Studies of Aging Patterns and Phosphatase Isozymes in the Life Cycle of the Nematode Panagrellus silusiae

Glenn Nicholas Doering

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STUDIES OF AGING PATTERNS AND PHOSPHATASE ISOZYMES IN THE LIFE CYCLE OF THE NEMATODE \textit{PANAGRELLUS SILUSIAE}

by

Glenn Nicholas Doering

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

June 1977
I wish to express my gratitude to my advisor, Dr. Edward E. Palincsar, and to Mrs. Palincsar, for their valuable guidance throughout this investigation.

Sincere appreciation is extended to Dr. Benedict J. Jaskoski and Dr. Clyde E. Robbins for serving as committee members and assisting in the preparation of this thesis.

For help with microscopy and photography, as well as loyal support, I wish to thank Jeanne E. Moldenhauer.

Special thanks to my parents, Miss Pauline J. Doering, and Dr. and Mrs. Jeffrey L. Doering for their constant concern, patience and encouragement.
VITA

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CHAPTER 1

INTRODUCTION

Old age and death are considered to be the termination of the life span. In many organisms including man, an increase in age is accompanied by a decline in the ability of self-maintenance and an increase in the probability of disease and death (Comfort, 1970). A new interest in the causes of aging has emerged, focusing on the modern techniques in molecular biology, seeking the controlling mechanism and the molecular basis for the life-long process of aging. Through these studies of senescence man hopes to alleviate the physical deterioration that accompanies age.

Despite the volume of research that has been done concerning aging, a single, universal mechanism has yet to be identified. A number of questions remain unanswered, and because of this lack of knowledge, a suitable definition of the aging process has thus far been difficult to formulate.

Based on the concept that aging is a universal phenomenon among the metazoans, Gershon (1970) considered nematodes to be suitable organisms for aging studies because (1) it is possible to obtain age-synchronized populations of nematodes and establish survival curves with 50% survival of about 25 days, (2) the nematodes' growth and life-span are not altered or affected by as much as 90% inhibition of
DNA synthesis, and (3) they yield large populations under easily controlled environmental conditions, making them fit for biochemical investigations. Since nematodes are eutellic animals, cell division and turnover are negligible and most cells are already differentiated after hatching (Hyman, 1951), making any deteriorative processes leading to senescence more easily observable. The nematode chosen for this study was the free-living form Panagrellus silusiae.

The lysosome has been implicated as part of the terminal lytic aging process (Brock and Strehler, 1968; Herold and Meadow, 1970). Lysosomes were discovered and first described by de Duve (1959), who called them "suicide bags", filled with powerful hydrolytic enzymes capable of breaking down most constituents of living matter, being present in a variety of animal cell types. The particular lysosomal enzyme chosen for this study was acid phosphatase (orthophosphoric monoester phosphohydrolase). Studies by Ghiretti (1950) and Hahn (1960) indicate that acid phosphatase activity may differ with the age of the organism, and Allison (1966) also suggested that the escape of hydrolytic enzymes from lysosomes seemed to be related to the aging process.

Therefore, the purpose of this study was to investigate the hydrolytic enzyme acid phosphatase, and its role in the biological mechanism of aging. The techniques used polyacrylamide gel disc electrophoresis, Triton X-100 to
study the membrane bound and unbound enzyme, and light microscopy, in the hope of better understanding the causes and mechanisms of the biological phenomenon of aging.
CHAPTER II

REVIEW OF RELATED LITERATURE

THE ORGANISM

Panagrellus silusiae (de Man, 1913), Goodey, 1945, is a free-living, ovoviviparous, dioecious nematode. It was first described by de Man (1914) from specimens found on the felt coasters placed under beer mugs in old German inns. It is believed that places where plant tissue was undergoing acetic fermentation were probably the primitive habitat of the genus, and *Drosophila* visiting these places would serve to disperse the nematodes (Lees, 1953).

The life span of this organism is divided into two major phases by Westgarth-Taylor and Pasternak (1973). The first or embryonic stage takes place in utero, and lasts about 20 hours. It starts with internal fertilization during copulation and continues with the complete embryogenesis of the worm, which supplies all of the somatic cells of the adult nematode. Before birth, the initial molt occurs within the egg, so that the first (L₁) stage gives rise to the L₂ stage, which is the first free-swimming form (Samoiloff and Pasternak, 1968, 1969). Length of worm is used to identify the different larval stages. The intrauterine (L₁) stage averages 200µ in length, as recorded by Samoiloff.
and Pasternak (1968).

The second or post-embryonic phase begins with the birth of the L₂ stage, which measures 350u in length (Gysels and van der Haegen, 1962). The post-embryonic period is characterized by a large increase in body mass, three molts and the development of the reproductive system. The different molts yield L₃ worms (500u in length), L₄'s of 850u in length and 30u in width, and the adult or L₅ worms whose males and females are 1400u and 1900u in length respectively. Both sexes are about 70u in width (Gysels and van der Haegen, 1962). Tortorello (1975) found that the molts occurred at 24 hour intervals, so that the entire growth period requires approximately 96 hours.

An outstanding anatomical feature of nematodes is their cell constancy or eutely, meaning cell division stops at hatching, except in the reproductive system (Hyman, 1951). This means that the increase in size which occurs during the post-embryonic period is due primarily to enlargement of the cells, rather than an actual increase in cell number (Pasternak and Samoiloff, 1970). Sin and Pasternak (1970) showed that a certain amount of cellular multiplication occurs during the growing period making P. silusiae about 74% eutelic, which falls within the accepted limits for being an eutelic organism. They also found a 26% increase in the number of tissue nuclei, from about 410 total nuclei in the L₂ stage to 553 in the L₅'s. There was also a 54% increase in the
mean length of worm, a 36% increase in the number of muscle tissue nuclei, a 9% increase in nervous tissue nuclei, and a 27% increase in the nuclei of the intestinal tract. This growth in *Panagrellus* seems to be regulated by the hindgut (Samoiloff, 1973).

Westgarth-Taylor and Pasternak (1973) found that gonads would not develop without normal continued growth and molting, but that growth and molting continued even when gonadogenesis was inhibited. The initiation of gonad development coincides with the final molt (Bird, 1971).

*Panagrellus silusiae* was chosen for this study because it is eutelic, is easily cultured, rapidly multiplies and has a short life span of about 28 days (Brokans and Palincsar, 1975) which affords easy examination of all stages of development.

**ELECTROPHORESIS**

Electrophoresis is defined as the movement of suspended particles through a fluid under the action of an electromotive force applied to electrodes in contact with the suspension.¹ Tiselius (1937) was the first to develop the moving boundary method for serum proteins, which established the basis for later simplified methods. There are numerous methods of electrophoresis in use today. They

include paper, starch-block, cellulose-acetate, agar gel, starch gel, column and polyacrylamide gel electrophoresis. Because of the nature of the process itself, electrophoresis is used to separate proteins. The protein that is to be separated, and the organism being used determine which form of electrophoresis should be utilized. Time is also an important factor in determining which method should be used, since some take only 30 to 45 minutes to run completely, while others (eg. paper electrophoresis) take as long as 18 hours.

In this study, disc polyacrylamide gel electrophoresis (developed by Ornstein and Davis, 1959, and Raymond and Weintraub, 1959) was used, since enzymes are often separated with this method, and it utilizes small samples. Disc electrophoresis employs discontinuity in both pH and gel size. In this system, the gel is formed inside a glass tube in 3 layers: (1) upper gel, (2) spacer gel with large pores, and (3) lower gel with small pores. Proteins first separate according to their electrophoretic mobility into highly concentrated discs of protein in the spacer gel (hence the name "disc" electrophoresis). When the proteins enter the small pore gel, separation is based on molecular size (Cawley, 1969).

Regarding the gels themselves, the shorter the gel length the more rapid the separation. Cawley (1969) states that the distance of movement of charged particles is in-
versely proportional to the length of the gel and directly proportional to the voltage used, as can be seen in the equation:

\[ d = \frac{vtu}{L} \]

where "d" is the distance of migration in cm, "v" is the voltage, "t" is the time, "L" is the length of gel in cm, and "u" is an expression of mobility in cm²/volt-second. Two more factors affecting migration distance are the pore size of the gel, and the adsorption (ie. the adhesion of the protein molecules to the gel itself).

The ability of a protein molecule to migrate in an electric field depends primarily on its net electrical charge. Since this can be varied by a change in the pH of the medium (Wilkinson, 1966) a protein can travel toward the anode (+) if in an alkaline solution, or toward the cathode (-) if in an acidic solution. The nature of the buffer used (ie. viscosity, ion strength, chemical properties), as well as the strength of the electric field, may also have an effect on the protein migration.

Wilkinson (1966) cautions that an increase in voltage and current will cause a rise in temperature which could denature the proteins being used. To overcome this possibility, he suggests keeping the samples cool (usu. around 4°C) while electrophoresis is in progress.
One enzyme system that has been implicated in the aging process is the lysosome (Brock and Strehler, 1968; Herold and Meadow, 1970). The discovery, physiology and morphology of lysosomes and their hydrolytic enzymes were described by de Duve (1959, 1963a, 1963b, 1964, 1965, 1970, 1971) and Novikoff (1961, 1963). De Duve called them "suicide bags" filled with powerful enzymes capable of breaking down most of the constituents of living matter. He also showed acid phosphatase to be one of a group of acid hydrolases found in the lysosomes.

The technical name for acid phosphatase is orthophosphoric monoester phosphohydrolase (Schaeg et al., 1972). Its presence was noted as long ago as 1935, when Schäffner and Bauer found it in yeasts. Powdery mildew was also shown to contain a strong concentration of acid phosphatase (Atkinson and Shaw, 1955). Since then, it has been found in a number of higher plants including the root tips of *Vicia faba* (Benes et al., 1961), the meristematic tissues of *Pisum arvense* (Barker et al., 1974), and the sweet potato (Uehara et al., 1974). Acid phosphatase has also been identified in certain bacteria (Schaeg et al., 1972), and molds (O'Day and Horgen, 1974) as well as in most animals from mosquitos to man.

It was soon realized that there was more than one
type of acid phosphatase. In 1935, Kutscher and Wolbergs first described prostatic acid phosphatase, and Gutman and Gutman (1938, 1940) extensively studied its possible functions. Herbert (1944, 1946) was the first to show that it could be differentiated from normal serum acid phosphatase by its instability in alcohol. The distinctive acid phosphatase of red blood cells was first described by King, et al., (1945) and has been studied extensively since then.

Folley and Kay (1936) found that three types of acid phosphatase existed which could be differentiated by their pH optimum, their sensitivity to Mg$^{2+}$ ions, and their relative activity towards alpha and beta glycerophosphates. Later studies (Roche, 1950) strongly suggested the presence of more than one acid phosphatase in the liver, and further work (Goodlad and Mills, 1957) produced evidence to support these findings.

Many studies have shown that species specific differences in the enzyme are present. Abul-Fadl and King (1948a, 1948b, 1949) were some of the first to do so, finding almost 100% inhibition of human liver acid phosphatase by 0.01 M tartrate between pH's 4 and 6 but only 40% inhibition in the rat.

More recent studies continue to support the idea that a number of separate acid phosphatases exist in single situations. For example, differences in the rate of activity of acid phosphatase in *Tetrahymena pyriformis* towards several
substrates (Connor and MacDonald, 1964) have been attributed to multiple forms of the enzyme. Much of the later work has been concerned with acid phosphatase activity in various tissues, and many investigators have developed elaborate procedures for its characterization and purification (for examples, Hendrikson, 1969; Timperley, Barson and Davies, 1971; and Uehara et al., 1974). These studies have demonstrated that acid phosphatase is actually composed of a number of isoenzymes (multiple molecular forms), which agrees with the early work done on numerous enzymes by Hunter and Markert (1957), Vesell and Bearn (1957) and Markert and Moller (1959).

Acid phosphatase was first separated electrophoretically by Estborn and Swedin (1959) who observed that it migrated as a single band on starch gel electrophoresis in a borate buffer of pH 8.9. More recent work has shown that their results were incomplete, since acid phosphatase is unstable at such a high pH and any minor bands present would have been destroyed (Sur et al., 1962). Since then, numerous investigators have successfully separated different isoenzymes of acid phosphatase from different animals, at various pH's and using different methods of electrophoresis (for example Sloat and Allen, 1969; Gonzales and Meizel, 1973).
Through the years, numerous theories have been suggested as possible explanations for the molecular basis of aging, but little agreement seems to exist regarding its true nature. One of the first theories of aging was based on the observation that a relationship exists between the life span and the metabolic rate in many animals (Pearl, 1928). It was shown that the greater the metabolic rate, the shorter the life span of the organism.

No other major publications appeared in the field of aging until the late 1950's when Comfort (1957) published some of his findings. He observed that aging is not a universal phenomenon among animals, since it does not seem to occur in organisms that continuously renew all of their cells throughout life (e.g., sea anemones). He theorized that a possible determinant of aging was the possession of cells that cannot be renewed by cell division and deteriorate in the course of metabolic life. He also proposed that all aging processes have one common factor—a progressive failure of adaptation and homeostasis over a period of time.

Another theory considers the everyday stress of life as the cause of aging (Selye et al., 1960). It suggests that stress produces organismal damage, and that less stress can be tolerated with increased age.
Strehler (1962) proposed four criteria for the biological mechanism of aging. He said that aging must be (1) universal, occurring in all old animals of a species and essentially absent in the very young, (2) time dependent, progressing gradually in an individual and in the population, (3) intrinsic, being a consequence of the action of time on the biological system rather than the result of a pathology or accident, and (4) deleterious, unfavorably effecting the survival capacity of the individual organism in its normal environment.

The collagen theory (Verzar, 1963) suggests that collagen tends to increase in amount and slowly but progressively cross-links during aging, causing the collagen fibers to shrink in length and choke the surrounding tissues. This causes the surrounding tissue to become damaged and wrinkled.

Walford (1964) offered an immunological theory, suggesting that mutated cells stimulate immunological reactions within an organism's system, which eventually degrade and destroy the organism.

Another important theoretical suggestion is the concept that aging occurs from the cellular damage caused by the lytic activity of hydrolytic enzymes that escape from the lysosomes (Allison, 1966).

The mutation theory (Curtis, 1966) states that the somatic cells of an organism develop spontaneous mutations as do germ cells. The mutations are propagated by subsequent
cell divisions, thus greatly increasing in number and effect. Since most mutations are harmful, the organism's cells become less similar to the original, and therefore less efficient.

Another possible cause of aging was suggested by Harmon (1966) in his free radical theory. Free radicals are chemicals which have unpaired electrons. They undergo spontaneous chemical reactions and tend to be self-propagating. Only a few free radicals are needed at any given time to create considerable damage.

Bjorksten (1968) proposed the cross-linkage theory, saying that aging is caused by the slowly increasing cross-linking of protein and nucleic acid molecules. This chemical combining frequently alters the chemical behavior of the original molecules, hindering them from performing their proper functions. This progressive cross-linkage of molecules in an organism is considered to lead to the progressive deterioration of chemical performance, and eventually will cause the organism's death.

Diffusion has also been hypothesized as a cause for aging (Carpenter, 1969). According to this theory, an organism may tend to produce some large and complex molecules at a rate which is much faster than that at which they can be removed.

Finch (1972) believes that aging involves changes in gene activity which are selective, not random. While
some genes will show no change in their activities, other genes may be activated or repressed. According to this idea, the selective changes in cell activity are controlled by changes in the pattern of gene activity, as in embryogenesis.

The more recent theories on the causes of aging are characterized by the attempt to synthesize the numerous aspects of the aging phenomenon into an integrated whole. An example of this is the two hypotheses presented by Hahn (1973). In his first hypothesis, he states that aging is a continuation of growth and differentiation, not a special biological phenomenon. His second theory proposes the existence of a distinct group of aging genes which are non-functional during growth and development, but become activated once adult homeostasis and the end of the reproductive period have been reached. Such genes would set a species specific limit to individual life spans. The function of the aging program within the cell is still a matter of speculation, and much research continues to be done in this field.

**ACID PHOSPHATASE AND AGING**

Hochschild (1971) has postulated that lysosomes may play a role in aging processes in at least three ways: (1) by carrying out injurious lytic activity within cells, either by excessive autophagy or by leakage of lysosomal hydrolases into the cytoplasm through damaged lysosomal membranes whose permeability has been changed, (2) by damage to extracellu-
lar structures through the extrusion of enzymes or enzyme leakage following membrane breakdown or cell death, (3) by inadequately carrying out their lytic activity as a consequence of becoming congested with undigestible material. Since acid phosphatase is considered to be a lysosomal marker, it follows that it too may be linked to the aging process. Much research has been done considering this possibility.

One of the early investigations conducted on acid phosphatase and aging, studied the different levels of the enzyme's activity during limb regeneration in the adult *Triturus cristatus* (Ghiretti, 1950). Hahn (1960) was also one of the earlier investigators, studying post-amputation acid phosphatase activity in the regenerating tail of *Xenopus laevis* tadpoles. Both of these studies found that although acid phosphatase appears to have little influence on regeneration, it did increase in activity during the process. Both also found evidence that the enzyme is associated with tissue and cell autolysis.

The acid phosphatase-aging link has also been studied in aging mice (Youhotsky-Gore and Pathmanathan, 1968; Elens and Wattiaux, 1970). Both of these investigations concluded that acid phosphatase activity increases with age; the first finding the enzyme's levels increased in the kidney, liver, diaphragm and the gastrocnemius muscle; the second finding liver acid phosphatase increased in total activity, per
gram activity and specific activity.

The histochemical studies of Herold and Meadow (1970) showed an age-related increase in acid phosphatase activity in the vitellarium, stomach, and dorsal gastric gland of the rotifer *Philodina acuticornis odiosa*, which was generally well correlated with an increase in the number of inclusion bodies. Their data suggested that the inclusion bodies were primary lysosomes that contain acid phosphatase, which explains the correlated increase.

Rats have also exhibited an increase in the activity of acid phosphatase with an increase in age (Bogart, 1970). This study also found a concurrent decrease in RNA concentration, while the concentration of DNA remained unchanged with aging.

While studying the nematode *Turbatrix aceti*, Gershon (1970) considered nematodes to be suitable organisms for aging studies, because (1) it is possible to obtain age-synchronized populations of nematodes and establish survival curves with 50% survival of about 25 days, (2) the nematode's growth and life-span are not altered or affected by as much as 90% inhibition of DNA synthesis, and (3) they yield large populations under easily controlled environmental conditions, thus making them fit for biochemical investigations. He continued to study *T. aceti* electrophoretically, and found a noted increase in activity of acid phosphatase in old populations (Erlanger and Gershon, 1970).
Chow and Pasternak (1969) also found nematodes to be well suited to aging studies. Their study of a number of enzymes yielded results indicating no change in acid phosphatase activity with increased age, and only one isoenzyme of acid phosphatase present in all the larval stages of *Panagrellus silusiae*.

Another nematode, *Nippostrongylus brasiliensis* was selected more recently to study the relationship between acid phosphatase and aging (Bolla, Weinstein and Lou, 1974). This electrophoretic investigation showed that the number of active enzyme subunits or isoenzymes depended on the developmental stage or age of the worm, indicating a definite correlation between the lytic enzyme acid phosphatase and the biological phenomenon of aging.

The present study adds to previous work with *Panagrellus silusiae*, by investigating acid phosphatase activity in the larval and adult stages, both electrophoretically and light microscopically.
CHAPTER III

MATERIALS AND METHODS

TEST ORGANISMS

The culture of *Panagrellus silusiae* was obtained from the laboratory collection of Edward E. Palincsar, Ph.D., Loyola University of Chicago, Chicago, Illinois.

CULTURING TECHNIQUES

The culturing method was a modification of Tortorello's (1975). Stock cultures of *Panagrellus silusiae* were maintained at 23-25°C in clear, plastic boxes measuring 12" long, 9" wide and 4" deep. The medium in which the nematodes were grown was Gerber Mixed Cereal for Baby. The weight to volume proportion of cereal to distilled water used was 1:5. The depth of mixed cereal which covered the bottom of the boxes was approximately 1.5 cm. The cereal, obtained from local food stores, contained a mixture of:

- oat flour
- barley flour
- corn flour
- wheat flour
- barley malt flour
- dextrose
- electrolytic iron
- niacinamide
- riboflavin
- thiamin mononitrate

In addition, the cereal was fortified with vitamins A, C, B-1, B-2, and B-6, as well as calcium and phosphorus.

Each culture was maintained for 14 days. The
nematodes tended to crawl up the sides of the containers in large numbers by the end of one week after inoculation, and could be harvested by scraping them off the sides of the boxes, relatively medium-free.

At the end of two weeks, subculturing was done by scraping 5 ml of nematodes off the sides of each container with a small spatula. Each 5 ml aliquot was placed in a 15 ml Pyrex centrifuge tube with 5 ml of room temperature (23-25°C) distilled water. The tubes were centrifuged at 2500 xg for 2 minutes in an International Clinical Centrifuge, model CL, in a swinging bucket rotor with a head radius of 9 cm. This forced the nematodes to the bottom of the tubes. The top water layer was removed with a Pasteur pipette and replaced, mixing the nematodes in with the clean distilled water. This process of centrifugation and water removal was repeated three more times to remove any adhering culture medium and waste. In order to avoid contamination of the cultures, 5 ml of a dilute antibiotic solution (0.6 ug of penicillin-G and 10 ug of streptomycin per ml) was then mixed in with the nematodes in each tube, and the worms were centrifuged once more (Krupp, et al. 1973). After removal of the supernatant, the washed animals were poured into containers of fresh culture medium.

**SAMPLE COLLECTION**

*Panagrellus silusiae* is an ovoviviparous animal with
five larval stages, the first of which (L₁, stage) is intrauterine. The first free-swimming stage (L₂) is followed by the L₃, L₄ and adult (L₅) stages, each being separated by a molt (Bird, 1971). The easiest way of defining the different stages of the life cycle is the length of the worm. The larval stages of the nematodes were identified according to the average lengths of worm, as recorded by Gysels and van der Haegen (1962), which are: L₂ = 350 μ, L₃ = 500 μ, L₄ = 850 μ, and the L₅ males and females are 1400 μ and 1900 μ respectively. These measurements were used to separate the worms by age with the aid of an optical micrometer in the ocular of a microscope calibrated to a stage micrometer.

Sixteen hours prior to sample collection, the 7 day old cultures were covered with an opaque vinyl tent in order to induce the nematodes to reproduce, so that more L₂ worms would be collected. This was done since P. silusiae tends to copulate more often while in the dark, (B. J. Jaskoski, Ph.D., personal communication). The nematodes were then removed from the cultures by using a small spatula to scrape off those individuals that crawled up the sides of the containers. The worms were then divided into 5 ml aliquots which were placed in 15 ml Pyrex centrifuge tubes with 5 ml of room temperature (23-25°C) distilled water. The nematodes were centrifuged and rinsed as in subculturing (see above), but the process was repeated 5 times, and no antibiotic wash was used. This washing procedure removed any adhering cul-
ture medium and most bacteria and other forms of contamination. A sample of the last water layer removed was placed on triple-sugar agar in petri dishes at 25°C and at 37°C for 48 hours, (Davis et al., 1973), to verify the fact that no significant contaminants were present.

**SEPARATION AND AGE SYNCHRONIZATION**

After washing, the nematodes were separated by age using the procedure outlined by Samoiloff and Pasternak (1969). Spherical glass beads of approximately 0.5 mm diameter (Micro bead Dept., Cataphote Division, Ferro Corp., P. O. Box 2369, Jackson, Miss.) were placed in a 125 ml separatory funnel to a depth of 6 cm. The worms plus 100 ml of distilled water were added and allowed to drip out of the funnel at a rate of 1 drop/second. The L2 stage, being the smallest in size, readily passed through the microbeads, while passage of older (and larger) worms was inhibited. More water was added and 10 ml samples continued to be taken, until larger nematodes passed through the beads. The L2's collected in this manner were concentrated by centrifugation at 2500 xg for 2 minutes.

The used beads were removed from the separatory funnel to be cleaned for reuse. First they were rinsed 10 times in tap water. Then the beads were placed in 12 N H₂SO₄ for 20 minutes, with occasional stirring. The H₂SO₄ was then poured off and 6N HNO₃ was added for 5 minutes.
The beads were then rinsed 10 times in cold tap water and 10 times in distilled water. A sample of the beads was then observed under a microscope at 30X to be sure that the beads were thoroughly cleaned. Then they were poured into a finger bowl and placed in a drying oven at 75°C until they were completely dry.

If the L₂ larvae collected were not to be studied at that stage in their life cycle, they were allowed to molt at 23-25°C to a later larval stage. Following the work of Chow and Pasternak (1969), the L₂'s were added to petri dishes containing 10 ml of clear 1% barley solution. They found that in so doing, the ensuing growth to maturation was highly synchronous. The 1% barley solution used in these experiments was boiled for 60 minutes, cooled and filtered to give a clear solution. It was made as needed and stored under refrigeration.

In the barley solution, L₃ larvae were obtained in 24 hours, L₄'s in 48 hours, and adults (L₅'s) in 72 hours (Tortorello, 1975). The nematodes were also kept in the 1% barley solution until they were 10, 15, 20 and 25 days old in order to study the aging adult worms. This part of the study was conducted in a Freas 818 dual program illuminated incubator (Precision Scientific Co., Chicago, Ill.) at 5°C, with a timed day/night cycle which corresponded to the daily sunrise and sunset. This allowed the adults to age, but not to reproduce, since cool temperatures greatly slow down
reproduction in *P. silusiae*, (B. J. Jaskoski, Ph.D., personal communication). Therefore, new L₂ larvae would not be born into the age synchronized cultures.

Every 24 hours, 1 ml of fresh 1% barley solution was added to the petri dishes in each experiment, to offer more fresh nutrients. Any individuals that were not in the same stage as the rest of the synchronized culture were removed manually with a Pasteur pipette before experimentation.

**SAMPLE PREPARATION**

The nematodes to be studied were rinsed 6 times in distilled water and concentrated by centrifugation at 2500 xg for 2 minutes. The resulting pellet of tissue was ground with a Foredom tissue grinder, series DD, at maximum speed (0.8 amps and 300 volts) for 5 to 15 minutes, depending on the stage being ground (L₂ worms required the longest grinding time in order to be completely macerated). The grinding was done on ice to minimize the denaturing of the isozymes. In each experiment 0.01 ml of the homogenate was used to measure the protein content, using the method of Lowry et al. (1951), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as standard (FIG. 1). This was done so that the protein content of all samples of the different stages studied would be identical. Results were read in a Bausch and Lomb Spectronic 20 spectrophotometer.
FIG. 1: STANDARD CURVE FOR PROTEIN DETERMINATION
DISC ELECTROPHORESIS

Polyacrylamide gel was selected as the electrophoretic medium due to its good sensitivity, fine resolution, ease of methodology and for use in densitometric quantitation. These reasons and methods were discussed and summarized by Brewer (1970).

The Research Disc Electrophoresis Standard Reagent Kit (RDS kit) from Canalco (Rockville, Md.), based on the methods of Ornstein (1964) and Davis (1964) was used. The procedure outlined in the Research Disc Electrophoresis Instructions was followed, with the following exceptions. The bridge buffer used was an 0.01M histidine-NaOH buffer of pH 7.5 which was suggested by Robinson (1972). The gels were 10% acrylamide and were made fresh on the day of use. The bridge buffer was never reused. An ice bath kept the bath temperature at a constant 6°C (Bolla, Weinstein and Lou, 1974). The number of tubes used for electrophoresis varied, depending on the volume of nematodes available. Sample size varied, but always contained 100 ug of protein per tube. Electrophoresis was run at 4 mamps/tube. The process was carried out using a Buchler Polyanalyst analytical temperature regulated electrophoresis apparatus and a DeVry dual variable power supply, model 28500. In the preliminary studies, a Heathkit regulated H.V. power supply,
model IP-17 was used. After approximately 100 minutes, the 0.005% bromphenol blue tracking dye was about 1 cm from the end of the tubes and the run was stopped. The sites of acid phosphatase on the gels were determined using the reaction method of Barka (1961), which utilizes a wash and stain buffer of 0.1M sodium acetate-acetic acid of pH 5.0 to rinse the gels 3 times before staining. The gels were incubated for 30 minutes at room temperature (23-25°C) in a solution of 1 mg/ml α naphthyl acid phosphate (aNAP) and 1 mg/ml fast garnet GBC (Sigma Chemical Co., St. Louis, Mo.) in the sodium acetate-acetic acid buffer. The staining reaction was stopped by rinsing the gels 5 times in distilled water. All pH's were measured on a Corning, model 12, pH meter with a Sargent-Welch model S-30072-15 combination electrode (Sargent-Welch Scientific Co., Skokie, Ill.).

After staining, the gels were photographed with a Polaroid model 450 land camera and a Polaroid close-up kit #563 attachment. Polaroid land pack type 108 color film was used, and the camera was mounted on a Phoreto-Phot apparatus (Canalco, Rockville, Md.), which is especially made for the photography of electrophoretic gels. Electrophoretic mobility (Ef) values were determined directly from the gels, by comparing the migration distance of the bands of acid phosphatase with that of the tracking dye. The gels were then stored in distilled water under refrigeration.
DENSITOMETRY

In order to analyze the isozymes separated electrophoretically more completely and accurately, and to quantify the results obtained, densitometric tracings of the gels were made immediately after the end of incubation on a Gelman ACD-15 densitometer (model 39430). The gels run for each nematode age group were scanned at 515 nm (Robinson, 1972; Bolla, Weinstein and Lou, 1974). In order to be scanned by the densitometer, the gels were placed in distilled water in clear glass tubes that were slightly larger than the gels themselves (Ken Hopkins, technical representative for Gelman Instrument Co., personal communication). The open end of each tube was sealed with parafilm, making sure that no air bubbles were present in the tubes. The instructions listed in the Operator Manual for Gelman ACD-15 were followed exactly when operating the densitometer. Heights of the peaks were measured directly from the tracings, and the relative activities were calculated.

ESTIMATION OF BOUND AND SOLUBLE ENZYME

Triton X-100 has been used extensively by biochemists to disrupt the lysosomal membranes and to release those bound acid hydrolases contained within the lysosomes (for example de Duve, 1959). Therefore, the amount of membrane bound and soluble or unbound acid phosphatase in the different nema-
tode stages was determined using a modification of the assay procedures suggested by Meany, Gahan and Maggi (1967).

Triton X-100 (Sigma Chemical Co.) was added in 9 different concentrations using 6 different methods, to a mixture of the stages of washed *Panagrellus silusiae*. The concentrations of Triton X-100 used were 0.1%, 0.5%, 1.0%, 2.5%, 5.0%, 10%, 25%, 50% and 100%. Triton X-100 was added to the nematodes in the following ways: (1) immediately before the nematodes were ground for electrophoretic experimentation, (2) 10 minutes before grinding the nematodes for experimentation, (3) 10 minutes at 37° C before grinding tissue for electrophoresis, (4) immediately after grinding the nematodes for electrophoresis, (5) to pre-ground tissue 10 minutes before experimentation, (6) to pre-ground nematodes 10 minutes at 37° C before running electrophoresis. Each concentration of Triton X-100 was added to the nematodes in all six methods, yielding 54 different possibilities. Each result was compared with the appropriate control of distilled water. In each case, equal portions of nematodes and Triton X-100 solution (or distilled water in the controls) were used.

In order to ascertain the quantity of acid phosphatase liberated in each trial, a total acid phosphatase test (Sigma Technical Bulletin #104) was run on a sample from each test. The instructions in Bulletin #104 were followed exactly, reading the results on a Bausch and Lomb Spectronic
20 spectrophotometer at 410 nm. Percent transmittance was read on the spectrophotometer and converted to units of optical density (O.D.) using a percent transmittance conversion table. Optical density was in turn converted to sigma units/ml of acid phosphatase, using a standard curve based on p-nitrophenol, as outlined in Sigma Technical Bulletin #104 (Fig. 2).

**LIGHT MICROSCOPY**

To ascertain what structures within *Panagrellus silusiae* contain acid phosphatase, a light microscopic study was done on each larval stage, staining the nematodes specifically for acid phosphatase.

The tissue was fixed in 1:10 commercial formalin with 2% formaldehyde (Harleco, Gibbstown, N.J.) for 60 minutes. The fixative was then poured off and the tissue was rinsed in 30% ethanol for 1 minute. This was removed and 50% ethanol was added. After 10 minutes, this was poured off and the tissue was rinsed for 1 minute in 70% ethanol. This alcohol was removed and fresh 70% ethanol was added for 10 minutes. The 70% ethanol was then poured off and the nematodes were rinsed for 1 minute in 95% ethanol. After removal of the 95% ethanol, a 6:1 mixture of 95% ethanol and Loeffler methylene blue was added to the tissue for 5 minutes and then poured off. The methylene blue was added so that the nematodes were more easily seen once they were
FIG. 2: TOTAL ACID PHOSPHATASE CALIBRATION CURVE

![Graph showing the relationship between absorbance and sigma units/ml.

Absorbance values range from 0.0 to 1.0, with markers at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9.

Sigma units/ml values range from 0.0 to 3.0, with markers at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0.

The graph shows a linear relationship between absorbance and sigma units/ml. The error bars indicate the variability in absorbance at different sigma units/ml values.]

SIGMA UNITS/ML

0.0 0.5 1.0 1.5 2.0 2.5 3.0

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
embedded in paraffin. Then, 100% ethanol was added for 5 minutes and removed. Fresh 100% ethanol was then added to the tissue and remained until the time of paraffin infiltration (Jensen, 1962).

The fixed-dehydrated tissue was infiltrated with paraffin at 15 atm and 60° C in a Tissue-Tek II vacuum infiltrator. The L2 nematodes were infiltrated for 1 hour, L3's were infiltrated for 2 hours with one paraffin change after 1 hour, and the L4 and L5 worms were infiltrated for 3 hours, with hourly paraffin changes. The infiltrated nematodes were then positioned in paraffin blocks. After solidification of the paraffin, the blocks were trimmed with a razor blade and sectioned at 10u with a Standard Rotary Microtome, model 820 (American Optical Co., Buffalo, N.Y.). Paraffin sections were affixed to slides with a thin coating of Meyer's albumin.

The sectioned tissue was stained using an acid phosphatase-lead sulfate procedure (Gomori, 1952; Jensen 1956). The slides were placed directly into a substrate solution made by dissolving 0.6g lead nitrate in 500 ml of 0.05 M acetate buffer at pH 4.5, to which was added 50 ml of 0.1M sodium glycerophosphate. Before using, the pH of the substrate was adjusted to pH 5.0. The sections were incubated at 37° C for 4 hours. The slides were then rinsed in distilled water, 2% acetic acid and distilled water again. They were then placed in a 4% solution of
ammonium sulfide. After 20 minutes, the sections were rinsed in distilled water, placed in 95% ethanol for 30 seconds, and then in 100% ethanol for 30 seconds. Deparaffinization was done with two changes of xylene followed by mounting of the tissue with Pro-Mount (Scientific Products, Inc.). The sites of acid phosphatase appeared as black deposits. The control for this study was heat killed tissue (boiled in distilled water for 5 minutes) carried through the entire process (Jensen, 1962).

The sections were studied under phase contrast at 128X (10X, optovar 1.60, ocular 8) and 320X (25X, optovar 1.60, ocular 8) on a Zeiss binocular microscope (#4303364). Photographs were taken with an Olympus-Tokyo C-35A camera, which was mounted on the microscope with an Olympus-Tokyo model 201008 attachment (color temp. reg. 3200, ASA reg. 1.2, color ASA speed 50/100, 6.5v) manufactured by Brinkman Instruments. Kodak 35 mm Kodachrome (KR 135-36) or Ektachrome (EHB 135-36) color film was used for photography.
CHAPTER IV

RESULTS

DISC ELECTROPHORESIS

This study showed that there is a relationship between acid phosphatase activity and age in the free-living nematode Panagrellus silusiae. Through electrophoretic separation, 10 different isoenzymes of acid phosphatase were obtained. The L2 and L3 stages each showed 4 isozyme bands on the polyacrylamide gels. Five separate isozymes were present in the L4's, and 6 distinct bands were found on the L5, 10 day, 15 day and 20 day gels. The oldest nematodes studied, those that were 25 days old, showed 7 isozymes of acid phosphatase, which was the largest number present in any stage of the life cycle.

After measuring the electrophoretic mobility (E_f) values of the bands, it was determined that 10 separate and distinct isoenzymes actually existed. Only 2 of these isozymes were present in all 8 of the stages of the life cycle studied. The average electrophoretic mobility values are listed in TABLE 1: a dash (-) indicates that the isoenzyme was not present at that particular stage.

The isoenzymes of acid phosphatase stained in one of three ways on the polyacrylamide gels. The bands appeared
TABLE 1: AVERAGE ELECTROPHORETIC MOBILITY ($E_f$) VALUES (measured directly from gels)

<table>
<thead>
<tr>
<th>BAND #</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>-</td>
<td>.1176</td>
<td>-</td>
<td>.2581</td>
<td>-</td>
<td>.3521</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.1427</td>
</tr>
<tr>
<td>L3</td>
<td>-</td>
<td>.1077</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.3529</td>
<td>-</td>
<td>-</td>
<td>.6452</td>
<td>1.1385</td>
</tr>
<tr>
<td>L4</td>
<td>-</td>
<td>.1176</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.3516</td>
<td>.5156</td>
<td>-</td>
<td>.6464</td>
<td>1.1406</td>
</tr>
<tr>
<td>L5</td>
<td>-</td>
<td>.1270</td>
<td>-</td>
<td>-</td>
<td>.3143</td>
<td>.3498</td>
<td>.5178</td>
<td>-</td>
<td>.6395</td>
<td>1.1471</td>
</tr>
<tr>
<td>10 days</td>
<td>.0366</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.3171</td>
<td>.3540</td>
<td>.5143</td>
<td>-</td>
<td>.6402</td>
<td>1.1427</td>
</tr>
<tr>
<td>15 days</td>
<td>.0342</td>
<td>-</td>
<td>.1370</td>
<td>-</td>
<td>.3093</td>
<td>.3501</td>
<td>-</td>
<td>-</td>
<td>.6461</td>
<td>1.1398</td>
</tr>
<tr>
<td>20 days</td>
<td>.0328</td>
<td>-</td>
<td>.1393</td>
<td>-</td>
<td>.3099</td>
<td>.3521</td>
<td>-</td>
<td>-</td>
<td>.6380</td>
<td>1.1406</td>
</tr>
<tr>
<td>25 days</td>
<td>.0351</td>
<td>-</td>
<td>.1405</td>
<td>-</td>
<td>.3170</td>
<td>.3513</td>
<td>-</td>
<td>.5956</td>
<td>.6453</td>
<td>1.1385</td>
</tr>
</tbody>
</table>
either red, faint red or yellow (FIGURES 3-10). To ascertain if these three colors each truly represented a band of the enzyme, the individual colored bands from each stage studied were sliced out of the gels and ground in 0.01M histidine-NaOH buffer of pH 7.5 (the bridge buffer). A total acid phosphatase test (Sigma Technical Bulletin #104) was run on the ground slices of gel, which showed that each colored band was truly acid phosphatase. A piece of blank gel was used as a standard.

One of the isoenzymes which occurred in each of the stages consistently travelled beyond the tracking dye, therefore, resulting in an $E_f$ value greater than 1.0 (see TABLE 1). It always appeared as a narrow yellow band near the bottom of each gel, which indicates that it is of a molecular weight less than that of the bromphenol blue tracking dye.

**DENSITOMETRY**

Densitometric tracings were made of the electrophoretic gels from each age group (FIGURES 3-10). Following the suggestion of Bolla, Weinstein and Lou (1974), the relative activity of each of the separated isozymes of acid phosphatase was calculated by dividing the height of each peak by the ug of protein applied to each gel. Peak heights were measured directly from the densitometric tracings. The values obtained appear in TABLE 2: a dash (-) indicates the isozyme was not present in that particular stage. By study-
KEY: □□□□□ = red; □□□□□ = faint red; □□□□□□□ = yellow; □□□□□□□□□ = bromphenol blue (TD)

ELECTROPHORETIC & DENSITOMETRIC RESULTS

FIG. 2

ELECTROPHORETIC & DENSITOMETRIC RESULTS

FIG. 3

ELECTROPHORETIC & DENSITOMETRIC RESULTS

FIG. 4

Ef values

0.3521

0.2581

0.1176

1.1427

0.3529

0.6452

1.1385

0.1077
ELECTROPHORETIC & DENSITOMETRIC RESULTS

KEY: ▢ = red; ▢ = faint red; ▢ = yellow; ▢ = bromphenol blue (TD)

Figure 4

Figure 5
KEY:  red;  faint red;  yellow;  bromphenol blue (TD)

10 DAYS ELECTROPHORETIC & DENSITOMETRIC RESULTS

15 DAYS ELECTROPHORETIC & DENSITOMETRIC RESULTS

FIG. 7

FIG. 8

Ef values

- 0.0366
- 0.6402
- 1.1427

- 0.6461
- 0.3093
- 0.1370
- 0.0342

0.5143
0.3540
0.3171
KEY: 
- red;  = faint red;  = yellow  = bromphenol blue (TD)

20 DAYS ELECTROPHORETIC & DENSITOMETRIC RESULTS

25 DAYS ELECTROPHORETIC & DENSITOMETRIC RESULTS

FIG. 9

EF values
0.357 1
0.367 0
0.1393
0.0328

FIG. 10

EF values
0.357 1
0.367 0
0.5956
0.0351
**TABLE 2: RELATIVE ACTIVITY OF ACID PHOSPHATASE (peak height/ug protein applied to gel)**

<table>
<thead>
<tr>
<th>PEAK #</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
</tr>
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<tbody>
<tr>
<td>L₂</td>
<td>-</td>
<td>2.50</td>
<td>-</td>
<td>2.02</td>
<td>-</td>
<td>2.26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.07</td>
</tr>
<tr>
<td>L₃</td>
<td>-</td>
<td>2.73</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.32</td>
<td>-</td>
<td>-</td>
<td>0.95</td>
<td>1.28</td>
</tr>
<tr>
<td>L₄</td>
<td>-</td>
<td>2.62</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.62</td>
<td>1.25</td>
<td>-</td>
<td>1.04</td>
<td>1.18</td>
</tr>
<tr>
<td>L₅</td>
<td>-</td>
<td>1.57</td>
<td>-</td>
<td>-</td>
<td>0.67</td>
<td>0.50</td>
<td>0.47</td>
<td>-</td>
<td>1.11</td>
<td>0.95</td>
</tr>
<tr>
<td>10 days</td>
<td>0.22</td>
<td>-</td>
<td>-</td>
<td>0.35</td>
<td>0.37</td>
<td>0.33</td>
<td>-</td>
<td>1.11</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>15 days</td>
<td>0.30</td>
<td>-</td>
<td>0.56</td>
<td>0.35</td>
<td>0.35</td>
<td>-</td>
<td>-</td>
<td>1.54</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>20 days</td>
<td>0.24</td>
<td>-</td>
<td>0.38</td>
<td>0.30</td>
<td>0.34</td>
<td>-</td>
<td>-</td>
<td>1.04</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>25 days</td>
<td>0.22</td>
<td>-</td>
<td>0.16</td>
<td>0.18</td>
<td>0.30</td>
<td>-</td>
<td>0.32</td>
<td>1.03</td>
<td>0.26</td>
<td></td>
</tr>
</tbody>
</table>
ing the values listed in TABLE 2, it can be seen that the relative activities of the isoenzymes of acid phosphatase are also related to the age of the individual *P. silusiae*.

In comparing the densitometric tracings obtained for each age group, it can be seen that the individual isoenzymes increase or decrease in relative activity with an increase in the age of the nematode. However, as the nematodes age, there is a general decrease in the relative activity of the enzyme itself, while there is a concurrent increase in the number of isoenzymes present.

**EFFECTS OF TRITON X-100**

Triton X-100 was added to the nematodes in 9 different concentrations, using 5 different methods, in order to estimate the amount of bound and soluble acid phosphatase present in *Panagrellus silusiae*. The amount of the enzyme liberated in each trial was measured using a colorimetric determination of acid phosphatase (Sigma Technical Bulletin #104). The results of each test are listed in TABLE 3.

Method I (the addition of Triton X-100 immediately before grinding the tissue) liberated only 2% more acid phosphatase than the distilled water control. Method II (the addition of Triton X-100 ten minutes before tissue grinding) showed no increase in the level of acid phosphatase obtained. The third method (the addition of Triton X-100 at 37° C, ten minutes prior to grinding the nematodes)
<table>
<thead>
<tr>
<th>METHOD USED</th>
<th>CONC. OF TRITON X-100</th>
<th>I Added and ground immediately</th>
<th>II Added and ground after 10 min.</th>
<th>III Added and ground after 10 min. (37°C)</th>
<th>IV Added immediately to ground tissue</th>
<th>V Ground 10 min after it was added</th>
<th>VI Ground 10 min after it was added (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0%</td>
<td>0.40</td>
<td>0.34</td>
<td>0.39</td>
<td>0.54</td>
<td>0.45</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>0.41</td>
<td>0.34</td>
<td>0.34</td>
<td>0.55</td>
<td>0.44</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>0.42</td>
<td>0.32</td>
<td>0.38</td>
<td>0.57</td>
<td>0.39</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>1.0%</td>
<td>0.40</td>
<td>0.30</td>
<td>0.39</td>
<td>0.57</td>
<td>0.39</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>0.35</td>
<td>0.27</td>
<td>0.41</td>
<td>0.52</td>
<td>0.40</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>5.0%</td>
<td>0.41</td>
<td>0.31</td>
<td>0.42</td>
<td>0.58</td>
<td>0.43</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>0.35</td>
<td>0.29</td>
<td>0.40</td>
<td>0.49</td>
<td>0.44</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>25%</td>
<td>0.39</td>
<td>0.27</td>
<td>0.39</td>
<td>0.52</td>
<td>0.44</td>
<td>0.44</td>
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yielded a 3% increase in acid phosphatase activity. The immediate addition of Triton X-100 to pre-ground tissue (method IV) also yielded a 3% increase in the enzyme present. Method V (the addition of Triton X-100 to pre-ground nematodes 10 minutes before experimentation) showed no change in the level of acid phosphatase when compared to the control. The sixth method (the addition of Triton X-100 at 37°C, to pre-ground tissue ten minutes prior to experimentation) liberated only 1% more acid phosphatase than the distilled water control.

The results of these 6 trials indicate that only a negligible amount of acid phosphatase is bound to membranes within the cells. Therefore, no concentration of Triton X-100 was utilized when preparing tissue samples for experimentation, since at least 97% of the acid phosphatase present in *P. silusiae* could be obtained without it.

**LIGHT MICROSCOPY**

In order to determine what structures within *Pana-grellus silusiae* contain acid phosphatase, the nematodes were specifically stained for the enzyme, and studied using light microscopy. Controls of heat killed tissue were run for each larval stage to prove that any staining which occurred in the experimental studies was truly due to the presence of acid phosphatase and not to something in the staining procedure itself. Examples of the controls are
seen in PLATES 1 and 2.

In the experimental studies, any structure that appeared black in color contained acid phosphatase. The only thing visible in the L₂ stage was the faint line of the digestive tract, indicating very little acid phosphatase was present (PLATE 3). No black stained areas were observable. The L₃ stage stained lightly throughout the length of its body (PLATE 4), indicating a small amount of the enzyme was present. Again, the faint line of the intestine was also visible.

In the L₄'s, a large amount of staining occurred (PLATE 5). For the first time, the esophagus and intestine were stained black, indicating large amounts of acid phosphatase were present. Also, the immature gonads which begin to develop in this larval stage (Bird, 1971), stained positively for acid phosphatase.

A number of internal structures stained black in the L₅ (adult) stage. The entire digestive tract, from esophagus to anus, was again stained, indicating the presence of acid phosphatase (PLATE 6). The reproductive system, which fully develops in this stage (Bird, 1971), also stained positively for the enzyme (PLATE 7). Eggs, visible within the bodies of sexually mature L₅ females, also stained (PLATE 8), as did the excretory canals (PLATE 9). L₁ worms waiting to emerge from the bodies of L₅ females also stained lightly, indicating the presence of a small amount of acid phosphatase, (PLATE 10).
PLATE 1: L₅♂ CONTROL (anterior end)

PLATE 2: L₅♀ CONTROL (posterior end)
PLATE 3: $L_2$ STAINED FOR ACID PHOSPHATASE (whole)

PLATE 4: $L_3$ STAINED FOR ACID PHOSPHATASE (whole)
PLATE 5: L₄♂ STAINED FOR ACID PHOSPHATASE (whole)

PLATE 6: L₅♂ STAINED FOR ACID PHOSPHATASE (anterior end)
PLATE 7: L_{5+}^{O} STAINED FOR ACID PHOSPHATASE (mid-section)

PLATE 8: L_{5+}^{O} STAINED FOR ACID PHOSPHATASE (mid-section)
PLATE 9: L5 ♀ STAINED FOR ACID PHOSPHATASE (mid-section)

PLATE 10: L1's INSIDE L5 ♀ STAINED FOR ACID PHOSPHATASE (mid-section)
CHAPTER V

DISCUSSION

The isozyme approach to the study of biological aging has several good points. For example, studies can be done on crude extracts, and enzyme purification is not necessary (Brewer, 1970). This method also allows the enzymes to be visualized directly. Due to the commercial availability of substrates of high quality, a number of the isozyme techniques have considerable specificity (Maurer, 1971). Many of these specific reactions are catalyzed by multiple forms of the enzymes (isoenzymes), and an understanding of the mechanisms which bring about this enzymatic diversity and the functions it serves, is important to the study of aging.

Electrophoresis was chosen as the isozyme technique for these experiments. Polyacrylamide gel was selected as the electrophoretic medium due to its excellence in resolving multiple molecular forms of protein (such as isoenzymes), its sturdiness in handling, its good keeping qualities, and its suitability for densitometric analysis (Brewer, 1970).

The question arose as to which isozymes of acid phosphatase were involved in the present electrophoretic study, since the experiments would be relatively meaningless if both the unbound enzymes of the cell as well as the membrane-bound isozymes were not accounted for. Since the
unbound enzymes could be easily assayed, a method had to be found to release those isoenzymes bound to membranes within the cells. Since the detergent, Triton X-100 has often been used to disrupt lysosomal membranes to release the bound acid hydrolases (as acid phosphatase) contained in the lysosomes (de Duve, 1959; Robinson, 1972), it was selected to release the bound isozymes of acid phosphatase in these experiments. Since the exact concentration which should be used, and the method in which Triton X-100 should be introduced to the tissue were unknown, a wide range of concentrations (ranging from 0.1% - 100%) and 6 different methods of introduction were used, modifying the technique of Meany, Gahan and Maggi (1967).

Depending on the method of introduction and concentration of Triton X-100 used, no more than 3% of the total acid phosphatase present in *Panagrellus silusiae* was found to be bound by membranes, meaning 97% of the enzyme could be assayed without the use of Triton X-100. This finding was not surprising since similar results were previously exhibited in other organisms, such as the hydroids *Campanularia flexuosa* and *Cordylophora lacustris* (E. E. Palincsar, Ph.D., personal communication). Therefore, the amount of bound acid phosphatase was considered negligible, and the use of Triton X-100 was abandoned.

The results of the electrophoretic study indicate that there is a relationship between the enzyme acid phos-
phatase and aging in *P. silusiae*. The biochemical changes exhibited by acid phosphatase appear to be related to development and aging, and these changes probably result from the involvement of several isozymes (molecular subunits) in the activity of the enzyme.

Ten different isozymes of acid phosphatase were separated electrophoretically in these experiments. The first isozyme (average $E_f = 0.0347$) did not appear until day 10 of the life cycle (i.e., 5 days after the nematodes became adults), and remained throughout the rest of the adult life of the worms. It attained its highest relative activity on the 15th day of the life cycle, indicating it may indeed be connected with the onset of senescence.

Isozyme 2 (average $E_f = 0.1175$) appeared only in the larval stages, disappearing after the nematodes became $L_5$'s (adults). Its greatest relative activity was reached during the $L_3$ larval stage, which seems to indicate that this particular isozyme of acid phosphatase is involved with the development of the nematode, instead of the actual aging process. The third isoenzyme separated (average $E_f = 0.1389$) made its first appearance on day 15 of the life cycle, when it also exhibited its highest value of relative activity. Since it remained throughout the rest of the adult stages studied, it appears to be involved with the aging of the organism.

The 4th isozyme ($E_f = 0.2581$) was only present in
the L₂ stage, with a comparatively high relative activity. Because of this, it seems that it may be related to some very early development in the young nematode larvae. Isozyme 5 (average \( E_f = 0.3155 \)) made its first appearance in the first adult stage \((L_5)\) and remained during the rest of the life cycle. Its greatest relative activity corresponded with its first appearance, which indicates a correlation between this isozyme and the onset of aging.

Isozyme 6 (average \( E_f = 0.3521 \)) was present in every stage of the life cycle that was studied. Its relative activity peaked in the L₄ stage, and continued to drop during the rest of the life cycle. This seems to indicate that it is more involved with development (perhaps with the start of gonadogenesis, since it exhibits its greatest activity during the L₄ stage, which is when this process begins) than with senescence. Isozyme 7 (average \( E_f = 0.5159 \)) exhibited its greatest relative activity concurrently with its first appearance on the gels, which was the L₄ larval stage. This isoenzyme was only present in the L₄, L₅ and 10 day old nematodes. Perhaps it is involved with the onset of the development of some particular structures and disappears upon their completion.

The 8th isozyme \((E_f = 0.5956)\) was only present in the last stage of the life cycle studied (the 25 day old individuals), which seems to indicate an involvement in the final aspects of aging, just prior to death. The 9th iso-
enzyme (average $E_f = 0.6430$) was first present in the $L_3$ stage and continued to appear throughout the rest of the life cycle. Its highest relative activity was attained in the 15 day old nematodes, which indicates that this is another isozyme connected to the senescence process.

The last isozyme (number 10) was present in each stage of the life cycle, but always travelled farther than the tracking dye, therefore, having an average $E_f$ of 1.1413. It attained its highest relative activity value in the $L_3$ stage. This indicates that this isozyme is of a molecular weight less than that of the bromphenol blue tracking dye, and may be involved in both development and aging, since it is always present.

Considering all 10 isozymes together, it can be seen that the $L_2$ and $L_3$ stages exhibited 4 isozymes, the $L_4$'s showed 5 isozymes, the $L_5$, 10 day, 15 day and 20 day old nematodes had 6 isozymes present, and the oldest worms (25 days) had 7 isozymes of acid phosphatase. This means that the older the individual $P. silusiae$, the greater the number of molecular forms of acid phosphatase present. However, the relative activities of the different isozymes reached their highest levels at different stages in the total life cycle, and the over-all enzyme activity level decreased with increasing age. These results are consistent with the observations of Erlanger and Gershon (1970) on $I. aceti$ and Bolla, Weinstein and Lou (1974) on $N. brasiliensis$. 
They concluded that these biochemical changes correlate with the morphological and physical changes that occur during the different stages of development and aging throughout the life cycle of the nematode.

However, these results are not in agreement with the findings of Chow and Pasternak (1969), who also studied *Panagrellus silusiae*. In their simultaneous study of LDH, MDH, esterase, acid phosphatase and alkaline phosphatase, they found that only a single band (i.e. one isoenzyme) of acid phosphatase was present in the same place on the electrophoretic gels in the L2, L3, L4 and L5 stages. In addition, Chow and Pasternak did not collect samples beyond the L5 stage because their measurements showed that young adults do not significantly differ in length from older and more mature worms. It was therefore concluded that because the nematodes did not increase in length as they aged, the enzyme levels would also remain the same. They also used a modified method of Gomori's to stain the gels for the presence of acid phosphatase. According to Weinstein and Mandel (1969), the Gomori technique for staining electrophoretic gels utilizes a non-specific reaction which often yields control gels that show similar staining patterns to experimental gels. Weinstein and Mandel also found that the incubation medium of fast garnet and alphanaphthyl phosphate (the staining procedure used for the electrophoretic gels in the present study) proved to be more specific than the
Gomori technique. These facts seem to question the results of the acid phosphatase work done on *P. silusiae* by Chow and Pasternak.

One of the most obvious facts which appears when glancing at the electrophoretic gels, is that the enzyme stained in three different ways. The isozyme bands appeared either red, faint red or yellow in color. Since each band tested positively for the presence of acid phosphatase, there must be a reason for the different colors. The difference between the two shades of red is easily explained by studying the relative activities of those bands. Those isozymes that stained faint red were always of a lesser activity than the isozymes that stained red on the same gel. This seems to indicate that there was less of each faint staining isozyme than of those which stained darker. However, the yellow bands are not so easily explained. Following the logic just used, it would seem that yellow bands represented isozymes of very low relative activity, but this showed not to be the case. Isoenzymes that stained yellow were often of equal or greater relative activity than the isozymes that stained faint red. MacIntyre (1971) helps provide an explanation. By studying the reactions of the staining procedure used in this research (Fig. 11), he found that although the final colored complex should contain two molecules of fast garnet, sometimes this does not
FIG. 11: CHEMICAL REACTIONS INVOLVED IN THE STAINING PROCESS

\[ \text{OPO}_3\cdot\text{Na} \rightarrow \text{acid phosphatase} \rightarrow \text{\(\alpha\) NAPHTHYL ACID PHOSPHATE} \rightarrow \text{\(\alpha\) NAPHTHOL} \]

\[ \text{\(\alpha\) NAPHTHOL} + 2 \text{FAST GARNET} \rightarrow \text{SPONTANEOUS} \rightarrow \text{MONOCOUPLED (YELLOW-ORANGE)} \]

\[ \text{DICOUPLLED (RED)} \]
occur as spontaneously as is theorized, and the end product may contain only one molecule of fast garnet. If this is the case, the colored complex appears yellow-orange and not red. Apparently the yellow bands found on the electrophoretic gels are due to a lack of spontaneity in the staining reaction, resulting in a yellow, monocoupled, colored complex instead of a red, dicoupled, colored complex.

There has been much controversy in recent years regarding the best technique to use for keeping adult nematodes alive and aging without allowing them to reproduce, in order to keep age synchronized cultures free of young larval stages. Hydroxyurea is often used in various concentrations to meet this purpose. Pasternak and Samoiloff (1970) first reported the effects of a 500 μg/ml concentration of hydroxyurea, stating that body growth in *P. silusiae* was only slightly retarded. Later, Westgarth-Taylor and Pasternak (1973) reported with a concentration of 2000 μg/ml, that body size in *P. silusiae* was normal after 196 hours, and that protein synthesis was reduced by 18%. They also found additional unexplainable effects of treatment, including surface blisters, large cracks and excessive growths in the adult cuticle after the final molt. Kisiel, Nelson and Zuckerman (1972) found that worms treated with chemical inhibitors were never more than 17% of the volume of untreated worms, and that treated worms had shorter lives than untreated ones.
Due to the ambiguity surrounding the actual effects of hydroxyurea, it was decided to approach the aging-without-reproducing problem from a different avenue. Instead of using such a harsh chemical, lowering the temperature of the nematodes' environment was selected as a less controversial technique since low temperatures simply slow down reproduction in *P. silusiae* (B. J. Jaskoski, Ph.D., personal communication). It was realized from preliminary cryptobiosis studies, that *Panagrellus silusiae* slows down its body processes when exposed to low temperatures (G. N. Doering, unpublished results), but it was decided that this was a much less radical method of attaining aging-without-reproduction than the use of hydroxyurea, and should therefore, introduce less error into the experimentation.

In order to ascertain what structures within the body of *P. silusiae* contain concentrations of acid phosphatase, a light microscopic study was done, staining the nematodes specifically for the enzyme. This study showed that the gastrointestinal tract and the reproductive system contain high concentrations of acid phosphatase.

Slides of the *L₂* stage showed very little staining for acid phosphatase indicating a low activity of the enzyme. However, the electrophoretic and densitometric studies indicated a high level of acid phosphatase activity, but very few isozymes. The *L₃* stage showed more staining, but again, not as much as was indicated by the electrophoretic
and densitometric results. Due to these somewhat conflicting results; further study of these two larval stages is necessary.

Complete staining of the gastrointestinal tract was obvious in the L₄ stage. The developing gonads also stained for acid phosphatase. In the L₅ (adult) stage, the digestive system as well as the complete reproductive system showed high concentrations of the enzyme. Eggs within the adult females, excretory canals, and unborn L₁ nematodes also stained positively for acid phosphatase.

This light microscopic study therefore seems to indicate that acid phosphatase is present in high concentrations in the gastrointestinal tract, the excretory canals, and the entire reproductive system of Panagrellus silusiae, which is consistent with previous findings in other nematodes (Cesari, 1974).

The results obtained in this research seem to lend support to the hypothesis that with increased age, acid phosphatase activity lowers the intracellular concentration of metabolically important phosphate esters and gradually shifts the equilibrium in the cell away from synthesis and towards catabolism, thus resulting in a general deterioration of the cells (Cristofalo et al., 1967).
SUMMARY

1. This study showed that there is a relationship between acid phosphatase levels and development and aging in the free-living nematode, *Panagrellus silusiae*.

2. Ten different isoenzymes of acid phosphatase were separated electrophoretically.

3. Relative enzyme activity peaked at different stages in the life cycle for the different isozymes.

4. As the nematode ages, there is a general decrease in the relative activity of acid phosphatase itself, while there is a concurrent increase in the number of isozymes present.

5. Through the use of Triton X-100, it was found that at least 97% of the acid phosphatase in *P. silusiae* is soluble (unbound).

6. Acid phosphatase appears to be present in large quantities in the gastrointestinal tract, the excretory canals, and the entire reproductive system of *P. silusiae*.

7. This study seems to question the previous work of Chow and Pasternak (1969) on acid phosphatase in *Panagrellus silusiae*, since they separated only one isozyme of the enzyme in the larval stages, and utilized a less specific staining reaction.


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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

May 5, 1977  E. E. Palinscar
Date Director's Signature