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Microelectrophoresis as a Means of Comparing Acid Phosphatase Isoenzymes in Aging *Campanularia flexuosa*

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MICROELECTROPHORESIS AS A MEANS OF COMPARING
ACID PHOSPHATASE ISOENZYMES IN AGING
CAMPANULARIA FLEXUOSA

by

Paul Joseph Meus

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

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1979

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VITA

The author, Paul Joseph Meus, is the son of Joseph F. Meus and Adeline (Lach) Meus. He was born February 16, 1952 in Chicago, Illinois.

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INTRODUCTION

Acid Phosphatase (APase) is a hydrolytic enzyme that increases in aging cell cultures, organs and animals. It has been suggested by Strehler (1961) and Brock (1970) that APase could cause regression in hydranths of Campanularia flexuosa. Electrophoresis has been done on the tadpole tail to compare the isoenzyme patterns of APase during regression (Filburn 1973). A micro procedure would utilize the small sample size of Campanularia for proper separation. The comparison of the location and size of the isoenzyme between the young and old hydranths of Campanularia could be beneficial in determining the role of APase during the aging process.

REVIEW OF RELATED LITERATURE

Campanularia flexuosa is a marine coelenterate. The animal is a colonial hydroid. The body wall consists of two cell layers, an ectoderm and a gastroderm. In the phylogenetic tree they are at the tissue level of organization. Each colony consists of hydranths attached to an upright stalk and new stalks are formed from stolon growth. The individual hydranths go through a growth cycle lasting approximately fourteen days. First a bud forms on the pedicel, which is the point of attachment of the hydranth to an upright stalk. See Figure 1. The bud increases in size and becomes cylindrical. During this time the tentacles are forming within. The hydranth is covered by a hydrotheca which is characteristic of this suborder of hydroids and serves to protect the hydranth. The developing animal receives nourishment from the feeding hydranths in the colony. In this hydranth the tentacles are extended and begin to feed for an average of 6.8 days (Crowell 1953). The tentacles, after this time, are retracted within the hydrotheca at the beginning of senescence. The hydranth appears to digest itself until an empty hydrotheca is all that remains. This is sloughed off and after a dormant period a new hydranth will form in the same position. See Figure 2. In this way an upright may contain ten or twelve hydranths in various phases of the life cycle. The growth is affected by environmental factors. Water temperature, and food supply have both been shown to affect the number of feeding hydranths and the length of the cycle (Crowell 1953).

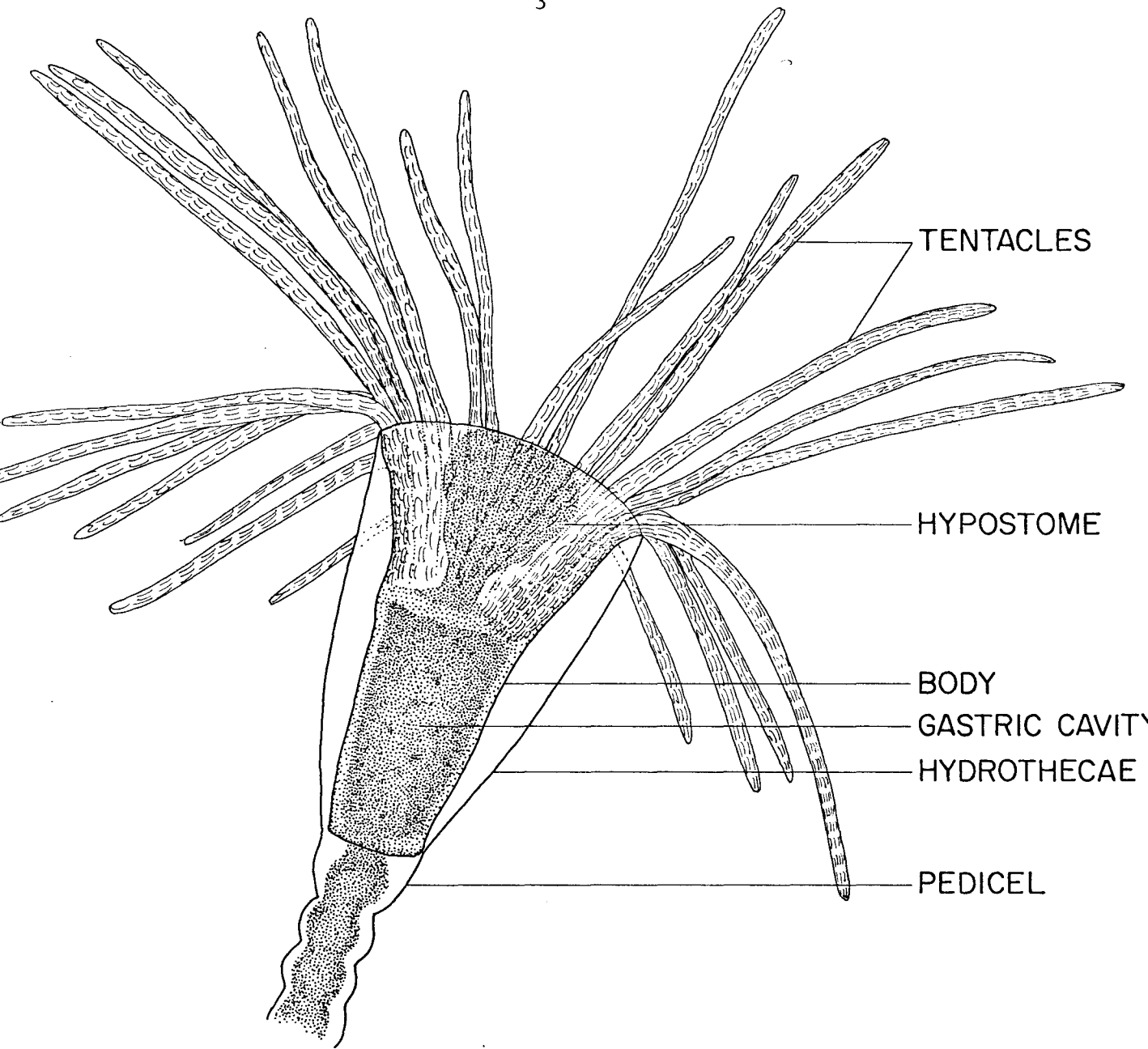


FIGURE 1
DIAGRAM OF
FEEDING CAMPANULARIA FLEXUOSA

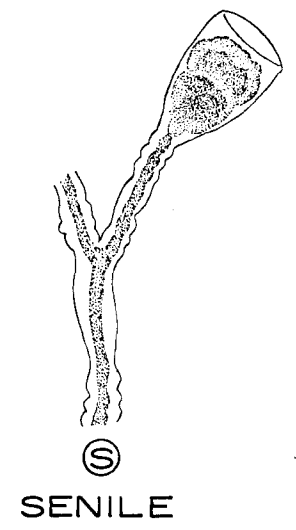
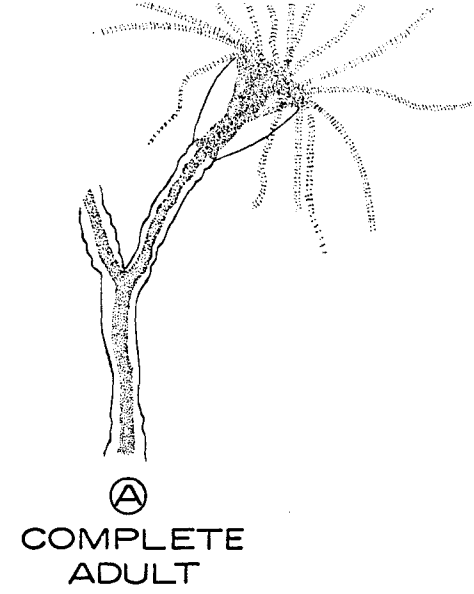
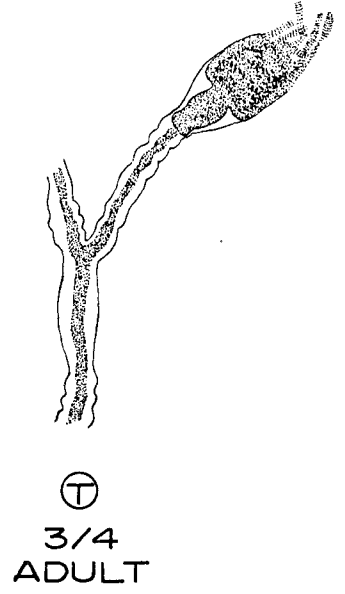
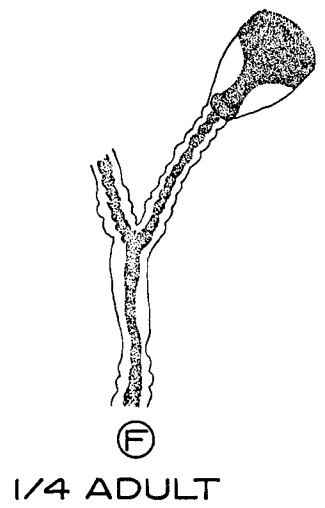
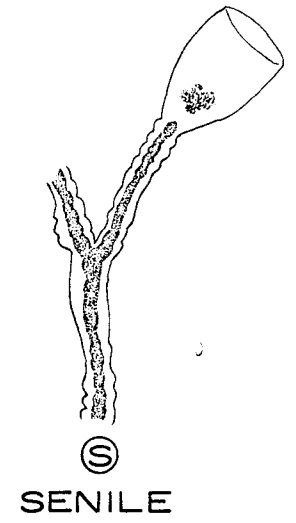
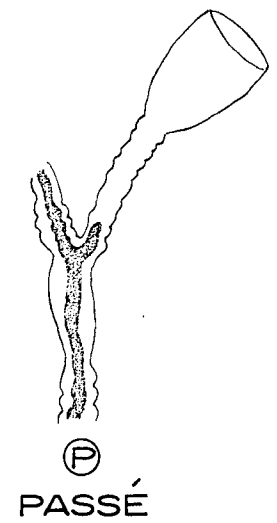
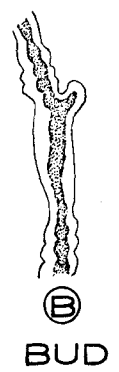
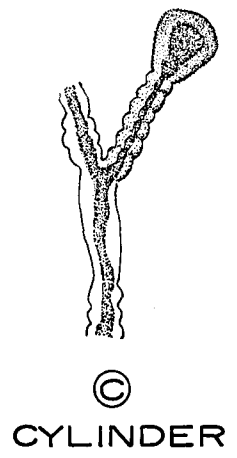


FIGURE 2
LIFE CYCLE OF
CAMPANULARIA FLEXUOSA



Strehler (1961) and Brock (1970) have both done work on Campanularia, with emphasis on aging. Strehler questioned whether activation of lysosomes was responsible for cell death in the regressing hydranth. Since the hydranth is digested during senescence there must be some type of hydrolytic enzyme present. Lysosomes are known to contain acid phosphatase. The Gomori staining method showed almost no APase in the young hydranth while after ten days the aging hydranth showed accumulations of acid phosphatase in the gastrodermal and ectodermal cells (Strehler 1961). Electron microscope studies comparing young and old hydranths showed bodies which appeared to be primary lysosomes, secondary lysosomes and residual bodies. The bodies were seen in the young but they were more numerous in the old hydranths (Brock 1970).

The lysosomes are cytoplasmic particles that take part in intracellular digestion. They contain acid hydrolases and function in defense, absorption and digestion. Acid phosphatase, ortho-phosphoric monoester phosphohydrolasê (E.C. 3.1,3.2) is membrane bound in the lysosomes. Their formation is not certain because they differ in kinetics and properties, such as activation, inhibition and optimum pH, from the plasma membrane of the cell (Kaulen 1970). Also, it has been shown in rat liver lysosomes that the lysosomal membrane contains APase and not the lysosomal content (Berzins 1975). This could be important when working with aging cells. Cristofalo (1969 and 1972) found that in human fibroblasts cultured through 45 passages, there was an increase in APase in the adult cultures. Cristofalo states there is either an increase in APase or the aging lysosomal membrane has become fragile. In this way more APase would be released during sample preparation. When the

membrane was stabilized the sample still showed an increase in APase activity.

The activity of APase in senescent cells has been observed in rat pituitary, (Smith and Farquhar 1966), rat liver (deDuve 1955), human lung (Cristofalo 1967 and 1972), human leukocytes (Li 1970), and in tadpole tails (Filburn 1973). In these cases APase was seen functioning in absorption or differentiation. Earlier work on the tadpole tails showed that regression could be controlled by the blocking of enzyme synthesis. Tata (1966) maintained cultures of tadpole tails. The regression could be initiated by the addition of triiodothyronine and observed by the uptake of C^{14} and H^3 during RNA synthesis. The regression could be stopped by the addition of actinomycin-D, puromycin and cycloheximide. Since one of the functions of APase is absorption, Filburn (1973) concentrated on its activity in tadpole tails during regression. Electrophoresis separated four isoenzymes of APase. An isoenzyme is a variation in molecular form of an enzyme in an organism, performing the same function. They may vary in their molecular weights so when placed in an electrophoretic chamber the individual isoenzymes will migrate at different rates. Differential staining highlights the isoenzymes for study. During the regression of the tadpole tail, two isoenzymes increased, one showed no change and one showed a slight decrease. When protein synthesis was inhibited, the regression was blocked. The APase was nonspecific in its hydrolysis of the substrates tested.

The isoenzyme banding varied depending on the organ and animal used (Lundin 1966). Generalizations could not be made on the number of

APase isoenzymes expected from a given location. Enzyme patterns would vary with the species being compared. In human leukocytes seven isoenzymes of APase were seen after electrophoresis (Li 1970). The testing showed that the isoenzymes varied not only in molecular weight but also pH range, substrate specificity and reaction to inhibitors. This study raises the possibility of one or more isoenzymes causing the autolysis of a cell or organelle.

In these studies electrophoresis was used to separate the APase into its isoenzyme patterns. The use of electrophoresis to separate charged particles began with the work of Tiselius in 1937 (Umbreit 1972 and Winfield 1972). His procedure was called the moving boundary technique. He utilized a U shaped tube containing a solution of charged particles. A buffer was layered on each side of the charged solution and an electrode inserted. The current passing through the U tube caused charged particles to migrate. The velocity and direction of migration depended on their charge and isoelectric point. The particles would form interfaces that could be observed with a complex optical system. The drawbacks to this system were that it did not use a solid support media for separation, it needed to be refrigerated and it needed a complex optical system to interpret the results. To improve separation of the charged particles a solid support media was used such as filter paper or cellulose acetate. The technique was called zone electrophoresis because the sample was placed in a particular zone on the medium and then migrated with an electric current. The particles were separated as to size and charge. In 1955 Smithies introduced starch gel as the support medium. He was followed in 1959 by the groups of Ornstein-Davis and Raymond-

Weintraub who introduced polyacrylamide gel as the support media. Polyacrylamide gel is used today because of its versatility in creating pore size. In this way, the separation of charged particles is by shape as well as molecular size and charge. Some of the other advantages are its thermostability, transparency, strength and that it is relatively inert chemically (Ornstein 1964).

A support media of polyacrylamide is formed by the addition of acrylamide, a basic monomer, to N,N'-methylenebisacrylamide (Bis) which is a crosslinking comonomer (Umbreit 1972). The ratio of these two chemicals determines the transparency, elasticity and pore size of the gel. The addition of a catalyst and initiator, ammonium persulfate and N,N,N',N'-tetramethylethylene diamine (TEMED), begins polymerization. Riboflavin when added with TEMED, accelerates the photopolymerization. To maximize the separation of charged particles consideration must be given to the buffer, pH, voltage and pore size in the system.

Polyacrylamide gel electrophoresis is usually performed in a glass tube approximately seven centimeters long and 0.5 cm in diameter. This test size can analyze a sample of 2×10^{-4} grams (Canalco 1969). The tube contains the polymerized polyacrylamide and the sample. The electrophoresis chamber suspends the tube so that both ends of the tube are within a buffer bath. The lower bath contains the positive electrode and the upper bath the negative electrode. The baths are separated so when a power source is applied, the tube will complete the circuit. The current passes through the tube causing the charged particles to migrate. The electrophoresis chamber has a circulating cold water sleeve around

the baths. This is to keep the temperature low within the tubes. The amino acids in the sample would denature if heat build up due to resistance to current flow were not controlled.

In 1966 Hyden and Bjurstam developed a procedure for micro-electrophoresis. This enabled them to separate the proteins in single nerve cells and glia from the rat brain. The range for sample size is 10^{-7} to 10^{-9} grams. Hyden and Lange in 1968 further developed this procedure. The electrophoresis was carried out in capillaries of 0.2 to 0.45 mm in diameter. The gels were stained with amido black in 7.5% acetic acid which is a general protein stain. The gels were then scanned with a densitometer to determine the number, location and relative amounts of protein present. In this way the protein content of cells could be compared within a single brain or between brains. Friz in 1970 utilized the procedure of Hyden and Lange (1968) to analyze the proteins in three individual amoebae species. He varied the number of animals per sample and found the results from the same species were identical.

Cordylophora lacustris is a member of the Phylum Coelenterata. It is a freshwater species and lacks a hydrothecae. Chandler Fulton (1960-61) did research concerning the culturing and growth of the colony. The colony grows best in brackish water. The asexual development consists of repeating units of hydranths, upright stems and stolons. The Cordylophora is an example of an invertebrate that does not have a regular replacing life cycle. This growth cycle differs from the suborder Calyptoblastea containing the hydrothecate animals. Crowell's (1953 and 1960) work on Campanularia found that the life cycle is regular. In

Campanularia the older hydranth does not increase in size and is reabsorbed within fourteen days of bud formation. It has been postulated from the study of Tubellaria suborder Gymnoblastera, which also contains the Cordylophora that under favorable conditions the animal will survive indefinitely (Mackie 1966). As the hydranth grows it will increase in size as well as regenerate any parts lost.

The Cordylophora was used as an animal control for my testing procedure. The Cordylophora was subjected to the test simultaneously with the Campanularia in this project. Theoretically, the Cordylophora should have the same quantity of APase whether it is tested right after development or in a long term adult. This should be the case since regression is not seen on a regular basis. The production of APase should remain constant.

My format was to find a workable microelectrophoresis procedure that could be used to identify and separate the isoenzymes of APase in Campanularia flexuosa. To do this, the variables in the procedure would be tested to find the optimum conditions for separation to occur. Different buffers were tested at different ionic strengths. The percent polyacrylamide gel was varied so the pore size or sieving effect was changed. The voltage as well as the run time were changed. Then after a reproducible procedure was found, it was used to compare the isoenzyme concentrations at the different stages of development.

MATERIALS AND METHOD

The Campanularia flexuosa, obtained from Woods Hole, Massachusetts, were cultured so that a large, replenishing sample population would be made available for testing. A stalk was secured by a thread to the slide. The slides were placed in boxes and submerged in salt water holding tanks (Instant Ocean, Aquarium Systems, Inc.). These tanks were maintained at a specific gravity between 1.020 to 1.024 using synthetic sea salts (Instant Ocean, Aquarium System, East Lake Ohio). The holding tanks were designed with built in aeration, filtration, and cooling units. The water temperature was constant at 20°C which was the optimum temperature for the growth and doubling of Campanularia (Crowell, 1957).

The colonies were feed daily for twenty minutes, on freshly hatched Artemia salina. The dried Artemia eggs were aerated for forty-eight hours in plastic containers containing salt water. The slide boxes were placed in feeding tanks and covered with water from the holding tanks. The hatched Artemia were poured into the feeding tanks. The feeding hydranths of Campanularia would trap the Artemia in their tentacles and withdraw the animal into the gastric cavity where digestion occurs. While this was proceeding, fresh salt water was placed in the holding tanks. After feeding, the individual slide boxes were rinsed in running water and returned to the holding tanks.

The growth cycle of the Campanularia was observed using a dissecting scope (Forty American Optical Co.). An individual upright stalk was singled out by marking the slide. This slide was then observed daily and the stage of development of the hydranths recorded. Figure

3 shows the diagram used to record hydranth growth and the means of differentiation. The temperature and specific gravity of the salt water holding tanks were also recorded. In this way the life cycle of Campanularia could be determined under our laboratory conditions.

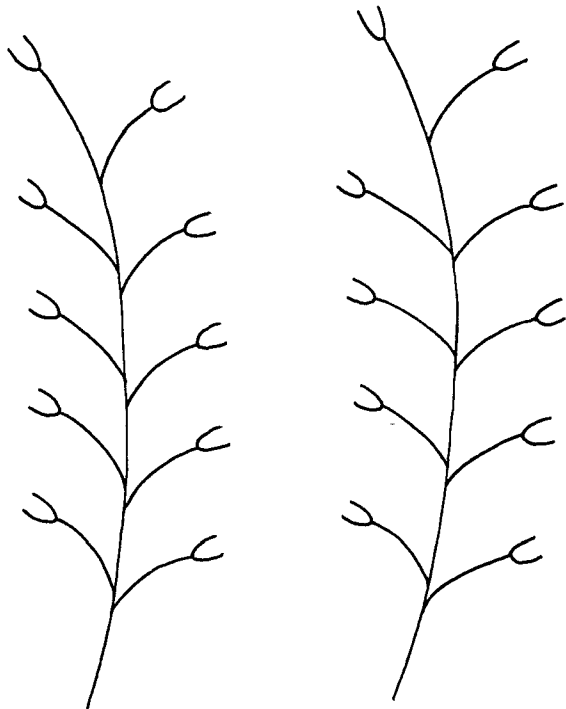
The individual hydranth was removed when preparing a sample for electrophoresis, using delicate forceps. Each sample contained between one and ten hydranths in a particular stage of development. The test was performed on three different age groups of Campanularia. Group one was the hydranth in the cylinder stage of development. The tentacles were only beginning to form within the hydrothecae. Group two was the feeding hydranth, with its tentacles fully extended. Group three was the regressing hydranth with the tentacles withdrawn in the hydrothecae. Ten hydranths of the adult Cordylophora were used as the control animal.

The Cordylophora lacustris were cultured in a similar manner to the Campanularia. The slide with the culture was placed in a beaker and filled with brackish water. The brackish water was prepared using the method of Fulton (1960):

Five gallons of distilled water

add: 1.2 g Versene (1.5×10^{-4} M disodium ethylene-
diamine tetracetate)
58.4 g sodium chloride
2.0 g potassium bicarbonate
15.8 g calcium chloride
20.4 g magnesium chloride

The beakers were maintained at a temperature of 22°C, and the water changed twice a day. The feeding was daily for twenty minutes using freshly hatched Artemia salina. The Cordylophora were observed and recorded in the same manner as the Campanularia.



CODE

- B - Bud
- C - Cylinder
- F - 1/4 Adult
- T - 3/4 Adult
- A - Complete Adult
- S - Senile
- P - Passé

DATE _____

TEMP. _____

SP. GR. _____

FIGURE 3
GROWTH CHART

A concentration of 0.25 M Sucrose with 0.5% Triton X-100 was used to prepare the sample. The Triton X-100 was used in the sample medium because it is a non-ionic detergent which has been shown to solubilize protein aggregates. This aids in the separation of proteins during electrophoresis (Eastman Kodak Co. 1976, Hyden and Lange 1968). A 0.5 ml of the sucrose-Triton X-100 solution was mixed with a varying number of hydranths, between one and ten. Micro tissue grinders, a high speed dental drill and sonication were all tested for maximum homogenization of the hydranths. The grinders, test tubes and capillary tubes were kept on ice while the hydranths were selected for testing. The hydranths were homogenized and observed under the dissecting scope. The sample was then centrifuged and placed on ice until the testing was begun.

The electrophoresis was performed in capillary tubes from Kimble Products (Kimax-51). The tubes were prepared by boiling in a detergent solution of Alconox (Scientific Products), then rinsed three times with distilled water, once with acetone and allowed to dry. The tubes were coated with 0.1% methyl cellulose and dried overnight at 90° C. This tube preparation aids in the prevention of electroendosmosis during electrophoresis (Friz, 1970). Electroendosmosis is a movement of buffer ions in the space between the gel and glass tube instead of the movement of the sample through the separating gel.

The ratio of acrylamide to N,N'-methylene-bisacrylamide was varied to find the polyacrylamide gel with the best sieving effect for APase. A three and a half, seven, ten, fifteen, and twenty-two percent separating gel was tested. The smaller the percentage the larger the pore size. A large pore size might have no sieving effect while too

small a pore size would inhibit the migration of the protein completely.

The buffers tested in the upper and lower baths of the electrophoresis chamber were a Histidine-sodium hydroxide buffer (Filburn, 1973) at a pH of 6.8 and a Barbital buffer (Nirenberg, 1966 and Cawley, 1969) at a pH of 8.6. Their ionic strengths were varied from a 0.01 M concentration to a 0.05 M concentration. They were tested to find the buffer and concentration needed for the best separation and resolution. The effect of concentration on migration is that the lower the concentration the better the protein migration. The higher the concentration the greater the heat build up in the tube.

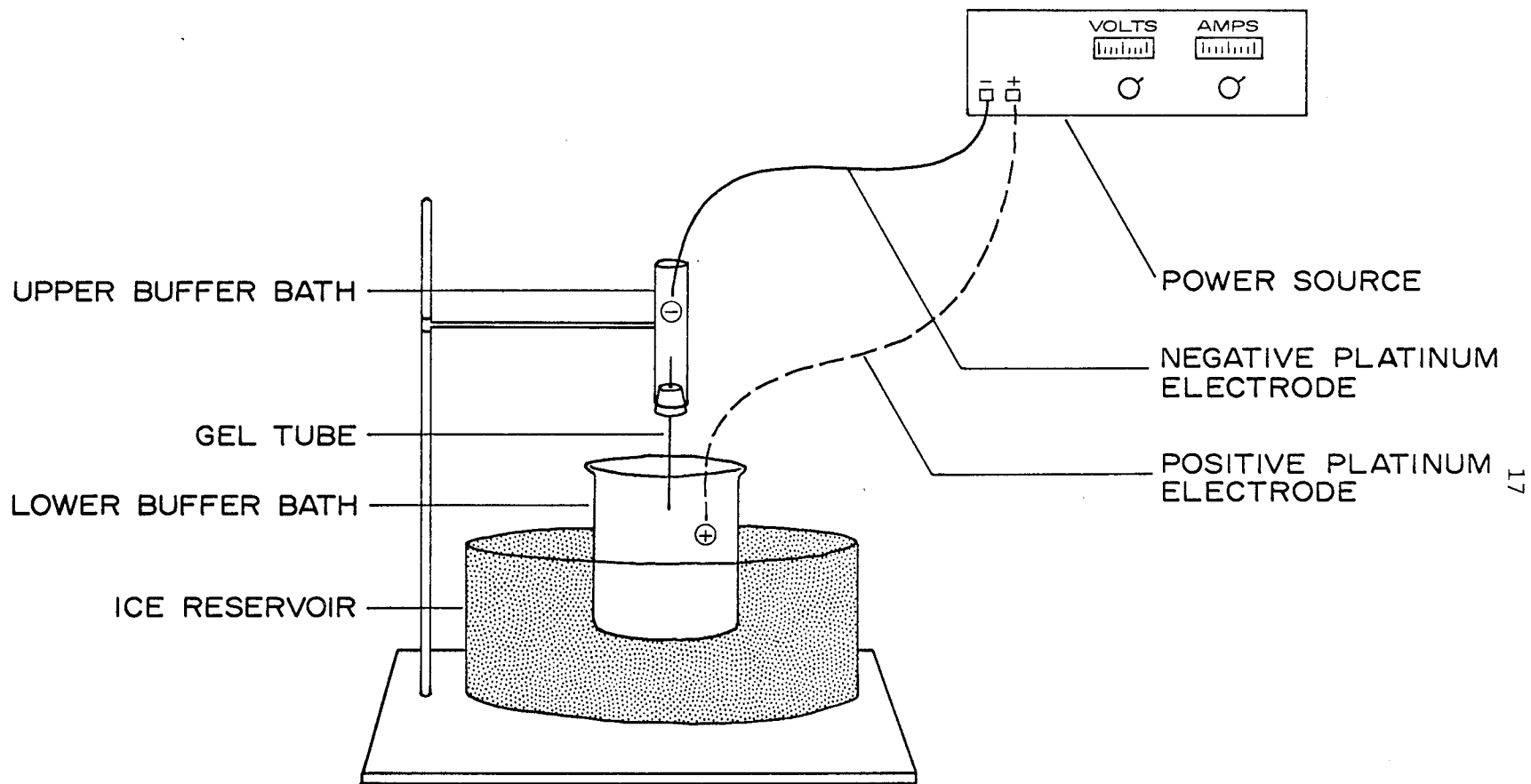
After the electrophoresis tubes were prepared, the separating gel was drawn up the tube by capillary action. The lower portion of the tube was capped, while the upper end of the separating gel was water layered using a microsyringe. The water layer smoothed the gel surface and prevented a meniscus from forming. The smooth interface between gels or gel and buffer aids in the clear separation of protein bands during electrophoresis. The tubes were placed upright in a rack and the gel polymerized for one hour in front of a fluorescent light source. Following polymerization of the gel, the water layer was removed by inversion of the tube and the cap removed. Dropwise, two microliters of sample were layered on the upper end of the separating gel using a microdispenser (Drummond Microdispenser, Drummond Scientific Co.). One drop of 0.005% Bromophenol Blue was layered on the sample to act as a tracking dye. The dye leads the migration of protein through the separating gel and was visible during electrophoresis. Thus, the current was stopped before the enzymes migrated completely through the gel. The

remainder of the tube length was filled with the buffer being tested.

The capillary tubes were tested singularly and in groups of eighteen at one time. The singular tube apparatus utilized a glass tube as the upper bath and a small beaker as the lower bath. See Figure 4. The lower bath was placed on ice. The capillary tube was suspended from a rubber stopper in the upper bath. A platinum wire was placed in each bath and connected to a power source. The tubes were tested under the varying conditions of electrophoresis and the results noted.

The commercial electrophoresis apparatus consisted of an upper and a lower buffer bath, a circulating cold water sleeve and space for eighteen tubes (Buchler Instruments, Fort Lee, N.J.). See Figure 5. The tubes were inserted through rubber stoppers. The stoppers enabled the electrophoresis tube to be suspended between the upper and lower baths. The baths were filled with cold buffer. To maintain the baths between 0-4°C, cold water was pumped from a reservoir through the sleeve by a stir pump (GCA/Precision Scientific Co. #11-AG-8). The positive platinum electrode was in contact with the lower bath while the negative was within the upper bath. The electrodes were hooked up to a Heathkit power source (Model #IP-17). When assembled the polyacrylamide gel completed the current loop. The siphoning of current through the gel would cause charged particles within the sample to migrate toward their respective poles.

The migration of the charged particles was effected by the constant voltage. The power source was adjusted to test voltages between 5 and 200 volts during a run. Also the length of time the voltage



17

FIGURE 4

SINGLE TUBE ELECTROPHORESIS APPARATUS

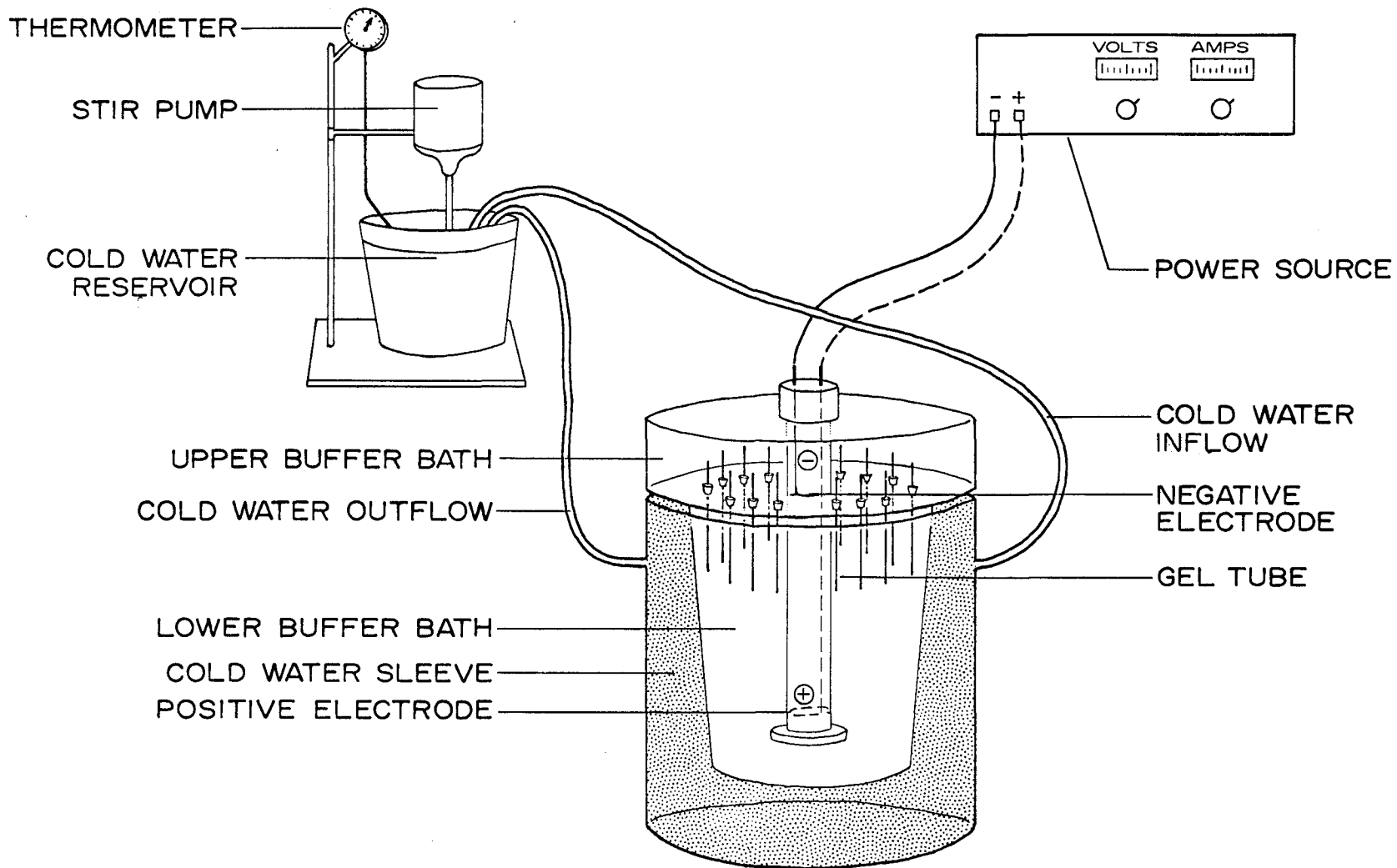


FIGURE 5
MULTIPLE TUBE ELECTROPHORESIS APPARATUS

was applied was varied from thirty minutes to three hours. The problem with prolonged run time or excessive voltage was the build up of heat that occurred. As the voltage increases the current increases. Since heat is equal to current squared times the resistance to flow times time ($H = I^2Rt$), then the longer the run time and the higher the voltage the greater the heat produced (Winfield, 1972). The heat would denature the protein and affect the results. Following the test, the tubes were removed from the chamber and the gels forced out of the tubes with water pressure from a syringe.

The enzyme specific stain for APase contained 1 mg/ml of alpha naphthyl acid phosphatase and 1 mg/ml of fast garnet red GBC salt in 0.1 M acetic acid-sodium acetate buffer at a pH of 5 (Li, 1971). The APase hydrolyzes the alpha naphthyl acid phosphatase sodium salt into naphthol and phosphorus. The diazonium salt, fast garnet red GBC salt, couples with the alpha naphthol to form the stained compound (Nerenberg, 1966). The gels were placed in the stain for six hours. The gels were then removed and placed in 7% acetic acid to remove any unbound stain. Following destaining the gel was inserted in a small glass tube and sealed. The gel was then scanned with a densitometer (Gelman ACD-15, Gelman Instruments Inc.) to show enzyme migration. The gel and graph were then photographed for a permanent record (Polaroid MP-3 Land Camera-Multipurpose Industrial View Camera).

The samples obtained from the three groups of Campanularia and the one from Cordylophara were assayed for total acid phosphatase activity. The concentration present was determined by the ability of the sample to hydrolyze p-nitrophenyl phosphate to p-nitrophenol. The

p-nitrophenol is colorless in an acidic environment and yellow in an alkaline environment. On completion of the assay time, sodium hydroxide was added to the test. This stops the reaction and produces an alkaline environment. The color change that occurs was then observed using a spectrophotometer (Spectronic).

Two tubes, each containing 0.5 ml of a substrate and 4 mg/ml of p-nitrophenyl phosphate disodium, were placed in a 37^o C water bath. To this was added 0.5 ml of an acid buffer in each tube. The acid buffer consisted of 0.09 mol/liter citric acid in 0.01 M HCl at a pH of 4.8. 0.2 ml of water was placed in one tube for the reference blank and 0.2 ml of the sample in the other tube for the test. The sample was prepared in the same fashion as those used in electrophoresis. The reaction proceeded for thirty minutes in the 37^o C water bath. The reaction was stopped with 6 ml of 0.1 N sodium hydroxide in each tube. If any p-nitrophenol was formed, the tube would turn yellow. The tubes were then read with a spectrophotometer at an absorbance of 410nm. The machine was zeroed using the reference blank and the test was read against that setting. The units of APase were determined from a calibration curve which was constructed previously, using known quantities of p-nitrophenol in the testing procedure (Sigma Chemical Co.).

RESULTS

The Campanularia were maintained in salt water holding tanks at a temperature of 20^o Celsius while the specific gravity was between 1.020 and 1.024. Under these conditions the life span of a single hydranth was fifteen days. Six to eight days were spent as a feeding hydranth. The regression and replacement of the hydranths occurred regularly as long as these conditions were maintained and the feeding was regular. When the feeding was reduced the replacement of the hydranth was slow and in some cases never occurred, depending on the position of the hydranth on the stalk. Crowell (1953 and 1957) states there is a movement of material from old hydranths to young hydranths. When the supply of food is reduced there is an inhibition of future growth on the stalk.

The testing showed that optimum separation could be achieved using a seven percent polyacrylamide gel as the support medium. The 3.75% acrylamide preparation would allow the APase to migrate in a large band. There was no separation seen within this band. Probably, there was no sieving occurring within the separating gel. The ten and twenty-two percent gels were not allowing the APase to migrate in the separating gel. After staining, regardless of the voltage or run time the staining was at the beginning edge of the separating gel. The enzyme was either too large to migrate or denatured because of the build up in resistance to flow. The composition of the stock solution for preparation of the seven percent solution were as follows:

RDS-A	RDS-C	RDS-G
24 ml 1N HCl	28 g Acrylamide	0.14 g Ammopersulfate
18.1 g Tris	0.0735 g Bis	H ₂ O to 100 mls
0.12 ml Temed	H ₂ O to 100 mls	
H ₂ O to 100 mls		
pH 8.8 - 9.0		

The proportions were one part RDS - A, one part RDS - C and two parts RDS - G (Canalco, 1969).

The buffer found to give the best separation was a 0.01M Histidine-NaOH buffer at a pH of 6.2. This buffer was used in the upper and lower baths of the electrophoresis chamber. The work of Filburn (1973) showed that this buffer was adequate for separation of the APase isoenzymes. The lower molar concentrations caused the APase isoenzymes to migrate too fast so there was no stacking of the isoenzymes seen. The isoenzymes appeared diffuse in the separating gel. The higher molar concentrations caused the APase to remain compact in a single thick band at the top of the separating gel. The reason for these changes are that the more ions in solution the less the charged particles, isoenzymes, will be migrated by the current through the apparatus. The less ions, the greater the current pull on the isoenzymes.

The Barbital buffer at a pH of 8.6 was also tested at different concentrations. 0.035M Barbital buffer was able to separate the isoenzymes of APase but better stacking and resolution were seen with the Histidine buffer. The same effect was seen with Barbital as you raise and lower the molarity, as was seen with the histidine buffer.

The single tube apparatus was useful in developing the basics of a microelectrophoresis procedure. The variables in this procedure could be tested on a smaller scale without spending time in the preparation of a large number of gel tubes. Sample size and preparation

polyacrylamide percentage, buffer strength, voltage and tube length were observed in this manner.

Initially, the known concentrations of APase (Sigma) placed in the polyacrylamide gel tube were electrophoresed and upon staining one large banded area could be observed. Knowing that APase could be migrated in this system, it was necessary to find a suitable hydranth sample size.

The 0.5 ml Sucrose Triton X-100 solution with ten hydranths had a sufficient quantity of APase for banding to be observed. The homogenization of this solution with hydranths was done easily and most effectively with the micro tissue grinder (Fischer Sci. Co.). The grinders were kept on ice while the hydranths were selected for testing. The hydranths were ground until homogenized as observed under the dissecting scope. The grinder was microcentrifuged (Misco Microchemical Specialties Co.) until the supernatant was clear. The speed was set at ten percent of maximum using a rheostat (Powerstat, Superior Electric Co.) for a period of three minutes. The sample was placed on ice until the testing began.

The glass capillary tubes were prepared in the manner described in the method section. The 33 mm tube length (Friz, 1970 and Hyden and Lange, 1968) contained a long enough separating gel for observable migration to be seen.

The commercial electrophoresis chamber was tested with the guidelines obtained from the single tube testing. Since eighteen tubes could be run at a time the electrophoresis procedure was performed on each group simultaneously to minimize differences caused

by changes in the test performance. This meant that a typical test had representative tubes for each group. The immature; the feeding, and the old hydranths of Campanularia as well as the Cordylophora were all subjected to the same current, run time and buffer concentration, as well as sample size and gel percentage. One of the tubes was always a blank containing only tracking dye on the gel. This was the control blank and the Cordylophora the animal control.

The application of 40 volts by the power source per eighteen tubes was sufficient for separation of the isoenzymes. The higher voltages probably denatured the enzymes while the lower voltages showed little or no migration.

The criterion for the length of time the voltage was applied was movement of the tracking dye. The Bromophenol blue was visible during the electrophoresis run and when the dye reached the lower end of the separating gel the voltage was stopped. The run time with eighteen tubes was approximately two hours and forty minutes. In the single tube apparatus the time was varied from thirty minutes to three hours. The decrease in time slowed the migration and compacted the isoenzymes. The longer time was necessary for adequate separation to be seen in the micro tubes.

Following the staining procedure using alpha-naphthyl acid phosphatase and the fast garnet GBC salt in 0.1M acetic acid-sodium acetate buffer, four bands were visible in the separating gels of the different tests of Campanularia. The general characteristics of the bands when viewed from cathode to anode end were as follows: The first and second bands were diffuse, the third was very thin and clearly

differentiated and the fourth was dense and thick. See Figure 6. The tube containing the Cordylophora sample had two bands stained. The first band was dense and diffuse and the second thinner but dense.

The stained tubes were then scanned using a densitometer set at an optical density of 0.5 and a wavelength of 525. The photographs and graphs are seen on the next pages. Figure 7 shows the stained polyacrylamide gel and its densitometric recording for the immature hydranth. Figure 8 are the results of the feeding hydranth and Figure 9 the results of the regressing hydranth. Figure 10 is the animal control, Cordylophora. The blank tube containing only tracking dye is seen in Figure 11.

Each age group was tested a minimum of ten times using this microelectrophoresis procedure and the commercial electrophoresis apparatus. The presence of four isoenzymes was seen in each test. When comparing the results seen from the densitometry readings of the three Campanularia age groups, the quantity of APase present within the hydranth increases as the animal ages. The individual isoenzymes all showed an increase in APase activity from the young to feeding stage and from the feeding to the senescent stage. Marked increases were seen in the second and fourth isoenzymes. The Cordylophora showed two isoenzyme bands on staining. Repeated tests using ten hydranths of Cordylophora gave no change in the number of isoenzymes.

The three samples of Campanularia hydranths were compared using the spectrophotometer for total APase activity. As the hydranth aged there was an increase in the total amounts of APase. Comparing Cordylophora by this method, there was no significant difference between the individual samples.

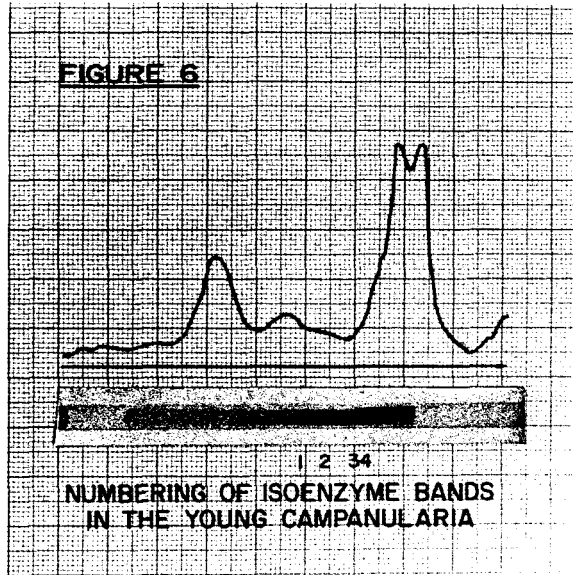
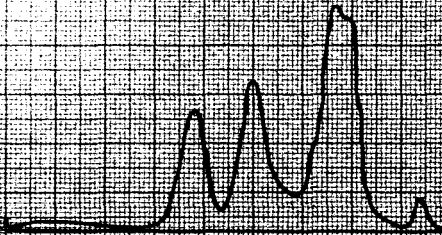


FIGURE 7



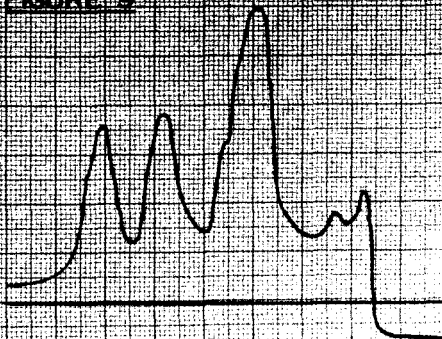
CAMPANULARIA - YOUNG HYDRANTH

FIGURE 8

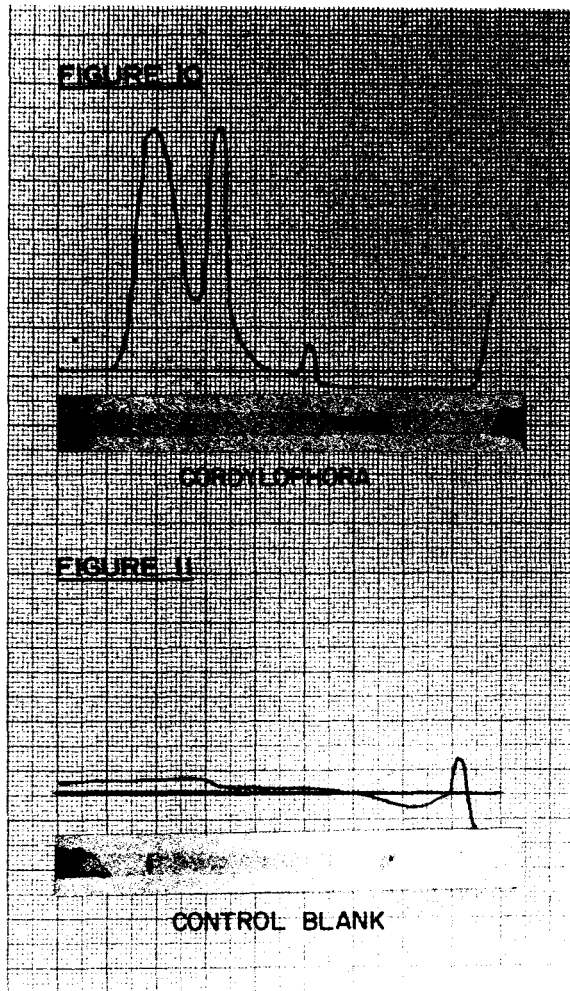


CAMPANULARIA - FEEDING HYDRANTH

FIGURE 9



CAMPANULARIA - OLD HYDRANTH



DISCUSSION

Daniel (1972) stated that aging somatic cells could not multiply indefinitely even under optimum conditions. The cells had inherent in them the mechanism to cause aging. Human diploid cell cultures (Cristofalo, Parris and Kritchevsky 1967) will not multiply indefinitely. These cultured cells after a period of time showed a decrease in proliferative ability unrelated to environmental conditions. This was the cellular response to aging. The growth cycle of Campanularia was in agreement with this process. The cultured slides kept at optimum conditions had an average lifespan of fifteen days at 20°C. The regression and replacement of the hydranths was on a regular basis. Any variation of conditions would cause a shortening of the lifespan.

Lysosomes are cytoplasmic particles that contain acid hydrolases and function in intracellular digestion. They function not only in digestion of food material but also as autolysosomes to remove damaged or aging cell structures. Secretory granules of the rat anterior pituitary are regulated by the action of lysosomes. Undischarged granules are absorbed and degraded by autolysosomes (Smith, 1966). Lysosomal membranes are found to contain acid phosphatase.

The presence of APase has been studied in the rat liver (Berzins, 1975), in the degradation of secretory granules in the rat anterior pituitary (Smith, 1966), in the absorption of tadpole tails (Filburn, 1973), in human leukocytes (Li, 1970), in human fibroblast cultures (Cristofalo, 1972) and in aging Campanularia flexuosa (Strehler, 1961 and Brock, 1970).

The primary means of identification of the enzyme was by the Gomori method and by electrophoresis. The Gomori method utilized by Novikoff (1963) located APase by the deposition of a black precipitate which is electron dense. This procedure was an indicator of the presence of any APase. Electrophoresis, by the use of an electrical field, separated the APase in regards to charge, molecular weight and size. This method separated the total APase into its isoenzyme components. An isoenzyme being a "molecular form of an enzyme from the same organism sharing a catalytic activity" (Brewer, 1970). In this way, the activity of the enzyme was monitored as a change in isoenzymes as well as total content.

The standard polyacrylamide gel electrophoresis procedure is adequate when testing a tadpole tail. A large sample size is expected after homogenizing. However, when the organism or cell is microscopic and the sample size is in the 10^{-9} to 10^{-7} gram range the standard procedure is less than adequate. Since the sample size is reduced, a micro procedure is developed to accommodate this size. The gel tube size and length is reduced, the current and gel quantities decreased. The microelectrophoresis procedure could then be adapted for the separation of proteins or specific enzymes.

Microelectrophoresis procedures have been successful in analyzing the proteins of single nerve cells (Hyden and Bjurstam 1966, and Hyden and Lange, 1968) and amoebae (Friz, 1970). Hyden (1966 and 69) found it necessary to develop microelectrophoresis so that protein from an individual rat brain cell could be separated. He then had a means of comparing single nerve cells from different brains. Friz (1970) used the microelectrophoresis procedure developed by Hyden to separate the protein in

amoebae. The protein bands were used to compare different species of amoebae.

This investigation utilized the microelectrophoresis procedure of Hyden (1966 and 68) in developing a procedure to separate the APase enzyme found in Campanularia flexuosa. The lifespan of fifteen days was divided into three observable stages: young, feeding, and old hydranth. The APase isoenzymes were determined for each age group and compared.

The significance in studying the aging of Campanularia flexuosa is the parallel that has been drawn between it and the cells of human myocardium (Strehler, 1961). In the human body the cells of the skin are in a constant state of renewal. This type of cellular replacement is seen in the growth of hydra. While the cells of the human myocardium will last for the lifetime of the individual, there is no growth or replacement of dying cells. This is the cellular growth seen in the Campanularia. Once the individual hydranth is formed there is no new cellular growth to replace aging cells. Any damage to the cells is detrimental to the existence of the hydranth.

Acid phosphatase operates actively during the aging of Campanularia. It is used in the absorption of food supplies entering the hydranth. The quantity of APase increases as the animal ages. During the senescent stage, the breakdown of the hydranth is accomplished by some hydrolytic enzyme. APase is a hydrolytic enzyme and the coding for the increased production of APase is a possible stimulus for aging.

The presence of APase in aging Campanularia had already been verified through the individual work of Strehler (1961) and Brock (1970). The question was whether this enzyme could be identified in a sample

containing a hydranth at a particular stage of development. Then the individual isoenzymes could be compared between stages for any increase or decrease in activity. Also, this information could be used to determine the effect of APase on aging Campanularia flexuosa.

In this investigation the single tube apparatus was similar to that used in the protein separation of nerve cells (Hyden) and amoebae (Friz). Their sample preparation differed in that a dental drill with a twisted loop of stainless steel was inserted into a capillary tube containing the sample and then homogenized. I found for my animal that the micro tissue grinder (Fischer Scientific Co.) was easier to use and as effective as the drill. Their capillaries were originally 55 mm long but later reduced to 33 mm in length. A 24 percent polyacrylamide separating gel was used. This had a much smaller pore size than the seven percent gel used in my procedure. Also an upper layer of either 5 percent polyacrylamide or Sephadex was placed on the separating gel to cause the proteins to stack themselves before separation began. After a period of time the upper part of the tube was cracked off to remove this stacking layer and the run would continue. This decreased the resistance within the tube. The buffer used in the upper and lower baths was a Tris-glycine buffer with a pH of 8.5. The gels were stained for the presence of protein. Hyden (1966 and 68) used Amido black 0.5% in 7.5% acetic acid and Friz (1970) used Coomassie brilliant blue to highlight the separated protein.

As a result of this investigation, the microelectrophoresis procedure has been shown to be capable of comparing minute quantities of a specific enzyme within an organism. Where sample size is too small for

testing on a macro level, adequate results can not be obtained. The microelectrophoresis procedure in conjunction with densitometric reading of the gels was able to separate and record the presence of four isoenzymes of APase in ten hydranths of Campanularia. The total quantity of APase increased as the animal aged in agreement with the work of Strehler (1961) and Brock (1970). In regards to the isoenzymes, each band increased as the animal aged. The specific activity of each isoenzyme level was not determined because the densitometer was used as a qualitative rather than a quantitative tool. The comparison of activity using the spectrophotometer showed an increase in activity in the aging hydranth. The small sample size made spectrophotometer analysis difficult. Large volumes of chemicals were used in relation to the small quantity of enzyme. The enzyme was excessively diluted so the reaction was variable and minimal. The absorbance read was on the lower portion of the calibration curve. For greater accuracy in recording, the procedure should be reworked for a micro level and the calibration curve expanded in the lower range of activity.

The question remains as to the role of APase during the aging process. Cristofalo (1972) suggested that the lysosomal membrane, with age, became more fragile and would release more APase during sample preparation. This would mean that the APase was present at a constant level during the growth cycle. He stabilized the lysosomal membrane with hydrocortisone and found the enzyme still increased with age. The finding of increased APase isoenzymes in Campanularia was not definitive proof of the role of APase in aging.

There is the possibility that RNA and protein synthesis inhibitors could be used to control regression and hydranth aging. These

blocking agents could theoretically inhibit the production of the enzyme which reabsorbs the hydranth. The chemicals L-tartrate, fluoride, and molybdate were all used in the study of tadpole tails and blood cells to inhibit the synthesis of APase and so affect cell death (Filburn, 1973 and Li, 1970). Tata (1966) using actinomycin-D, puromycin and cycloheximide was able to disrupt RNA and protein synthesis and thus stop regression of the tadpole tail cultures. If these blocking agents were used in conjunction with the microelectrophoresis procedure, it may be possible to determine which inhibitors affect which isoenzymes. The inhibitors could then be introduced into the hydranth and the effect monitored daily. In this way there could be correlation with the individual isoenzymes. The possibility of blocking senescence early enough and increasing the lifespan of Campanularia flexuosa should be investigated. Future studies to elucidate the role of APase in the aging of Campanularia might emphasize the approach utilizing microelectrophoresis.

SUMMARY

- 1) This study shows that microelectrophoresis is a valuable means of identifying the presence of small amounts of proteins or enzymes.
- 2) In this investigation the use of differential staining with alpha-naphthyl acid phosphatase and fast garnet GBC salt in 0.1M acetic acid sodium acetate buffer highlighted the isoenzyme of acid phosphatase.
- 3) Four isoenzymes of acid phosphatase were separated in a seven percent polyacrylamide gel microelectrophoresis procedure using ten hydranths of Campanularia flexuosa. The buffer was a 0.01M Histidine-NaOH buffer at a pH of 7.5 and the histological stain was 1mg/ml of alpha naphthyl acid phosphatase and fast garnet GBC salt in 0.1M acetic acid sodium acetate buffer at a pH of 5.
- 4) The data comparing the isoenzymes from a young to an old hydranth showed an increase in all four of the isoenzymes.
- 5) The role of acid phosphatase in aging Campanularia has not been clearly determined.
- 6) Future directions based on this study indicate that microelectrophoresis procedures in conjunction with inhibitors of enzyme synthesis could possibly elucidate the role of acid phosphatase in the aging of Campanularia flexuosa.

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

4-11-79

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