonly found in the soil (Baum, 1971). It is feasible that, because soil and leaf litter constitute the habitat, A. kochi easily could become infected with Pseudomonas. Bacteria, however, may be selective and show preference for particular tissues in an organism (Colwell and Liston, 1961). The purpose of this study was to determine whether fluorescence of pseudomonad origin is preferentially distributed in the body of A. kochi.

No fluorescence was seen in the foot in the muscular layer. Michejda and Turbanska (1958) reported that homogenates of foot muscle of Lymnaea failed to fluoresce on chromatograms while those of the skin fluoresced vividly. They suggested the reason for this was due to a lack of mucus-secreting glands in the muscular tissue. Mucus glands were never seen to occur in the muscular layer of the foot of Anguispira kochi nor did the necks of any of the glands pass through the muscular tissue.

Observation of the visceral mass of A. kochi revealed that the internal organs generally did not exhibit fluorescence; the nephridium and the digestive gland always failed to fluoresce, although

Meyer (1925) and Mahdihassen (1960) indicated that Pseudomonas was invariably present in the nephridia of Cyclostoma. Baum (1971) established that Pseudomonas occurs in non-fluorescent as well as fluorescent snails, which suggests that the physiology of the snail may be the factor which determines fluorescent pigment production. If Pseudomonas occurs in the nephridium, as suggested by Meyer (1925), and Mahdihassen (1960), conditions therein may not permit pigment production. Previous investigation has shown that certain compounds such as sulfates and magnesium are required by pseudomonads to enhance pigment production (Baum, 1971), and it is reasonable to assume that these compounds might be lacking in certain organs but present in others.

If the assumption is made that certain parts of the organism are uninhabitable by Pseudomonas bacteria, this could be used to explain why fluorescence is not found everywhere in the body of the organism. However, no evidence has been offered that pseudomonads do not exist in other portions of the body. The gut of Anguispira kochi, for example, fluoresces only at the oral and anal openings, but it is possible that pseudomonads could be found elsewhere in the digestive tract; the pH in that portion of the gut could be such that the fluorescence of the pigments is quenched.

Since all mucus glands and the necks of the gland cells opening

to the epithelium were fluorescent, it is reasonable to suggest that the physiology of these structures is conducive to pigment production. The epithelial covering of the mantle fluoresces vividly because it is rich in mucus cells, but fluorescence found in the mantle cavity offers a different problem because there are no mucus glands; the pigment produced by Pseudomonas is diffusible however, and could easily move from gland cells into the mantle cavity. The bacteria themselves are motile, also, and could move into the cavity.

Seasonal variation in occurrence of the fluorescent material was noted in two areas of the body; the reproductive tract and the hemocoelic fluid. No fluorescence was observed in the reproductive organs of specimens collected in the spring or summer collections, but in snails collected in the fall these organs did exhibit the phenomenon. Wright (1958) suggested that differences in chromatograms of compounds derived from snail tissues were due to changes in metabolic activity; preparation for hibernation in the fall and increased sexual activity in the spring. These observations might apply to the distribution of fluorescence in the reproductive tract of A. kochi. Copulation occurs in these snails mainly in the spring and summer with little sexual activity taking place in the fall. Increased sexual activity may be coupled with increased concentrations of compounds which inhibit fluorescent pigment production in the reproductive organs;

conversely, compounds might be present in the fall and winter which would enhance pigment production.

Fluorescence seen in the hemocoelic fluid of the spring and summer specimens, but not those collected in the fall, would give an indication that a change in metabolic activity might be related to the presence or absence of the fluorescent compound. Since the hemocoel is in close proximity to the oral and anal apertures, the possibility exists that the diffusible pigment produced by Pseudomonas passes directly into the hemocoel. The fluorescence seen in the hemocoelic space was never as vivid as that of the external tissues, and this suggested a somewhat lower concentration of the pigment.

The specimens collected in the fall were held in a terrarium until used in early February, and the artificial environment may have induced changes in metabolic rate in these specimens. Work by Colwell and Liston (1962) revealed that bacterial floras of different marine tissues in vertebrates are different when taken from different environmental locations. Therefore, a lengthy time in a different environment may have had an effect.

It was established by Kirk, Main and Beyer (1954) that no significant differences between young, intermediate and mature land snails could be detected in the characteristic ultraviolet patterns of chromatograms. This study has determined that no differences exist

in the sites of fluorescence in different age groups also, based on shell size. These results would suggest the possibility of a genetic factor based on metabolism of the specific tissues which does not change throughout life.

The blue-green fluorescence seen in the center of the digestive gland proved not to be of Pseudomonas origin. However, it seems likely that other bacteria possessing the ability to produce fluorescent pigments could be the cause of this fluorescence. The solubility properties exhibited by this could be entirely different than those of Pseudomonas and could be mistaken for a naturally fluorescent compound in the organ.

SUMMARY AND CONCLUSIONS

The anatomical distribution of fluorescence caused by pigments produced by pseudomonad bacteria in Anguispira kochi was investigated by means of gross dissection and study of frozen sections. An attempt was made to determine whether this fluorescence is limited to the mucus secretions of the snail, or is distributed throughout the body of the animal.

As a result of this investigation, it is apparent that the fluorescence caused by ultraviolet stimulation of pigments produced by pseudomonads living in the body of A. kochi is confined to the fluid of the hemocoel and to the subepithelial mucus glands, the necks of which pass through the epithelium to open at its surface. The variable fluorescence in the reproductive tract seems directly correlated with seasons of the year, but no satisfactory explanation for this apparent correlation can be given at this time. It is also possible that the portions of the body of Anguispira kochi which do not exhibit fluorescence either are unsatisfactory for the survival of Pseudomonas bacteria or the physiological conditions of those tissues are such that the pigments produced will not fluoresce; this could be a function of pH, but is more likely to be related to available magnesium ions as suggested by Baum (1971).

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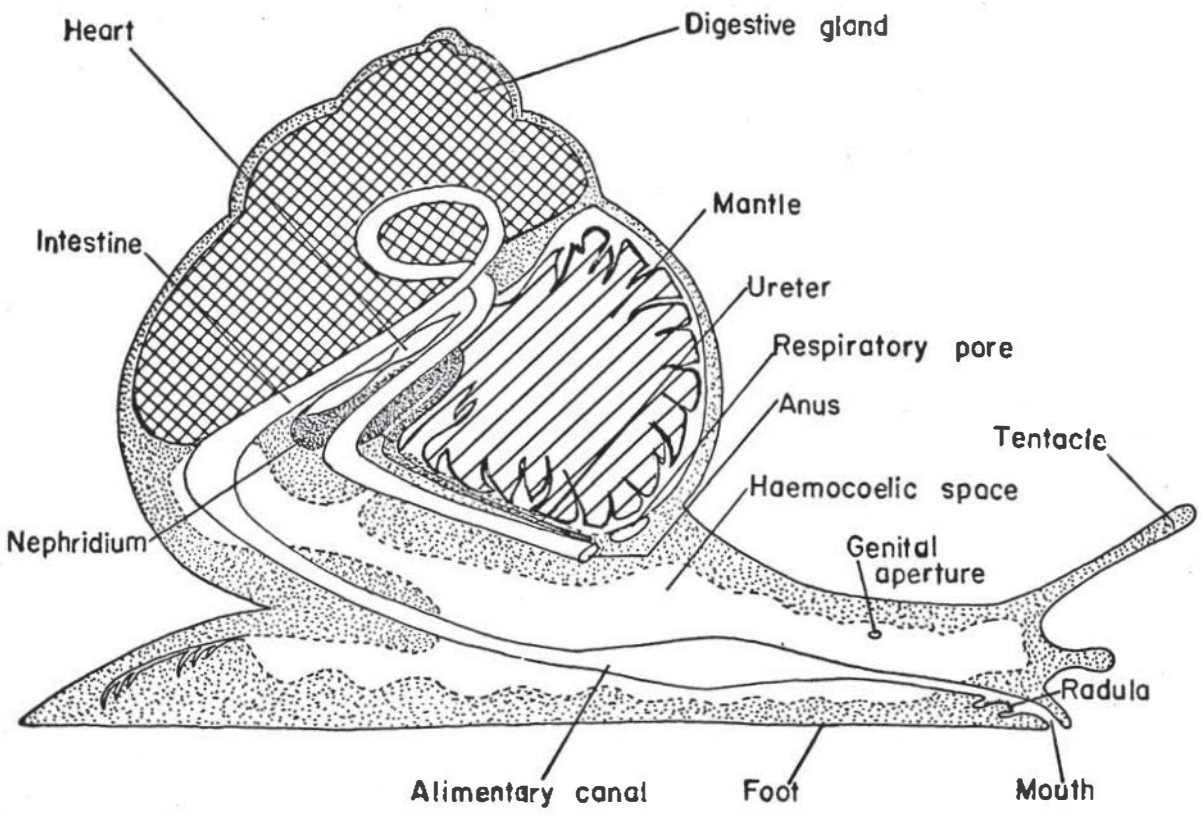

Figure 1. Internal anatomy of a land snail. The reproductive system is not shown. (After Burch, 1962).