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Microelectrophoretic Separation of Isozymes in Relation to Aging in the Hydroids Campanularia Flexuosa and Cordylophora Lacustris

Patricia Marie Brown
Loyola University Chicago

MICROELECTROPHORETIC SEPARATION OF ISOZYMES IN RELATION TO AGING IN THE HYDROIDS *CAMPANULARIA FLEXUOSA* AND *CORDYLOPHORA LACUSTRIS*

by

Patricia Marie Brown

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VITA

The author, Patricia Marie Brown, is the daughter of Richard James Brown and Emy Lou (Dunne) Brown. She was born in Chicago, Illinois on July 21, 1954.

She attended Chicago and suburban parochial grade schools and Sacred Heart of Mary High School in Rolling Meadows, Illinois. A member of the National Honor Society, she graduated in 1972.

In September of 1972, she entered Loyola University of Chicago where she was a member of the Beta Beta Beta Biological Honor Society. She received the Bachelor of Science degree with Honors in June of 1976 with a biology major.

Graduate studies began in September of 1976 also at Loyola University of Chicago. In May of 1981, she was awarded the Master of Science degree in Biology.

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CHAPTER I

INTRODUCTION

The purpose of this study was to investigate and characterize the aging process by comparing total protein and specific metabolic enzymes in a non-aging organism and in the stages of the life cycle of an aging organism.

The organisms chosen were Cordylophora lacustris which is unusual in that it shows no evidence of aging (Crowell, 1961) and Campanularia flexuosa which has a definite life cycle (Hammett, 1943). Both animals are phylogenetically similar and are easy to culture and maintain.

The enzymes used in this research were lactate dehydrogenase, malate dehydrogenase, and glutatmate dehydrogenase. These enzymes were chosen because they are indicative of various cellular metabolic processes. Lactate dehydrogenase is a glycolytic enzyme and is indicative of oxidative and hypoxic respiration. Malate dehydrogenase is a citric acid cycle enzyme and it is thus an indicator of oxidative respiration. Glutamate dehydrogenase is an enzyme linking carbohydrate and nitrogenous metabolism.

As organisms age, there is evidence that isozyme band presence and intensity vary as shown by the technique of electrophoresis (Pappas et al., 1971; Hu Chow and Pasternak, 1969; Lin and Zubkoff, 1969). A micro-modification of electrophoresis was used in this research.

Therefore, by examining the presence and intensity of electrophoretic isozyme bands this study investigates and compares the cellular processes of respiration and nitrogenous metabolism in a non-aging organism and in the stages of the life cycle of an aging organism.

CHAPTER II

REVIEW OF RELATED LITERATURE

AGING

Aging is a phenomenon to which no generally accepted definition has yet been assigned. A cycle of development, maturity, and apparent degeneration, is evident throughout the animal kingdom; but the threads linking these processes are as yet unelucidated. Aging is seen grossly as physical changes and sometimes microscopically as structural changes. The process of aging often manifests as loss of function.

There is little published research on this subject before 1960. Pearl (1928) noted that the higher the metabolic rate of an animal, the shorter its lifespan when compared to other organisms of the same genus and species. This relationship is referred to as the 'rate of living' phenomenon and many studies which involve decreasing food intake or metabolic rate support it. Loeb and Northrup (1917) found that the lifespan of Drosophila larva increased in a yeastless medium. The lifespan of ticks increased from its normal two weeks to up to two years by food deprivation (Bishopp and Smith, 1938). More recently, McCay (1952) did similar extensive research on a vertebrate. He found that by severely underfeeding rats in the first half of life, their lifespans were greatly increased. The results of these and other related studies

seem to indicate that the growth process is delayed while the animals are immature. The stress theory of aging (Seyle and Prioreschi, 1960) is a variation of the lifespan lengthening work as applied to humans. They theorized that an organism has a limited amount of energy to utilize throughout its lifetime and that any increased stress depletes some of this energy thus shortening the lifespan.

Much research done since 1960 has focused on characterizing the aging process through the examination of aspects of molecular genetics or metabolic processes occurring at the cellular, tissue, organ, or organismic level. Many theories of aging have evolved from the resultant data.

One of the earliest of these was proposed by Orgel (1963) who suggested that aging is caused by the accumulation of errors in protein synthesis. Related to this is the cross linkage theory by Bjorksten (1941) which states that aging occurs as protein is immobilized by cross linkage. Support for these theories comes from Verzar's (1963) study of the aging of collagen and from Holliday and Tarrant's (1972) research with human fibroblasts. In both of these studies protein cross linkage was found to increase as the organism ages. The work of Carpenter (1969) further supports the cross linkage theory by mathematically describing the phenomenon of diffusion as it relates to cross linkage. Research involving human liver cells (Kahn et al., 1977) and granulocytes (Rubinson et al., 1976) failed to show evidence of a decrease in the accuracy of protein synthesis with age. Further discussion and clarification of Orgel's theory followed (Baird et al., 1975; Holliday,

1975). Two other observations may also prove to be valuable in understanding the correlation, if any, between protein synthesis and aging. The first is a 1970 study by Gershon and Gershon which demonstrates that as an organism ages it produces more and more inactive enzymes. The second, by Adelman in 1971, showed that enzyme induction decreases with age.

Aging theories specifically involving DNA and RNA are numerous and are comprehensively reviewed by Hayflick (1975). Allison (1966) suggests a cause of abnormal DNA. He hypothesizes that when lysosomes rupture, regardless of the cause, they release DNAases which can damage the chromosomal DNA resulting in abnormal growth potential in progeny cells.

Some researchers have looked at cellular processes that gradually change with the age of the organism. Walford (1964) states that gradual diversification of the immunological process results in failure of the organism to recognize some of its own cells. This may lead to a prolonged autoimmune phenomena which could cause the process of aging to occur.

Changes seen at the molecular level are the basis of a number of aging theories. Tappel (1968), in his free radical theory, suggests that free radicals formed from the peroxidation of polyunsaturated fats are very easily involved in random rather than appropriate reactions resulting in possible damage to the cellular membranes and organelles. Strehler (1959) found that lipofuscions accumulate as the cell ages. Bjorkerud (1964) confirmed Strehler's research and noted that lipofuscion

accumulation may be related to lysosomal activity. The 'hourglass hypothesis' by Menzies (1976) suggests that the irreversible loss of certain molecules will lead to the initiation of aging.

The idea that a physiological clock mechanism controls the process of aging was presented with substantial data in 1967 by Brunning.

Many recent investigators have tried to produce a more eclectic theory of aging by combining a number of the above theories. These include Cutler (1976), Comfort (1970), Hahn (1973), and Goldstein (1974).

Two very recent reviews on aging research include an exhaustive compilation by Comfort (1978) and a thorough review by Finch and Hayflick (1977).

THE ANIMALS

The animals used in this study are *Cordylophora lacustris* and *Campanularia flexuosa* which are members of the phylum Cnidaria, class Hydroida. They are useful for research because of ease in culture and maintenance, thus keeping variations due to food and environment to a minimum. Lenhoff (1971) notes the following factors as making laboratory rearing particularly successful: suitable culture solutions have been found, a live nutritional food supply is known, the animals grow attached to a solid surface, and they reproduce asexually.

C. lacustris and *C. flexuosa* were chosen for this study because even though they are phylogenetically closely related *C. lacustris* exhibits no signs of aging (Crowell, 1961) while *C. flexuosa* has a regular aging cycle (Hammett, 1943). Both have a simple structure and are considered to be primitive in relation to most animals.

Cordylophora lacustris was first described by Allman (1853). Hymen (1940) states that these are the only brackish water colonial hydroids. Fulton (1960) reported a simple carefully controlled culture method. Further research by Fulton (1962) showed that moderate variations in light, PH, temperature, and oxygen do not affect the growth of this animal.

The gross structure of Cordylophora lacustris colonies was presented by Fulton (1961). The colonies are a series of interconnecting uniform diameter tubes, each composed of a tubular perisarc surrounding a cylinder of tissue. Stolon tubes grow on the substratum and give rise to both secondary stolons and uprights. Secondary stolons are formed at right angles to the parent stolon tubes while uprights form at right angles to the substratum. Side branches may also be formed; exiting the uprights at forty-five degrees. Hydranths develop only just behind the growing tip of the colony and only on the uprights and their side branches. PLATE 1. Overton (1963) found that the uprights arose from areas of mitotically active interstitial cells, which were always located approximately three millimeters behind the growing tip of the stolon.

When cultured, secondary colonies of Cordylophora go through a lag phase of one or two days before entering a period of exponential growth which lasts from about day three to day six. Then growth slows to a constant linear rate (Fulton, 1963).

Cordylophora lacustris was the subject of a 1970 study by Mace and Mackie which describes their natural habitat, food, and repoduction,

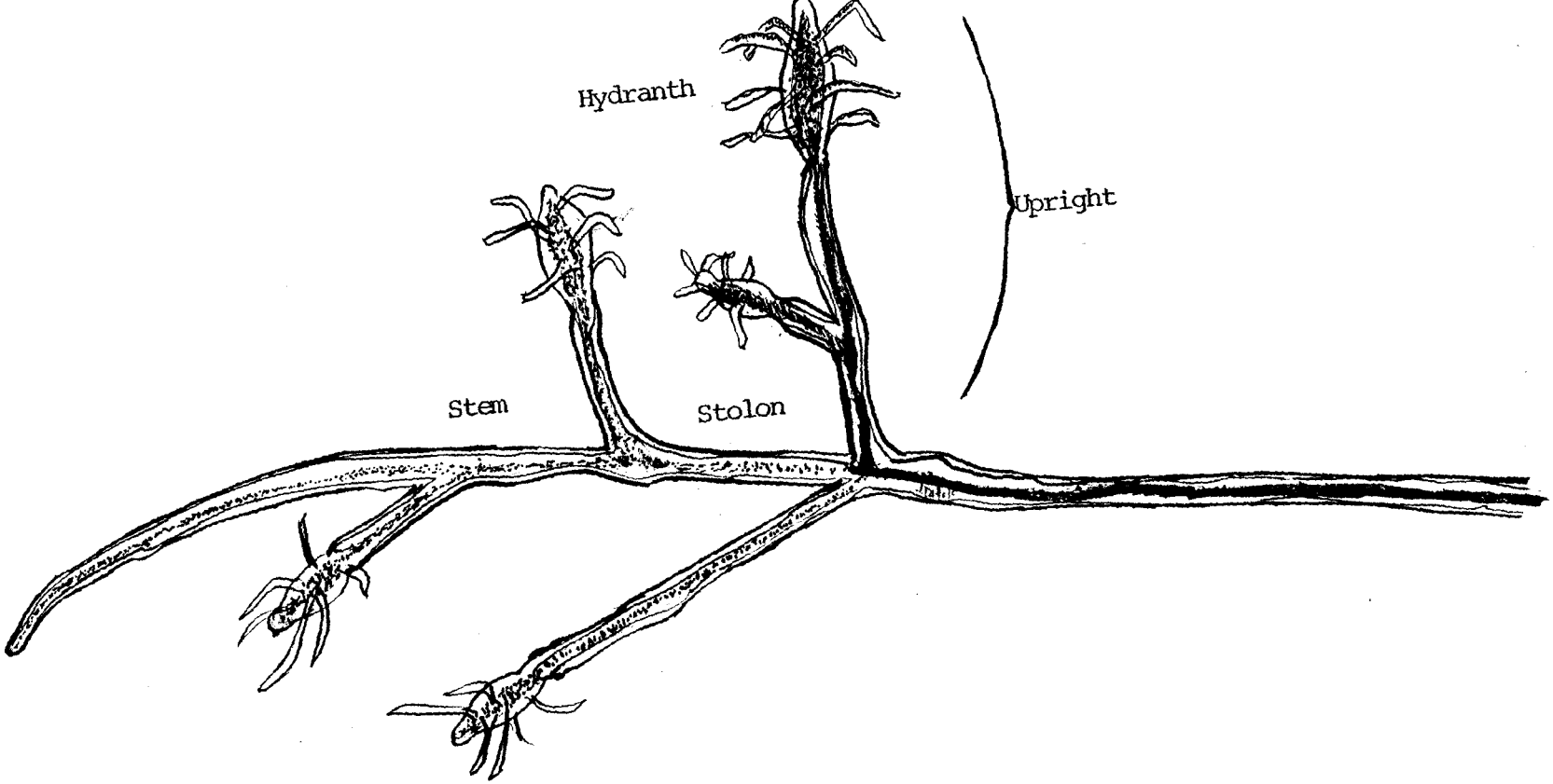


PLATE 1. Colony of Cordylophora lacustris.

in an estuarian lagoon. C. lacustris, being at the tissue level of organization, was chosen to observe the formation of endoderm from ectoderm (Zwilling, 1963) and to study cellular differentiation and morphogenesis (Diehl, 1968).

Cordylophora was chosen for this study because it is one of the coelenterates that show no overt signs of aging (Crowell, 1961). It was found that as long as the animals were fed, new hydranths were produced and old ones did not regress. Only if they were starved would old hydranths be resorbed and new ones formed from the materials of the old. Healthy properly fed Cordylophora hydranths have been kept alive for many months (Fulton, 1962).

The non-aging property of Cordylophora seems to be characteristic of all other athecate (Gymnoblastic) species as well (Crowell, 1961). In these organisms, the oldest hydroid is in the terminal position indicating monopodial growth. Other properties of this group include the regeneration of removed parts and growth of the fully developed hydranths. These properties are exactly opposite to the ones seen in Campanularia flexuosa which is a thecate (Calyptoblastic) species.

It is also interesting to note that a group of sea anemones, members of the class Anthozoa, were carefully kept alive and observed for a period of eighty years (Strehler, 1961). The fact that they all died the same night indicated that the cause of death was probably not senescence.

Campanularia flexuosa was first described by Hincks (1868). He gave a detailed account of the gross anatomy and mentions its habitat.

Loeb (1900) describes resorption of the hydranths into the stolon of the colony. In 1903, Thatcher expanded on this work by following resorption on a cellular level. He reported that endoderm and gland cells are the first to enter the digestive fluid. Then the ectoderm and hypostome disintegrate followed by the remaining structures. In 1923, Huxley and deBeer, reported hydranth resportion in greater cellular detail and broke down the process into stages.

Hymen (1940) described the habitat of C. flexuosa as shallow marine water. She mentions that they grow fastened to a substrate, are carnivorous, and luminesce throughout the hydranth and stalk. Also described is their colonial structure.

The life cycle of this animal was probably first studied and described in detail by Hammett (1943) who photographed the animals every fifteen seconds for a period of seventy-two hours. Time-lapse photography by Strehler (1961) and my own regular observations confirmed that Hammett's description of the life cycle was accurate.

Colonies begin as embryos that attach themselves to a substrate such as rock or the algae Ascophyllum. These produce stolons (hydrorhiza) which periodically send out upright stems (hydrocauli) which bear the hydranths on pedicels. PLATE 2. An annulated perisarc surrounds all parts of the colony. It is modified into a bell shaped covering over the hydranth (hydrotheca).

Hammett (1943) found the entire colonies to be colorless or pale in early spring with the chitinous hydrotheca becoming darker towards

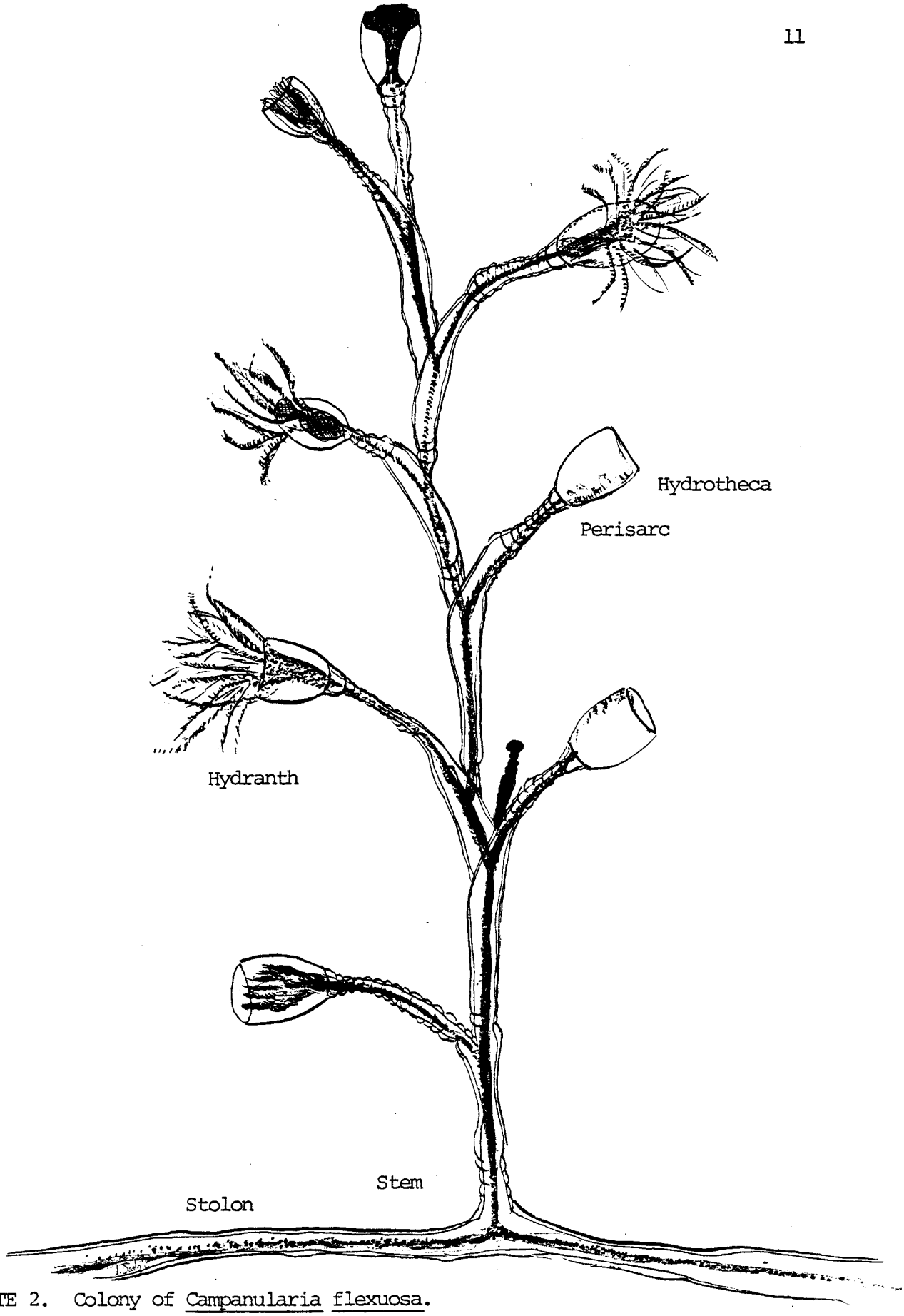


PLATE 2. Colony of Campanularia flexuosa.

autumn. He suggests that this is due to the attachment of brown and red algae during the summer.

To differentiate stages of the life cycle, the names assigned by Hammett (1943) were utilized in the research. PLATE 3. Hydranth formation begins with the development of a bud on a pedicel. This is the bud stage. It then elongates to form a large cone designated as the cone stage. The cone then transforms into a structure known as the cylinder stage. Formation of the tentacles and hypostome signals the end of the growth cycle; thus the complete adult stage is reached. These adult hydranths actively feed and survive for a few days. Regression begins and gradually all remnants of the hydranth are distributed to the rest of the colony. During the regression the senile stage is seen. The hydrotheca then drops off and a new hydranth begins to form on the pedicel if sufficient food is available to the colony (Crowell, 1953). Gonangia are rarely produced and were not included in this research.

It is important to note at this point that although Hammett called his animals Obelia geniculata, Crowell (1957) states that a number of invertebrate specialists later identified these hydroids as Campanularia flexuosa.

The animals were cultured following the procedure of Crowell and Rusk (1950). They found that by maintaining the animals in standardized conditions, hydranth development took twenty-four hours at twenty to twenty-two degrees centigrade and took thirty-seven hours at seventeen degrees centigrade. They also found that at twenty-two degrees centigrade colonies doubled their mass in six days, while at

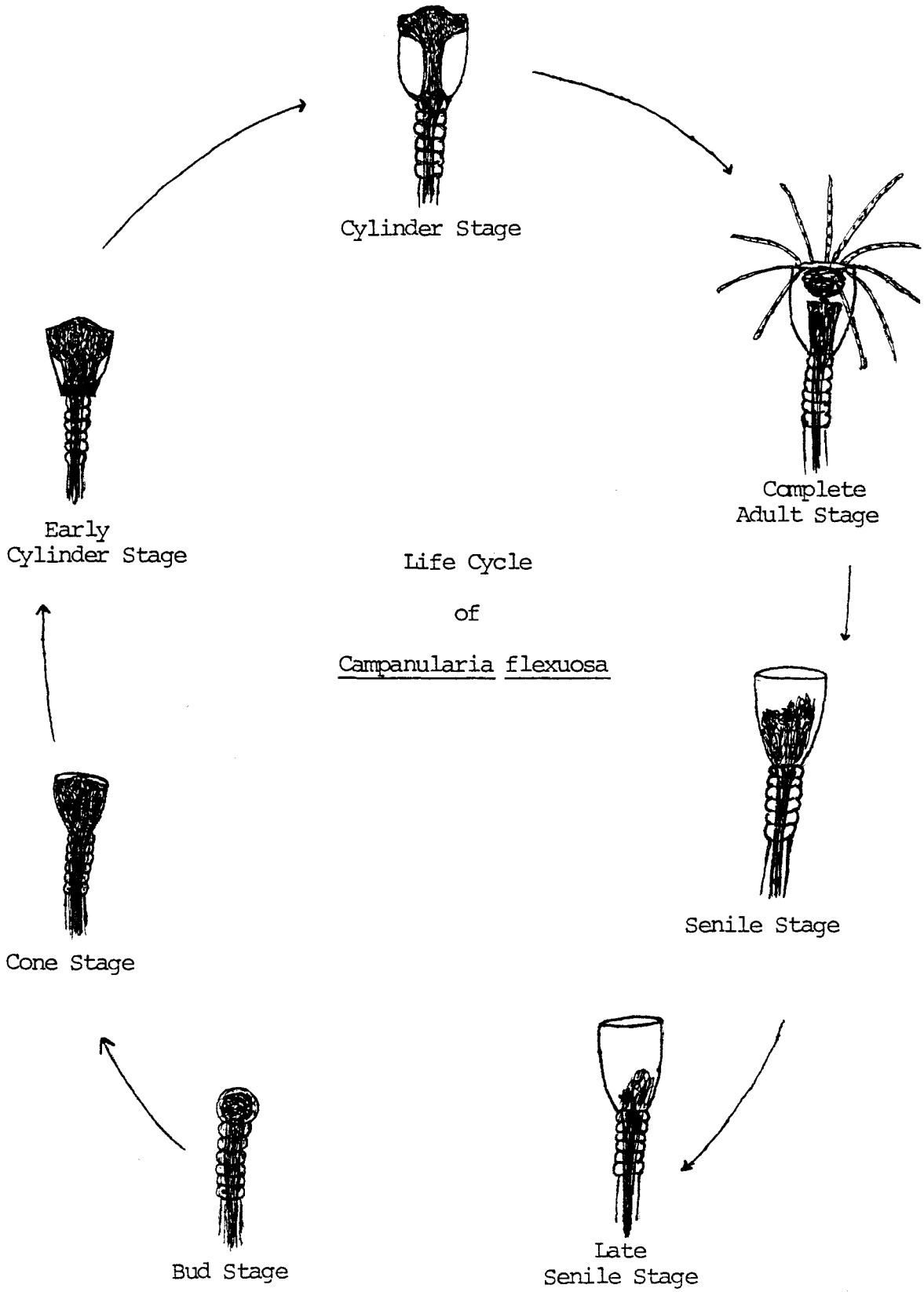


PLATE 3. Life Cycle of Campanularia flexuosa

seventeen degrees centigrade, mass doubled in three and one-half days. Thus, more hydranths are produced at the cooler temperature.

Crowell (1953) reports that individual hydranths live for about four days at twenty-one degrees centigrade and for about seven days at seventeen degrees centigrade. Older hydranths usually regress before younger ones. Regression will occur earlier under conditions of starvation. The materials from the regressed hydranths will be used for new hydranth growth (Nathanson, 1955).

Colonies of C. flexuosa were found to have 'circannual rhythms of growth'. These are considered to be analogous to circadian rhythms with a period of one year rather than twenty-four hours (Brock, 1974). These were recorded as periods of sparse growth interspacing longer periods of profuse growth. These rhythms were more evident at cooler temperatures than at warmer ones.

For this study, the ages of the hydranths were identified by developmental stages rather than by elasped chronology. The developmental stages may be only six to eight hours apart and therefore difficult to follow. More importantly, variables such as slight differences in temperature, food consumption, and circannual rhythms were eliminated.

Campanularia flexuosa is a thecate or calyptoblastic species exhibiting sympodial growth (oldest hydranth or pedicel at base). The adult hydranth does not grow and will be resorbed in a few days, nor can it regenerate lost parts (Crowell, 1961).

C. flexuosa has been utilized for a number of other studies. The dynamics of stolon elongation have been extensively researched by Crowell and Wyttenbach (1957), Wyttenbach (1968, 1974), and Suddith (1974). Brock, Strehler, and Brandes (1968) did electron microscope studies of the young adult and Brock (1970) also did one on the old adult. Earlier, Lunger (1963) did electron microscope studies to elucidate the fine structural aspects of digestion. O'Rand (1974) reported gamete interaction during fertilization.

THE ENZYMES

The enzymes chosen for this study were lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and glutamate dehydrogenase (GDH). All three were present in multiple molecular forms in Cordylophora lacustris and Campanularia flexuosa.

The term isozyme designates multiple molecular weight forms of a single enzyme catalyzing the same reaction. The first isozyme studies involved the separation of LDH and MDH isozyme bands in several tissues (Markert and Moller, 1959). Later, a molecular basis for isozymes was postulated using the LDH sub-units and their combinations in various tissues as a model (Markert, 1968).

Lactate dehydrogenase catalyzes the following glycolytic reaction:

$$\text{pyruvic acid} + \text{DPNH} \rightleftharpoons \text{lactic acid} + \text{DPN}$$

Using this information the spectrophotometric methods to quantify LDH were elucidated (Henry et al., 1960). Vessell and Bearn (1957) were early reporters of activity peaks which were later termed isozymes in human serum.

Other early work on LDH isozymes included data suggesting that the five electrophoretic bands represent two distinct types of enzymes, M and H, with three intermediate hybrids (Fine et al., 1963). LDH also began to be used to study development during this time. Isozymes of LDH were reported during the development of the rat, chick, and human by Wiggert and Villee (1964). A survey of LDH isozymes in the sperm of vertebrate and invertebrate species showed from one to five isozymes present in varying relationships, but failed to suggest taxonomic ties (Goldberg, 1964).

The isozymes of LDH have been studied in many species for a variety of research purposes. These include reports of LDH isozyme bands in Staphylococcus pyogenes (Keller, 1965), of various trypanosomes (Bayne and Roberts, 1969), of the mollusc Helisoma antrosum (Rodrick et al., 1971), of various species of Drosophila (Pappas and Rodrick, 1971), and of livers from various fresh water snails (Coles, 1969). Utilizing heart, skeletal muscle, and brain tissue to compare diving and non-diving animals, LDH isozyme patterns were found to change in animals that undergo long periods of hypoxia during diving (Blix and From, 1971). Long, (1976) reporting on LDH isozymes of forty-eight species in six invertebrate phyla suggested an alternative evolutionary sequence. Very recently, Lin (1979) reported that two or less LDH isozymes were seen during starvation or changing environmental conditions in four anemones. These cnidaria were chosen because with their low evolutionary position, physiological adaptation strategies were hoped to be elucidated. For more highly evolved animals, LDH depletion would lead to accumulation of toxic

lactic acid. However, for an animal capable of performing facultative anaerobiosis LDH depletion may be advantageous. Further cnidarian research on LDH is needed to substantiate these ideas.

LDH activity has been correlated with aging in numerous research reports. A study of LDH activity and distribution during development using Drosophila was described by Rechsteiner (1969). They have also been traced through the development of various lung tissues in mice (Azzopardi and Thurlbeck, 1967). When LDH activity was traced through the ontogeny of the mouse, rat, rabbit, and gerbil, there was evidence that ontogenetic changes were correlated with changes in structural and functional specializations of organs and tissues (Bengtsson and Karlsson, 1976). A study on human skeletal muscle showed that LDH activity decreased with age (Norlund and Borrebaek, 1978). In the human fetus, Diebler et al., (1979) reported that neural tissue LDH activity was highest in the early fetal period, declined until the end of neurogenesis, and then rose until the end of the first year of life.

The isozymes of LDH have been related to aging in various studies. In Drosophila, the evidence suggested that different loci may be used for LDH synthesis in different stages and that a combination of these may be used in the adult (Pappas et al., 1971). In a study of human skeletal muscle involving males ages twenty-two to sixty-five LDH activity of heart specific isozymes did not change with age, while the LDH activity of muscle specific LDH isozymes decreased (Larsson et al., 1978.) LDH isozymes were also traced through the life cycle of the nematode Panagrellus silusiae (Hu Chow and Pasternak, 1969).

Malate dehydrogenase catalyzes the following reaction which is the final step in the citric acid cycle:

$$\text{malate} + \text{NAD} \rightleftharpoons \text{NADH}_2 + \text{oxaloacetate}$$

One of the earliest MDH isozyme studies was done on sperm. Isozyme bands were found to be similar for vertebrate species while heterogenous for invertebrate species (Goldberg, 1964). A study of forty-six members of the vertebrate and invertebrate phyla found MDH isozyme bands in similar locations to be present in closely related species but not in distant ones. An analysis of the data suggested some congruence with evolutionary theory (Thornber et al., 1968).

Vertebrate studies of MDH isozymes include one on various lung tissues in developing and adult mice (Azzopardi and Thurlbeck, 1967), and one on various tissues of the rat, chick, and human (Wiggert and Villee, 1964).

Invertebrate research includes a comparison of MDH isozyme bands in four sea anemones during starvation or environmental changes (Lin 1979). Isozyme patterns were found to vary in relation to temperature changes in only one sea anemone. The suggestion was made that cnidarians may respond to the changes in oxygen tensions rather than to the different temperatures. In the estuarine anemone Diadumene leucolena, Beattie and O'Day (1971) found seven MDH isozymes and noted that low oxygen tension caused changes in the amount of activity per band. They proposed the idea that under low oxygen tension, anemones derive their energy from facultative anaerobic respiration. Lin and Zubkoff (1976) found no

changes in the MDH isozymes of scyphozoan scyphistomae acclimated to cold temperatures. Other invertebrate research includes a study of snail livers in which from one to four MDH isozyme bands were seen; these varied from species to species (Coles, 1969). MDH isozymes have also been reported in trypanosomes (Bayne and Roberts, 1969).

Looking at the mitochondria of mouse liver, the activity level of MDH was found to decrease slightly with age (Wilson and Franks, 1975). Using the muscle, kidney, heart, liver, and brain of the gerbil throughout its life cycle, data suggested that changes in MDH activity are correlated to changes in the structural and functional specialization of developing tissues and organs (Bengtsson and Larlsson, 1976).

A study involving MDH isozymes and the aging process in a vertebrate demonstrated that the inner membrane of the mitochondria increased in permeability in an old rat in comparison to an adult rat (Spencer and Horton, 1978).

Aging studies involving MDH isozyme patterns in invertebrate species are numerous. They include one on the stages of various Chesapeake Bay jellyfish (Lin and Zubkoff, 1976), one involving the development of Strongylocentrotus purpuratus (Ozaki and Whiteley, 1970), one involving the development of the molluscs Argobuccinum oregonesis and Ilyanassa obsoleta (Goldberg and Cather, 1963). MDH isozymes have also been reported throughout the life cycles of three nematodes; Tubatrix aceti (Erlanger and Gershon, 1970), Panagrellus silusiae (Hu Chow, 1969), and Ascaris lumbricoides (Barrett and Fairbain, 1971).

Glutamate dehydrogenase catalyzes the following reaction which links carbohydrate and nitrogen metabolism:

$$\text{glutamate} + \text{NAD(NADP)} + H_2O \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NADH(NADPH)}$$

One of the earliest studies involving GDH isozymes resulted in the finding of six bands in human myocardium tissue (VanderHelm, 1962). This was followed by a survey of GDH isozymes in forty-six animals which showed similar bands in phylogenetically related species (Thornber et al., 1968).

GDH isozymes have been reported for a number of invertebrate species. Pappas et al. (1971) reported results of a study on a number of Drosophila species. He found from four to six GDH isozymes per species with one band common to all. GDH isozymes have also been reported for Mycoplasma laidawii (Yarrison et al., 1972) and in multiple species of fresh water snails (Coles, 1969).

A study involving GDH activity in many coelenterate species revealed that all GDH in this phylum is NADP specific (Hoffman et al., 1978). The same results were reported specifically for the sea anemone Metridium senile (Bishop et al. 1978). GDH activity in the brain of the human fetus was found to be very low until near birth and then to increase until the end of the first post-partum year (Diebler et al., 1979).

Studies of vertebrates have been done following GDH isozyme patterns during aging. Azzopardi et al. (1967) reported GDH isozymes for various tissues during the development of the mouse. GDH bands were used to indicate that the inner mitochondrial membrane increases in permeability as the adult rat ages (Spencer and Horton, 1978).

CHAPTER III

MATERIALS AND METHODS

CULTURE TECHNIQUES

The organisms Campanularia flexuosa and Cordylophora lacustris were obtained from the Woods Hole biological station, Woods Hole, Massachusetts.

Cordylophora lacustris were cultured according to the techniques of Fulton (1962). They were maintained in a Sargent-Welsh model 505 incubator which was kept at twenty degrees centigrade. The brackish water medium (CCS-5) contained the following per five gallons:

1.2g Versene	15.8g $CaCl_2$
58.4g NaCl	20.4g $MgCl_2$
2.0g $KHCO_3$	

Versene was always added before the other reagents and the medium was kept at a pH of 6.8. A small group of animals was attached to a slide using clear nylon thread. Two such slides were placed back-to-back in a one hundred milliliter beaker filled with the CCS-5 solution.

Campanularia flexuosa were cultured according to the technique of Crowell (1953). Refrigerated twenty-five gallon aquariums from instant ocean culture systems, model CS-25, were employed. The windows and top of the aquariums were always darkened to prevent algal growth. Synthetic sea water was made from instant ocean synthetic sea salts (Aquariums Systems, Inc., Wickliffe, Ohio). The sea water was maintained at seventeen degrees centigrade, with a pH of 7.9. The specific gravity

was 1.025 and the salinity was 33.31 parts per thousand. The Campanularia were cultured by attaching small groups of animals to a glass slide using clear nylon thread. Five of these slides were held in a four inch rectangular plastic frame. These animals were subcultured on alternate weeks.

Both Cordylophora lacustris and Campanularia flexuosa were fed fresh brine shrimp daily. Hatching was accomplished using glass gallon containers that were three-quarters filled with instant ocean sea water to which one-quarter teaspoon of dried brine shrimp eggs was added. The mixture was aerated with a silent giant aquarium pump for forty-eight hours, and then collected by straining the contents of the containers through a cotton cloth.

Cordylophora were fed in their beakers by the direct introduction of the brine shrimp. After one-half hour the CCS-5 was emptied from the beaker. The animals were quickly rinsed with fresh water and new CCS-5 was added filling the beakers.

To feed Campanularia, two gallons of sea water were removed from each aquarium and poured into a plastic container. The animals in their plastic frames were removed from the aquariums and placed in the plastic container. The brine shrimp were introduced and the animals were allowed to feed for one-half hour. During this time two gallons of fresh sea water were put into each aquarium. The animals were then rinsed in fresh water and returned to the aquariums.

PROTEIN DETERMINATION

To maintain a constant quantity of protein in each sample for electrophoresis it was necessary to determine the amount of protein present in Cordylophora and in each stage of Campanularia. First a standard curve was determined using bovine serum albumin (Sigma Chemical Co., St. Louis, MO.) according to the method of Lowry et al. (1951).

Samples containing various numbers of Cordylophora or of a stage of Campanularia were prepared. Hydranths were identified under a five power magnification stereoscopic microscope. They were separated near their base from the upright stems using a fine forceps, placed in one and two-tenths millimeter diameter capillary tubes filled with distilled water, and suspended in an ice bath where mechanical homogenization took place. This procedure utilized a Foredom tissue grinder, series DD, with a thirty gauge wire inserted and operated at maximum speed for five minutes. The results of the protein determination procedures were read on a Bausch and Lomb Spectronic 20 spectrophotometer at 540 mn.

DISC ELECTROPHORESIS

The disc electrophoresis procedure (Ornstein and Davis, 1964) was modified to minimize the sample size thus significantly reducing the number of animals used per run. Microelectrophoretic procedures have been utilized by Grossbach (1965) who described micro-modifications, Hayden and Lange (1968) as well as Hazama and Uchimura (1970) utilizing nervous tissue, and Friz et al. (1970) working with individual amoebae.

The reagents used were taken from the research disc electrophoresis reagent kit (RDS kit) from Canalco, Rockville, MD. Glass non-heparinized capillary tubes, seventy-seven millimeters in length by one and two-tenths millimeters inside diameter, were cut to a length of sixty millimeters and fire polished. Next they were coated with a one-tenth of one per cent methyl cellulose and dried overnight in a fifty degree centigrade oven. The tubes were filled to a height of forty-five millimeters by capillary action with a seven per cent polyacrylamide gel solution. The tube bottoms were capped and a water layer was syringe applied to the gel solution. After one-half hour the water was removed by syringe and replaced with a ten per cent solution of four degree centigrade sucrose filling the tube to a height of fifty-five millimeters.

The appropriate number of animals, all of which were unfed in the previous twenty hours, were added directly using a thirty gauge wire loop and homogenized in the capillary tubes using the same procedure as for protein determination. Care was taken to avoid scratching the surface of the gel. A stacking and loading gel was omitted (Dietz and Lubrano, 1967; Hyden and Lange, 1968; and Fritz et al., 1970). The sample was covered with a drop of the seven per cent gel solution, layered with the buffer, and allowed to set for five minutes.

The tris-glycine buffer was prepared from the RDS kit on the day it was to be used and refrigerated at four degrees centigrade. It was used in both the upper and lower baths and maintained at a constant four degrees centigrade using an ice bath.

Electrophoresis was carried out in a Buchler Polyanalyst analytical temperature regulated electrophoresis apparatus. The grommets were replaced with rubber stoppers in which a hole was burned to tightly accomodate the capillary tubes. PLATE 4.

Before beginning a run of electrophoresis, one milliliter of five-thousandths of one per cent bromphenol blue tracking dye was added to the upper bath. Each run consisted of eighteen tubes including some with samples of serum albumin or brine shrimp rather than Cordylophora or Campanularia. The time for each run was about ninety minutes. The power source was a Heathkit Regulated Power Supply, model PS-4 which was used to supply one milliampere to the electrophoresis apparatus. Each capillary tube received one-eighteenth of this or fifty-five microamperes. At the finish of each run the gels were removed by rimming the tubes with a #27 syringe and forcing the water down the side of the tubes. Staining began immediately.

The stain used to indicate total proteins was coomassie blue prepared according to the instructions of the RDS kit. The staining procedure of Deitz and Lubrano (1967) was followed specifically for lactate dehydrogenase and the method of Brewer (1970) was used for malate dehydrogenase and glutamate dehydrogenase. All three of these staining procedures involved the tetrazolium system. The enzyme reduces the pyridine nucleotide. The electron carrier phenazine methosulfate is reduced by the pyridine nucleotide. The phenazine methosulfate then reduces a tetrazolium dye which is precipitated as a formazan with a blue color.

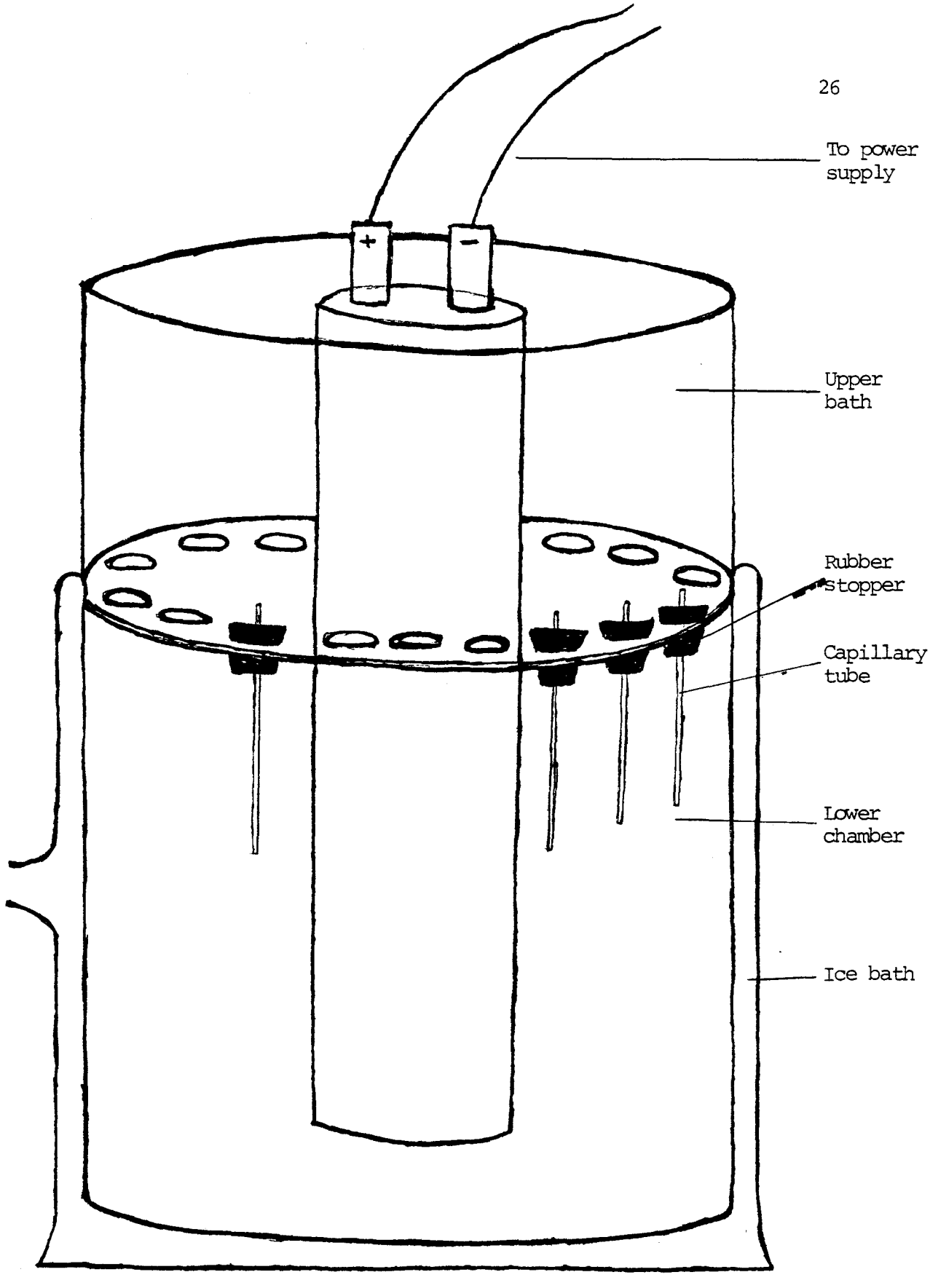


PLATE 4. Electrophoresis equipment

DENSITOMETRY

To accurately analyze the electrophoretic bands, densitometric tracings were made using a Gelman ACD-15 densitometer model 39430. Each gel was placed in a two and four-tenths millimeter diameter glass tube which had been filled with distilled water. Standard operating procedures for this instrument were followed using a wavelength of 540 mn. using the resultant tracings, electrophoretic mobility values (E_f) were determined by dividing the distance of the migration of the protein bands by that of the tracking dye.

CHAPTER IV

RESULTS

PROTEIN DETERMINATION

The results of the protein determination were unfortunately significantly variable and therefore it was only possible to estimate the protein present in a group of hydranths. The averages of ten trials indicated that an equivalent amount of protein, approximately .030 mg., was present in the following quantities of organisms:

Organism/Stage	Quantity
Cordylophora	2
Campanularia (cone)	16
Campanularia (cylinder)	15
Campanularia (complete adult)	14
Campanularia (senile)	17

By trial and error, it was experimentally determined that this quantity, approximately .030 mg., was sufficient to do an electrophoretic run which was stained for total protein. However, this quantity was insufficient for lactate dehydrogenase, malate dehydrogenase, and glutamate dehydrogenase staining. By further experimentation, double this initial quantity, or .060 mg., was found to be necessary for these enzymes. Therefore, double the quantity of organisms shown above were used for each electrophoretic run on these animals.

ELECTROPHORESIS

The results from the electrophoretic gels stained for total protein are presented in table 1 and figure 1. For Cordylophora, twelve protein bands were found, while for Campanularia eleven were found. It is a noteworthy occurrence that the shape of the densitometric tracings, figure 1, for Cordylophora and for all stages of Campanularia were quite similar. The locations of the bands (E_f value) while very similar for the various stages of Campanularia, are seemingly unrelated to the locations of those in Cordylophora. Looking at the various stages of Campanularia, band height and position was found to be nearly identical for all stages. Therefore, the same size proteins are present throughout the life cycle of this animal.

Table 2 and figure 2 present the results of the gels stained for lactate dehydrogenase. Nine bands were seen in Cordylophora and twelve bands were seen in all stages of Campanularia. Band locations (E_f values) are unrelated between the two animals.

A comparison of the stages of Campanularia show little difference in band intensity (the darker the band the higher the peak on a densitometric tracing) between the cone and cylinder stages. However, in the complete adult bands I, II, and III decrease in intensity while bands IV, V, and VI increase in intensity in comparison to the first two stages. In the senile stage, bands I, II, and III increase in intensity to a level above what they were in the cone and cylinder stages.

Malate dehydrogenase results are presented in table 3 and figure 3. In Cordylophora only three bands were found, while in Campanularia ten bands were found in all stages. No relation was seen in band location between Cordylophora and Campanularia. However, the areas of greatest band intensity, band II in Cordylophora and band IV and V in Campanularia, are located in the same general area (E_f values).

In regards to the different stages of Campanularia, the intensity of all bands was similar for the cone, cylinder, and senile stages. In the complete adult stage, bands VI, VII, and VIII showed a significant increase in intensity in relation to those bands in the other stages.

The results for glutamate dehydrogenase are presented in table 4 and figure 4. For Cordylophora four bands were seen, while for Campanularia from three to five bands were seen depending on the stage. Bands IV and V, very prominent in the complete adult stage and also seen in the senile stage, were absent in the cone and cylinder stages. There was no correlation of band location between the two animals.

TABLE 1: AVERAGE ELECTROPHORETIC MOBILITY (E_f) VALUES FOR TOTAL PROTEIN

(measured from densitometric tracings)

Cordylophora lacustris

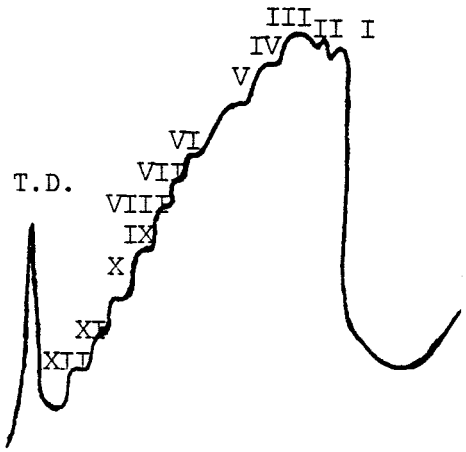
Band #	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
	.2093	.2566	.3161	.4050	.4791	.5318	.5912	.6375	.6999	.7763	.8478	.9099

Campanularia flexuosa

Band # / Stage	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Cone	.1681	.2174	.2514	.2914	.3568	.4433	.5101	.5838	.6497	.7336	.8192
Cylinder	.1568	.1925	.2475	.2996	.3546	.4448	.4947	.5517	.6336	.7368	.8180
Complete adult	.1455	.1821	.2389	.2815	.3567	.4301	.5029	.5552	.6283	.7221	.8190
Senile	.1508	.1951	.2428	.2931	.3529	.3755	.4950	.5483	.6254	.7191	.8069

FIGURE 1. Ddensitometric tracings of total protein

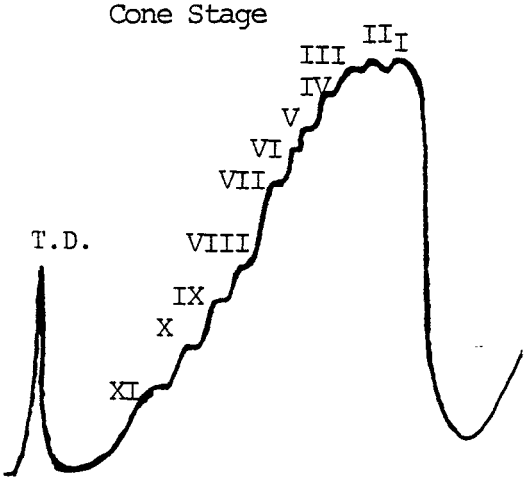
Cordylophora lacustris



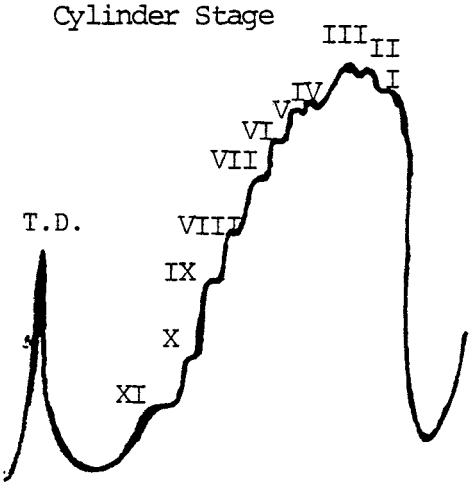
T.D. = Tracking Dye

Campanularia flexuosa

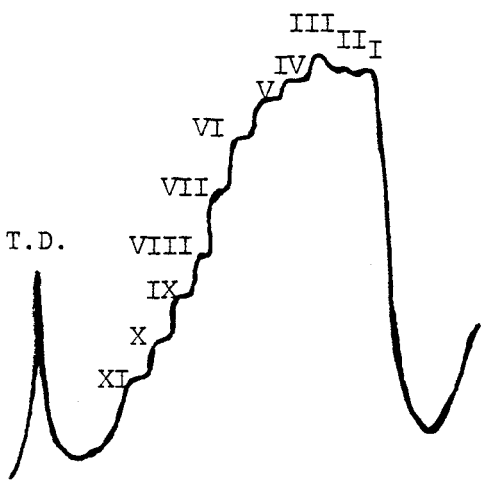
Cone Stage



Cylinder Stage



Complete Adult Stage



Senile Stage

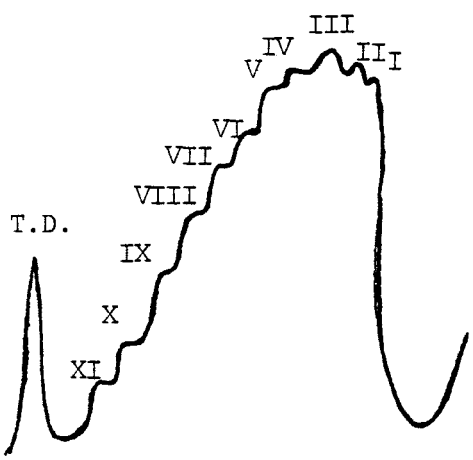


TABLE 2: AVERAGE ELECTROPHORETIC MOBILITY (E_f) VALUES FOR LACTATE DEHYDROGENASE

(measured from densitometric tracings)

Cordylophora lacustris

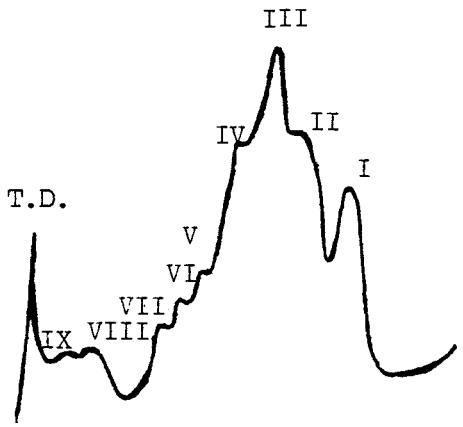
Band #	I	II	III	IV	V	VI	VII	VIII	IX
	.2867	.3738	.4291	.4900	.5804	.6295	.7040	.8342	.8830

Campanularia flexuosa

Band # / Stage	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Cone	.1251	.2044	.2825	.3369	.3828	.4510	.5088	.5781	.6521	.7197	.7761	.8450
Cylinder	.1298	.1783	.2428	.3176	.3721	.4390	.5021	.5674	.6364	.7168	.7985	.8592
Complete adult	.1075	.1785	.2417	.3152	.3659	.4304	.5078	.5665	.6252	.7068	.7530	.8183
Senile	.1180	.1836	.2597	.3272	.3963	.4468	.4962	.5827	.6571	.7285	.7711	.8476

FIGURE 2. DENSITOMETRIC TRACINGS OF LACTATE DEHYDROGENASE

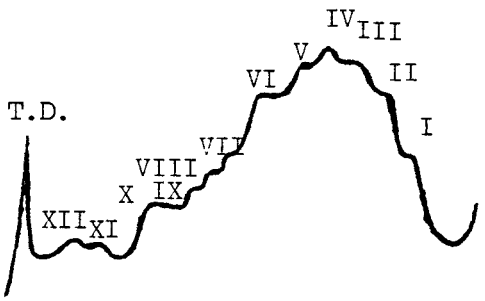
Cordylophora lacustris



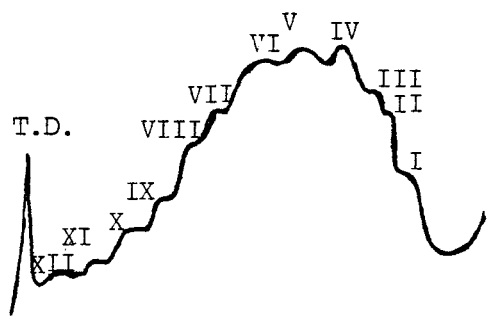
T.D. = Tracking Dye

Campanularia flexuosa

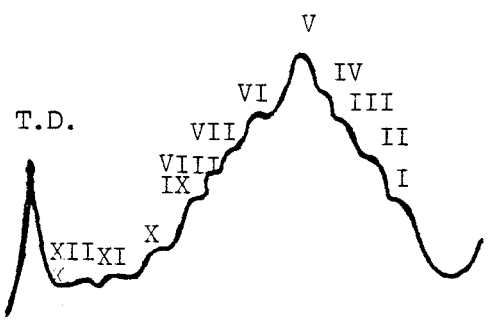
Cone Stage



Cylinder Stage



Complete Adult Stage



Senile Stage

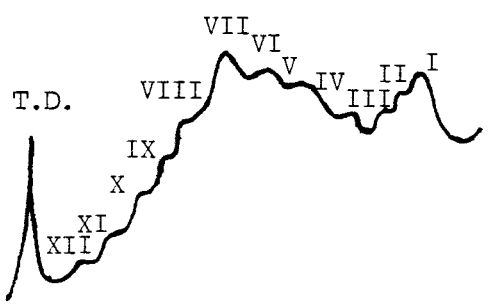


TABLE 3: AVERAGE ELECTROPHORETIC MOBILITY (E_f) VALUES FOR MALATE DEHYDROGENASE

(measured from densitometric tracings)

Cordylophora lacustris

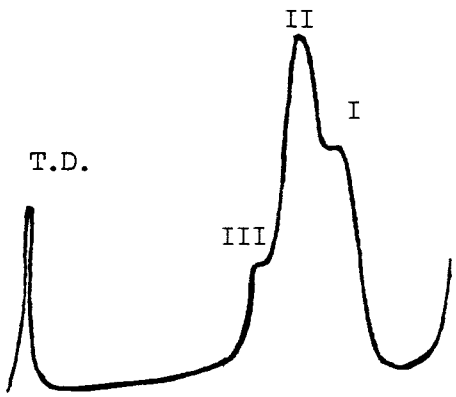
Band #	I	II	III
	.2583	.3339	.4214

Campanularia flexuosa

Band # / Stage	I	II	III	IV	V	VI	VII	VIII	IX	X
Cone	.2353	.2898	.3416	.3938	.4949	.5632	.6222	.6974	.7827	.8476
Cylinder	.1880	.2770	.3371	.3994	.4984	.5696	.6452	.6902	.7887	.8166
Complete adult	.1900	.2511	.3313	.3956	.4824	.5515	.6075	.6974	.7864	.8417
Senile	.1943	.2818	.3314	.3949	.4921	.5503	.6432	.6951	.7802	.8467

FIGURE 3. DENSITOMETRIC TRACINGS OF MALATE DEHYDROGENASE

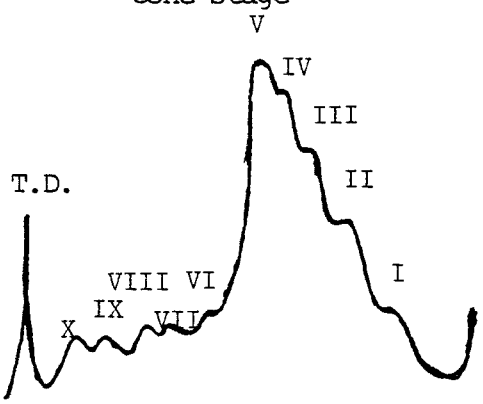
Cordylophora lacustris



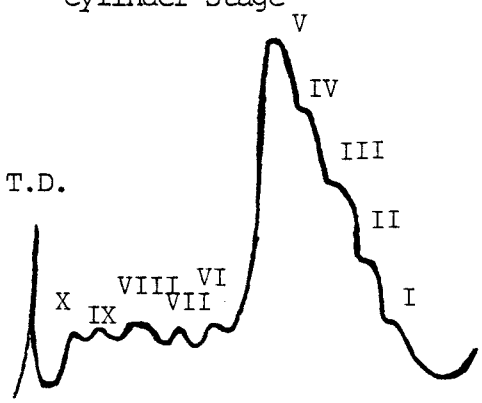
T.D. = Tracking Dye

Campanularia flexuosa

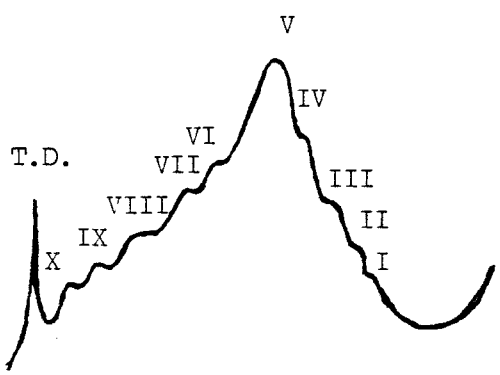
Cone Stage



Cylinder Stage



Complete Adult Stage



Senile Stage

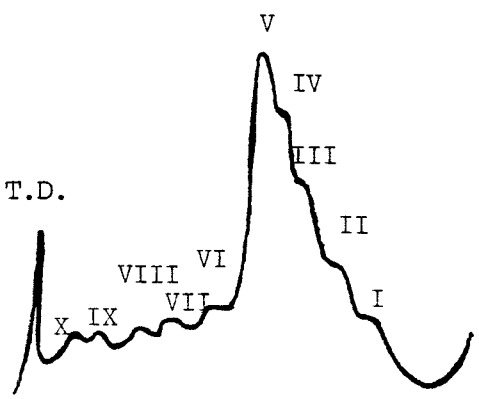


TABLE 4: AVERAGE ELECTROPHORETIC MOBILITY (E_f) VALUES FOR GLUTAMATE DEHYDROGENASE

(measured from densitometric tracings)

Cordylophora lacustris

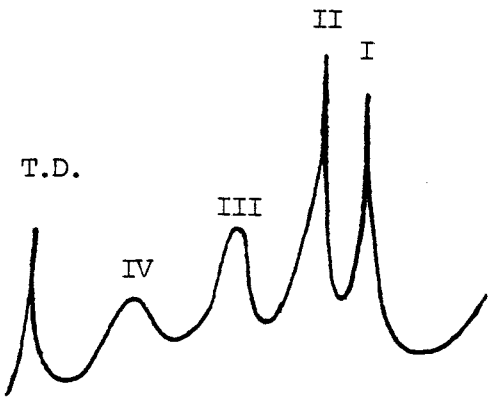
Band #	I	II	III	IV
	.1620	.2385	.3304	.6032

Campanularia flexuosa

Band # / Stage	I	II	III	IV	V
Cone	.2358	.3877	.4413	--	--
Cylinder	.2155	.3745	.4328	--	--
Complete adult	.2241	.3710	.4310	.4716	.5297
Senile	.2041	.3789	.4359	.4847	.5432

FIGURE 4. DENSITOMETRIC TRACINGS OF GLUTAMATE DEHYDROGENASE

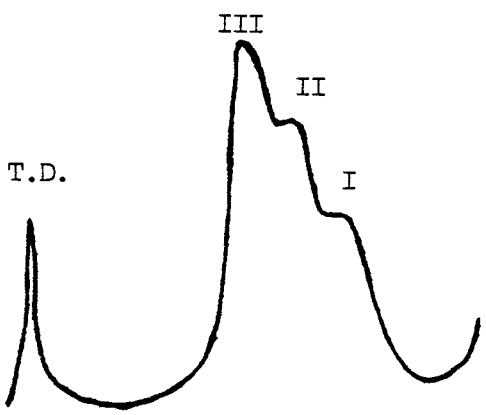
Cordylophora lacustris



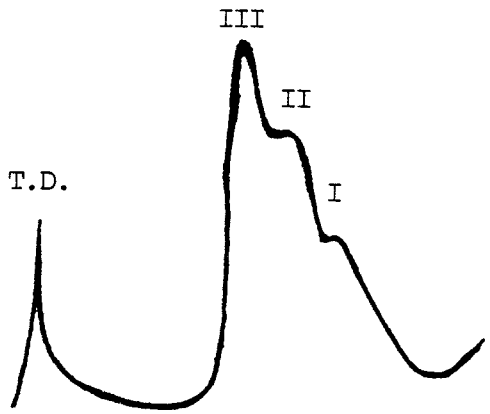
T.D. = Tracking Dye

Campanularia flexuosa

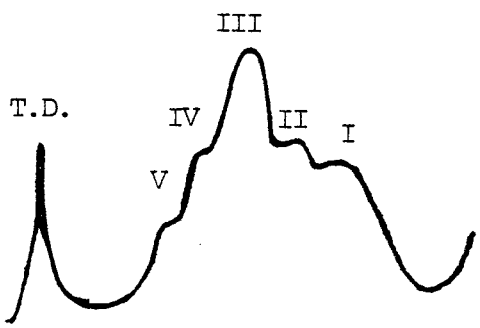
Cone Stage



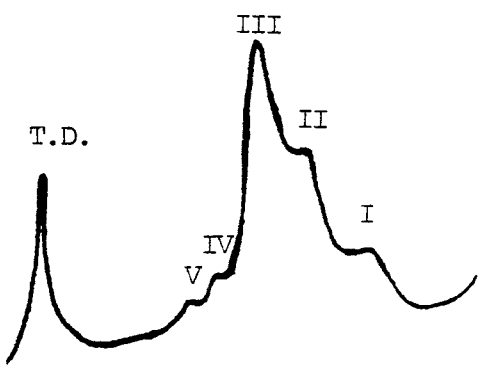
Cylinder Stage



Complete Adult Stage



Senile Stage



CHAPTER V

DISCUSSION

The animals chosen for this study included one that shows no physical evidence of aging, Cordylophora lacustris, and one that shows a clear cyclic sequence of senescence, Campanularia flexuosa. First, a comparison of total protein band locations (E_f values) was performed. Next, three enzymes were studied electrophoretically in an attempt to find evidence of differences in cellular processes in phylogenetically closely related animals.

Looking at total protein for the animals it was most interesting to note that the shapes of the densitometric tracings are very similar for both animals, as well as for all stages of Campanularia. Recognizing that molecular weight is a factor in the distance of electrophoretic migration, this indicates that for these closely related animals approximately the same size proteins are present. However, band locations for the two animals do differ indicating that protein content in Campanularia and Cordylophora is not identical.

All stages of Campanularia had the same number and locations of the protein bands. As the organism ages, bands IV, V, and VI increase in intensity. These results indicate that approximately the same size proteins are present throughout this animal's life. Hu Chow and Pasternak (1969) reported similar results for the nematode Panagrellus silusiae. They found the same number of protein bands at identical

locations in all stages of the life cycle but with changes in intensity in some bands. In Drosophila, Pappas and Rodrick (1971) found some protein bands to be lacking in some stages of the life cycle.

Lactate dehydrogenase is a glycolytic isozyme that has been extensively studied. It is active during oxidative and hypoxic respiration and it has been widely used to quantitate these processes.

For lactate dehydrogenase, only nine isozymal bands were seen in Cordylophora, while twelve were seen in Campanularia. No numeric correlation between band locations was noted when comparing the two animals. However, the general shape of the densitometric tracings for the Cordylophora and for the complete adult stage of Campanularia are very similar and suggest that isozymes of similar molecular weights are active in both.

It is also worth noting that all stages of Campanularia have all isozyme bands present, although there are some changes in intensity in bands I, II, III, and IV. These results differ from Hu Chow and Pasternak's (1969) work with Panagrellus and Pappas and Rodrick's (1971) work with Drosophila. These researchers reported differences in the number, location, and intensity of bands throughout the life cycle.

Malate dehydrogenase is involved in the citric acid cycle and is thus indicative of oxidative respiration. Only three isozymal bands were found in Cordylophora while ten were seen in Campanularia. The locations of the bands between the two were not closely related indicating somewhat different molecular weights of the isozymes present. The densitometric tracings were also dissimilar between the two.

For MDH in Campanularia all stages had all isozymes present. This corresponds to the data of Goldberg and Cather (1963) in their study of the molluscs Argobuccinum oregonesis and Ilyanassa obsoleta. In similar research, Barrett and Fairbairn (1971) reported three MDH bands at identical locations for the developing and adult Ascaris lumbricoides but only one of these bands was found in the egg. Ozaki and Whiteley (1970) reported only two isozyme bands of MDH to be present in the sea urchin Strongylocentrotus purpuratus; both were present throughout life. Erlanger and Gershon (1970), using the nematode Turbatrix aceti, stated that one of the three isozymes present in the early stages was not seen in the later stages. Hu Chow and Pasternak (1969) reported differences in quantity, location, and intensity in MDH bands throughout the life cycle of Panagrellus. In a study of three different jellyfish (same phylum as Cordylophora and Campanularia), Lin and Zubkoff (1976) found that in one jellyfish no MDH band differences with age were seen; for the other two, some stages lacked bands present in other stages. Each genus examined had its own characteristic isozyme band pattern.

Glutamate dehydrogenase is an enzyme linking carbohydrate and nitrogen metabolism. Four isozyme bands were found to be present in Cordylophora and from three to five in Campanularia depending on stage. In the cone and cylinder stages, bands IV and V were lacking. Thus, there seems to be a more significant change in isozyme activity with age than was evident in either LDH or MDH where little change was seen. Possibly, this difference indicates a variance in the amounts or specific reactions or metabolism between carbohydrate and nitrogenous compounds during

development. The four bands of Cordylophora do not correspond in location or intensity to those found in Campanularia.

For all three enzymes involved in this research, Campanularia flexuosa, the aging hydroid had more isozymes present. This may be a necessity for an animal with many life stages for proper physical development. It may also serve in the adaptation of the animal to changing environments. Further research could be directed at evaluating the above ideas as well as at furthering the understanding of the biochemistry of the metabolism in both the aging and non-aging cnidarians.

SUMMARY

1. A micro-modification of electrophoresis suitable for a small number of hydroids or other small animals or tissues was presented.

2. Electrophoretic gels stained for protein resulted in twelve isozyme bands in Cordylophora and in eleven isozyme bands in all stages of Campanularia.

3. Electrophoretic gels stained for lactate dehydrogenase resulted in nine bands in Cordylophora and twelve bands in all stages of Campanularia.

4. Electrophoretic gels stained for malate dehydrogenase resulted in three bands in Cordylophora and in ten bands in all stages of Campanularia.

5. Electrophoretic gels stained for glutamate dehydrogenase resulted in four bands in Cordylophora and in from three to five bands in Campanularia depending on the stage of development.

6. Cordylophora lacustris, a non-aging animal shows evidence of having distinct isozymes from a closely related aging animal, Campanularia flexuosa. However, some similarities do exist indicating relaxed metabolism.

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APPROVAL SHEET

The thesis submitted by Patricia M. Brown has been read and approved by the following committee:

Dr. Edward E. Palincsar
Professor, Biology, Loyola

Dr. Benedict J. Jaskoski
Professor, Biology, Loyola

Dr. Clyde E. Robbins
Associate Professor, Biology, Loyola

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.

April 8, 1981
Date

Edward E. Palincsar
Director's Signature