1960

The Biology of a Colonial Hydroid

Chandler Montgomery Fulton

THE BIOLOGY OF A COLONIAL HYDROID

A thesis submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy at The Rockefeller Institute

by

Chandler Montgomery Fulton, A.B.

15 April 1960

The Rockefeller Institute

New York, New York

Acknowledgements

This work was done during the tenure of a graduate fellowship at The Rockefeller Institute, and I am especially indebted to Dr. Detlev Bronk and Dr. Frank Brink, who have worked unceasingly to create an atmosphere of zealous research and study and to provide every facility needed for the execution of that research and study.

It has been a special privilege to work under the guidance of my faculty and thesis adviser, Dr. Norton Zinder. He has made himself available for discussion, had patience with a two-year unsuccessful attempt to cultivate hydroid cells *in vitro*, and most generously allowed me to explore an avenue of research rather distant from his own.

Dr. W. F. Loomis has offered encouragement throughout the course of the thesis research, and provided careful criticism of Chapters III and V of the dissertation.

The various services of The Rockefeller Institute have aided greatly in the research and preparation of the dissertation. In particular, I wish to acknowledge the help of Mr. Anthony Campo, Miss Muriel Fadrizio, Miss Sylvia Fullerton, and Miss Daphne Morse. The typescript of the dissertation was carefully prepared by Mrs. Georgia Fisher.

To these, and to others whose specific services are acknowledged in the text, I extend my heartfelt thanks.

Abstract

Simple invertebrate metazoa offer many potentialities for study of growth and differentiation - potentialities which have not been realized because the organisms usually have been difficult to handle in the laboratory. A simple coelenterate, the brackish-water colonial hydroid Cordylophora lacustris, was selected for the present work, and was domesticated so that it could be grown and manipulated under controlled laboratory conditions. Methods have been developed permitting exponential increase in hydranth number with a doubling time of about three days. Five ions - Na^+, K^+, Ca^{++}, Mg^{++}, Cl^- - have been found to be required for growth in the defined aqueous environment, and other variables influencing growth rate have been systematically studied. The colonies are fed daily with living larvae of the brine shrimp, Artemia.

The control of the feeding reaction has been studied. It has been found that, as in Hydra (Loomis, 1955), a single molecular species released from captured prey induces the feeding reaction. The active compound has been isolated from Artemia extract and identified as the imino acid proline, and reagent proline has been found to induce the feeding reaction at 10^{-5} molar. Magnesium, and possibly phosphate ions, have been shown to influence the response to proline. Perhaps the most interesting aspect of this discovery is from the point of view of comparative biochemistry and evolution, for previous workers have found that the feeding reactions of three other Hydrozoa are controlled by reduced glutathione.

Time-lapse cinematography of the colonies has revealed proximally-oriented peristaltic waves which apparently act to circulate nutrients through the colonies. These waves are rhythmic, and occur about once every twenty-five minutes in resting colonies. After feeding, the frequency of the waves increases to one every eight minutes, and falls back to the resting rate over the course of several hours. The most striking feature of the peristalsis is that it is synchronized throughout a colony, in that the waves begin at the tip of each hydranth simultaneously. The time-lapse movies have also elucidated a series of rhythmic movements which occur during the reconstitution of hydranths from tissue fragments.

The major emphasis of the study has been an attempt to gain an understanding of the asexual development of a Cordylophora colony. While at first glance a colony appears to be a forest of little trees, on closer examination one finds that a colony may be considered a series of tubes of uniform diameter. The shape of a colony may be considered to result from the (1) relative rates of growth, (2) spacing, and (3) angles of these tubes. A descriptive study of the development of shape under one set of culture conditions has been made. On the basis of the three parameters, three types of tubes may be distinguished: stolon tubes, upright tubes, and branch tubes. These tubes were all found to grow at constant, but different rates. This linear growth of tubes posed a paradox, since the number of hydranths in a colony had been found to increase exponentially. It was shown that the dry weight of a colony also increases exponentially, and a model was developed permitting the resolution of linear growth of parts into exponential growth of the whole. Study of the development of individual colonies using a marking technique showed that the development of shape in these colonies proceeded essentially as predicted by the model.

Contents

Chapter I

Introduction

Among the invertebrate metazoa there are many organisms which offer special advantages for studies of growth and development. Among these, the colonial hydroids are particularly rich in unexploited potentialities. The coelenterate phylum, of which the colonial hydroids are representatives, is unique among animals in the possession of a tissue level of organization (Buchsbaum, 1948, p. 70). Unlike the sponges, which may be viewed as a cell colony, and higher metazoa, in which the component tissues are partitioned into organs and organ systems, the coelenterates are built around the basic plan of a hollow cylinder, the wall of which is composed of two tissue layers. There are no separate organs, and the relatively few cell types are distributed throughout the organism. Thus the organization of a coelenterate is at least conceptually simple, yet at the same time shows the same order of integration of cells into tissues as is found in higher metazoa.

Colonial hydroids are aquatic, and do not have a complex, homeostatic milieu intérieur such as is found in higher metazoa. They are therefore quite sensitive to the external environment, much as are microorganisms or metazoan cells in tissue culture. In contrast to the milieu intérieur, the external aqueous environment is readily accessible to manipulation by the investigator.

In colonial hydroids, the colonies are formed by asexual budding, a process which leads to increase in the number of countable units rather than simply increase in size of a single unit. The units of a hydroid colony are arranged with respect to one another in a simple and regular pattern. Because of these features, it is quite feasible to study quantitatively the three measures of growth - size, shape and age (Medawar, 1945).

In addition to asexual growth, hydroids reproduce sexually, providing a potential source of excellent material for embryological or even genetic studies. The asexual colonies appear to become sexual in response to seasonal environmental conditions, so that it should be possible to induce sexual differentiation and to study the changes accompanying it at will.

Bits of tissue isolated from most hydroids have the capacity, if large enough, to transform into entire organisms without growth. In fact, most experimental studies of colonial hydroids to date have been confined to the phenomena of regeneration and reconstitution.

These and other properties of colonial hydroids make possible the study of problems of development using approaches which are difficult if not impossible with the more commonly used protozoan and vertebrate material. This potentiality has been recognized by many students of development, and the organisms have been studied extensively as found in nature. As yet, however, the colonial hydroids have contributed less to our understanding of development than the potential of the organisms would promise. Several factors have undoubtedly frustrated the progress of previous studies. The organisms are seasonal in nature and generally short-lived in the laboratory. Since they are marine, the investigator who wishes to study them must be able to work at a marine laboratory when they are in season. Because they must be brought in from nature for each experiment, there is much uncontrollable variability, both physiological and genetic.

The necessity for seasonal research, the variability of the material, and the lack of understanding of environmental conditions could all be eliminated if the organisms could be grown in clonal cultures under controlled conditions.[1] In a compendium on "Culture Methods for Invertebrate Animals" (Hyman, 1937), the only hydroid listed as being grown in the laboratory is the solitary hydroid Hydra, which has been reared since the work of Trembley (1744). Even for hydra, however, the conditions were poorly understood, and cultures frequently passed into a state of depression. "In spite of every care, hydra cultures will pass into this state at intervals and, unless prompt measures are taken, will die out" (Hyman, 1937, p. 141). Hydroids are carnivore and Daphnia and other small organisms were recommended as food for hydra; the

---

[1] The philosophy of many students of the development of aquatic metazoa is not sympathetic to this point of view. One of the best students of colonial hydroids, N. J. Berrill (1949), includes in a paper on the development of one of these organisms the comment that: "All of the observations were made upon healthy colonies growing in their original sites on floats or weeds, and not under less natural laboratory conditions." Other students, while willing to grow the organisms in the laboratory, are of the conviction that manipulations of the environment such as attempting to define the aqueous medium (e.g., sea water) in which they grow can only lead to unnatural conditions and the production of artifacts. Suffice it to say that the work to date amply demonstrates that if any meaningful understanding of these organisms is ever to be obtained, it will only be through the introduction of the artifact of controlled and manipulatable laboratory conditions, and the examination of cause and effect which such conditions will permit (cf. Rae, 1958).

methods for rearing these also gave erratic results. A major advance came with the studies of W. F. Loomis (1953, 1954) which resulted in the culture of hydra under controlled conditions in a defined aqueous environment. The use of Artemia larvae as food was an important part of this development, for as will be seen in Chapter III these organisms provide a uniform source of nutrients, available in unlimited quantities.

Important studies using hydra have already been possible since Loomis's advance: studies of the asexual growth of hydra and environmental factors controlling growth, of sexual and nematocyst differentiation, of regeneration, nutrition and digestion, of respiration and the feeding reaction. These contributions will be discussed in ensuing chapters.

In recent years, workers have been successful in growing three species of colonial hydroids in the laboratory. The achievements of Crowell (1953) with the laboratory growth of Campanularia and of Hauenschild and Kanellis (1952, 1953) with Hydractinia represent major accomplishments, since prior to their efforts these hydroids had been difficult to keep alive in the laboratory However, the methods which succeeded are elaborate, making controlled study difficult and providing little manipulatability. A third hydroid, Cordylophora lacustris, is considerably more hardy under laboratory conditions, and has many other features to recommend its selection, as will be described in Chapter II.

This thesis is concerned with the biology of Cordylophora. The organism has been domesticated (Chapter III), and the factors influencing its growth studied (Chapter IV), so that for the first time a colonial hydroid is availabl in a controlled, manipulatable system.[2] The first steps in an experimental analysis of Cordylophora comprise Chapter V, a study of the control of the feeding reaction, and Chapter VI, a study of certain rhythmic movements observed in Cordylophora. Chapter VII describes the asexual development of pattern in colonies growing under controlled conditions, and attempts to dissect this development into its component events. The studies described in Chapters III to VII provide most of the necessary background for an analytical study of the development of Cordylophora, the prospects for which are discussed in Chapter VIII.

---

[2] To the author's knowledge, Cordylophora is the only colonial metazoan available in controlled laboratory culture.

Chapter II

The Static Biology of *Cordylophora*

The study of colonial hydroids has had a most unorthodox history. As is normal in any scientific inquiry, the early invetigations were observational in character. There were the great monographs on structure, on distribution, and on taxonomy. From these, at about the beginning of the twentieth century, attention turned to an experimental inquiry into the phenomenon of regeneration in these hydroids. Although it may perhaps be said that little was learned from these investigations, there was great enthusiasm, and decapitating hydroids was a favorite enterprise of such notables as Jacques Loeb, T. H. Morgan, Hans Driesch and C. M. Child. But more general studies of the biology of colonial hydroids, studies which would seem so essential a prelude into intelligent inquiry into specialized phenomena such as regeneration, are almost nonexistent.

In contrast, studies of the biology of the well-known solitary hydroid, Hydra, which began with the remarkable monograph of Abraham Trembley (1744), have embraced every aspect of the life of hydra. Trembley had no microscope and did not realize that the organisms were cellular, but in spite of this there was no major departure from his approach until Loomis (1953).

It is not proposed to attempt to review here the literature on hydroids, or even on Cordylophora itself, for that formidable task would contribute little to the work to follow.[1] Rather, opportunity is taken to present a descriptive view of Cordylophora, providing background for the present work and defining the terminology to be used. The more dynamic aspects of Cordylophora are discussed in appropriate sections of the ensuing chapters.

Taxonomy

The coelenterates, or Cnidaria (Hyman, 1940), are generally considered to be among the simplest of metazoa, their cells are organized at the tissue level and they have no organs. Two epithelial layers of cells are formed into a radially symmetrical tube, the cavity of which is the coelenteron. The outer

---

[1] Although some information on colonial hydroids can be found in treatises of invertebrate zoology such as Hyman (1940), no serious attempt has been made to survey our knowledge of the group since the monograph of Allman (1871-72). Uncounted hundreds of papers have appeared since that time, but except for the studies of regeneration our understanding of the group is essentially unchanged.

layer, the ectoderm, is in contact with the aqueous environment, while the endoderm encloses the coelenteron. Between these two layers of cells is a supporting lamella, the mesoglea. Variations in the thickness and structure of the mesoglea account in large part for the diversity of coelenterate form. In the free-swimming medusae, the mesoglea is thick and of jelly-like consistency, hence the name jellyfish; in the sessile polyps the mesoglea is thin and more viscous.

Another characteristic feature of the coelenterates is the possession of stinging organelles, the nematocysts, borne on tentacles. The Cnidaria are carnivores, and when a prey organism comes in contact with the tentacles batteries of nematocysts are released. The spears of the nematocysts penetrate the prey, and inject via minute hollow needles a poison which quickly paralizes the prey organism. The poison remains unknown, as does the mechanism of nematocyst discharge.

The captured organism is brought to the mouth by the tentacles, engulfe and taken into the coelenteron. There the food is digested extracellularly, and taken into the endodermal cells, from which it is passed from cell to cell throughout the organism. Indigestible material is discharged through the mouth, for coelenterates have an opening only at the oral end.

Modifications of this basic organization, in almost every conceivable way, yields the plethora of coelenterates found in nature. The diversity of living types (about 10,000 species), coupled with a very scanty fossil record (R. C. Moore, 1957), has made the logical development of coelenterate taxonomy virtually impossible, although valiant attempts are still being made (e.g., Rees, 1957).

The cnidarian of special interest here is a colonial hydroid -- a member of the class Hydrozoa, order Hydroida. The organisms of this order are, in general, sessile during the bulk of their life. Most Hydroida form colonies in which the two-layered tube - the coenosarc - is surrounded by a supporting membrane, the perisarc. The colonies grow asexually by budding, and in addition produce sexual gametes which develop in a diversity of ways to yield colonies again. The individual members of a hydroid colony are polyps; the order also contains a number of solitary polyps, including hydra.

Allman (1871) has divided the Hydroida into two great suborders, the Gymnoblastea and Calyptoblastea. In the calyptoblasts or thecate hydroids

the perisarc forms a cup which surrounds the hydranth (oral body bearing tentacles), whereas in the gymnoblasts the perisarc stops below the hydranth.

In turn, the gymnoblastic hydroids are subdivided into two families, the Capitata and the Filifera, based on the morphology of their tentacles. The tentacles of the capitate hydroids are surmounted at the tip by a single large battery of nematocysts; the tentacles of the Filifera have their nematocysts distributed all along their length.

Cordylophora is a member of the family Clavidae, a group of filiform hydroids in which the tentacles, instead of having a regular arrangement, are scattered irregularly over the body of the hydranth. The classification of Cordylophora, together with that of other species considered in the thesis, is given in Table 1.

Structure

The genus Cordylophora was established by Allman (1843, 1853) to designate a colonial hydroid unique in that it grows in fresh or brackish water, while all other colonial hydroids are marine.[2] Cordylophora, derived from the Greek "a club - I bear," refers to the form of a hydranth with its stem. Although Allman and others made observations on Cordylophora, it was F. E. Schulze (1871) who first made a detailed study of its structure, which remains the most careful study of the histology of any colonial hydroid.

Figure 1 illustrates the basic pattern from which a colony develops. Stolons[3] grow along the substratum, to which they are attached. At intervals they produce perpendicular branches, so that eventually the stolons yield a compact reticular network attached to the substratum. Behind their tips, stolons give rise to uprights (Crowell, 1957), each of which soon bears a

---

[2] Cordylophora grows throughout the world, in habitats varying in their salinity from fresh to almost sea water (Roch, 1924). The species used in the present study agrees in every particular with the descriptions of C. lacustris (Allman, 1853; Schulze, 1871; Hand and Gwilliam, 1951), and Cordylophora will refer to that species.

[3] The terms stolon and stem are used here in preference to the more commonly used hydrorhiza and hydrocaulus, because of their simplicity and lack of ambiguity.

TABLE 1

A classification of Cordylophora, and a partial classification of other Hydrozoa referred to in this work

Phylum
- Class
  - Order
    - Sub-order
      - Super-family
        - Family
          - Genus

Cnidaria
- Hydrozoa
  - Hydroida
    - Gymnoblastea
      - Filifera
        - Clavidae
          - Cordylophora
        - Hydractiniidae
          - Hydractinia
        - Hydriidae
          - Hydra
          - Chlorohydra
      - Capitata
        - Tubulariidae
          - Tubularia
    - Calyptoblastea
      - Campanularia
  - Siphonophora
    - Physalia

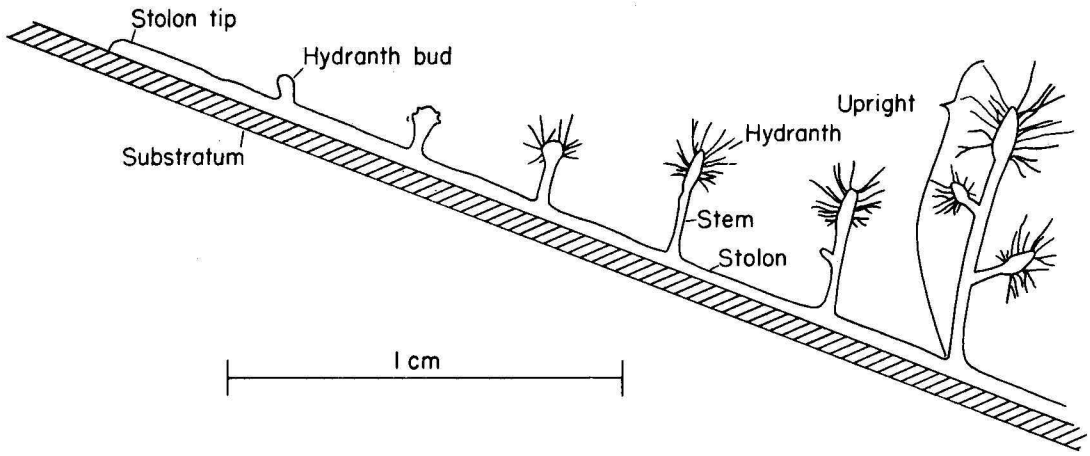


Figure 1. Pattern and macroscopic structure of an asexual Cordylophora colony. Outline of a portion of a young laboratory colony.

hydranth at its apex. These uprights increase in length and branch at intervals, and the branches in turn bear hydranths, grow and branch to produce secondary or tertiary branches. These processes, continuing indefinitely, yield a dense, interwoven bush.

The major morphological units of such a colony are stolons, stems and hydranths. All of these are tubular in cross section, though the stolon is flattened slightly at the surface in contact with the substratum. In cross section the stems and stolons form a series of concentric rings; from outside to inside, these rings are: perisarc, coenosarc (ectoderm, mesoglea, endoderm), and coelenteron. The body of the hydranth is identical except that it is not enclosed in a perisarc. The tentacles have no coelenteron, being filled with a single row of highly vacuolate endodermal cells. The mouth sits at the apex of the hydranth, atop the oral cone. The coenosarc tube is closed throughout, so that ectoderm and endoderm meet only at the mouth. Nematocysts are found at the surface only on the tentacles, where they form a spiral.

The perisarc is secreted by the ectodermal cells, and forms a rigid supporting structure for the stems and stolons of the colony. The perisarc is generally considered chitinous, and while chemical analysis is lacking tests have indicated that perisarcs of several hydroids fulfill the criteria used to identify chitin in arthropods (e.g., Richards, 1946). Schulze (1871) made a study of the perisarc of Cordylophora, and found it to have properties characteristic of chitin (cf. Tracey, 1955). Schulze found, however, that when the perisarc was left in cold concentrated sulfuric acid it separated into thin concentric lamellae, suggesting layers of some other substance between the chitin. The perisarc forms a thin membrane near hydranths and stolon tips, becoming thicker in older parts of colonies, indicating continuous deposition.

The ectoderm is made up of cuboidal epithelial cells which cover the organism as a single layer. Interspersed among these cells are nematoblasts, which carry nematocysts in various stages of development. Except in the tentacles, the nematoblasts are found at the base of the ectoderm, next to the mesoglea, where they are found in groups. Other groups of small cells, termed interstitial cells, are also found in the ectoderm next to the mesoglea. It is these interstitial cells which give rise to nematoblasts; some workers have also tried to implicate them as undifferentiated cells which give rise to all other cell types (for Cordylophora cf. Kirchner, 1934; J. S. Moore, 1952b).

Cordylophora has two morphological types of nematocysts, desmonemes and microbasic euryteles (P. Schulze, 1921; Hand and Gwilliam, 1951). While 17 nematocyst types have been recognized (Weill, 1934), the types have been of little value in classification. Lenhoff, et al. (1957, 1958) have found the nematocyst capsules of Hydra, Tubularia and Physalia to contain considerable hydroxyproline, suggesting a collagenous structure, but electron micrographs of Hydra (Slautterback and Fawcett, 1959; Chapman and Tilney, 1959) have failed to reveal any structure, collagenous or otherwise, in the capsule.

The mesoglea of Cordylophora is seen as a thin line separating the two cell layers, thicker in the hydranth region than in the stem. Slautterback (personal communication), in his electron microscope study of hydra, has observed minute pores in the mesoglea; these pores would permit the membranes of ectoderm and endoderm cells to come in contact at intervals.

The endodermal epithelium of Cordylophora is composed of at least two types of cells, generally termed gland and digestive cells. These cells are columnar, and bear flagellae on the surface in contact with the coelenteron.

The cells of Cordylophora and other hydroids are fairly small; the ectodermal cells of Cordylophora, for example, have a diameter of about 10 micra. The nuclei are spherical, and contain a single large nucleolus. Details of the cytology of Cordylophora may be found in the monograph of Schulze (1871) and in papers by Pauly (1900, 1902) and Morgenstern (1901). The electronmicrographs of hydra (Slautterback and Fawcett, 1959) show a fine structure similar to that of other organisms.

Sexuality

Although the experimental studies in this thesis are concerned exclusively with asexual Cordylophora, sexuality is a major aspect of the life of these organisms, and an aspect which would offer much to study could it be brought under laboratory control.

In Cordylophora, the two sexes are found on separate colonies. On side branches only, outgrowths appear which develop into knoblike appendages, the gonophores. Female gonophores each produce from 3 to 20 eggs, depending on conditions; the eggs are about 0.1 mm. in diameter. As male gonophores mature, clouds of sperm are released which travel through the aqueous environ-

ment to fertilize the eggs. The eggs develop in situ to planula larvae, two-layered ciliated ovals which then break free of the gonophore, swim or crawl to a suitable substratum, and there attach and differentiate into a hydranth, stem and stolon: the beginning of a new colony. Static observations on the gonophores and embryonic development of Cordylophora have been made by many, including Allman (1853), Schulze (1871), Morgenstern (1901), and Pauly (1902).

Comparison of laboratory hydroids

In comparison with other hydroids currently available for laboratory study, Cordylophora offers greater potentiality for certain developmental studies but would be unsuitable material for others. Hydra differs from Cordylophora in that it is a solitary organism which is never permanently fixed to the substratum. Populations of hydra can be treated as microorganisms; they can be collected, pipetted, centrifuged, etc. (cf. Lenhoff and Loomis, 1957), making hydra excellent material for research not possible with colonial hydroids. But Cordylophora forms an organized colony, making the organism suitable for studies of pattern, interaction of parts, and control of proportion. Hydra, having no perisarc and being completely motile, is too plastic for quantitative studies of shape (but cf. Rulon and Child, 1937; Li and Yao, 1945).

The other colonial hydroids available, Hydractinia and Campanularia, differ markedly from Cordylophora. Hydractinia colonies are polymorphic, in that they have separate morphological polyps for different functions. While the study of this polymorphism could be rewarding, Hydractinia lacks a regular colony pattern, which makes it less suitable than Cordylophora for studies of asexual development. Campanularia has an altogether different growth pattern than Cordylophora (Crowell, 1957), and its hydranths undergo a continuous cycle of development, aging, regression and replacement (Crowell, 1953), whereas the hydranths of the other hydroids are essentially permanent.

Of these four hydroids, only Cordylophora and Hydractinia produce, when sexually mature, large numbers of eggs which develop directly into polyps again. Hydra females carry few eggs, and after these begin to develop they encyst and remain encysted for variable periods of time. Campanularia colonies develop degenerate, sessile medusae; these medusae produce eggs which yield polyps again. Hydractinia eggs have already been used for embryological

(Tessier, 1931) and genetic (Hauenschild, 1954) studies; sexual Cordylophora should be equally useful.

Virtually all hydroids have considerable regenerative capacity, but one of the factors influencing the choice of Cordylophora is that, of all hydroids known to the author, it has by far the most remarkable ability to form a new organism from minute and mutilated tissue fragments. In fact, E. Zwilling (personal communication) has shown that bits of ectoderm removed from the coenosarc of Cordylophora can regenerate, first a new endoderm and subsequently an entire polyp.

The major features of Cordylophora which led to its selection are the simple and regular colony pattern, based on repetition of like units, the considerable ability to reconstitute polyps from tissue fragments, and the unusual hardiness under laboratory conditions.

Chapter III

Growth of *Cordylophora* under Controlled Conditions

The preparation of an organism for contribution to experimental biology begins with its domestication -- the accomplishment of cultivation under controlled conditions. Among other studies, those of Crowell, Hauenschild and Kinne are important in this regard, for they succeeded in growing three colonial hydroids in the laboratory.

Cordylophora has been kept in the laboratory since Allman (1872) reported that colonies survived for a while in dilute sea water, but died if placed in drinking water. Hargitt (1897) kept a colony in a pint jar next to a window for almost two years without changing the water or adding food; at the end of this time a few hydranths still survived. Schmalz (1913) also gave a brief report of survival of Cordylophora in the laboratory, but it was Roch (1924) who made the first extensive observations on the laboratory growth of Cordylophora. Roch's results, as well as the more extensive studies of Kinne (1956, 1958a, b), are discussed below.

All of the work on the growth of colonial hydroids in the laboratory has been done under conditions which are elaborate, involve the use of ocean water, and lack sufficient versatility to permit extensive variations of conditions. This chapter describes simple methods for the controlled cultivation and study of Cordylophora, similar to the methods for hydra developed so successfully by W. F. Loomis (Loomis, 1953, 1954; Loomis and Lenhoff, 1956). Cordylophora colonies are grown on microscope slides slanted in beakers. The culture solution is defined, and contains five required ions and a buffer. The cultures are grown at a constant temperature, fed on a daily schedule, and the culture solution changed shortly after feeding and again some hours later after the cultures have regurgitated undigested food.

MATERIALS AND METHODS

Cordylophora

All of the work discussed herein was done with the descendants of a single hydranth isolated from a colony growing Nye Pond, North Falmouth, Mass., in August 1957 (Clone A).[1] The sex of this clone is unknown.

---

[1] The writer is indebted to the Supply Department of the Marine Biological Laboratory, Woods Hole, Mass., and especially to Mr. Milton Grey, for repeatedly taking him to the sites where Cordylophora grows.

Four other clones were isolated in the summer of 1959 for experiments on sexual differentiation. Clone B (male) and C (female) were also isolated from Nye Pond, while clone D (male) and E (female) were isolated from Squiburcket Pond on Martha's Vineyard. These clones all grew under the conditions described below.

Culture container

The growth of the cultures on microscope slides slanted in beakers provides a satisfactory environment under the simplest possible conditions, with maximum ease of handling and observation, and with each colony in a separate container. For most purposes cultures were grown on 1 x 3 inch microscope slides slanted in 100 ml. beakers.

This method is superficially similar to the gradient tissue culture method of Osgood and Krippaehne (1955), in which tissue cultures are grown on slides slanted in bottles to create gradients in oxygen tension, etc. While slight gradients undoubtedly develop in the beaker-slide cultures, they never become pronounced due to the twice daily medium change, and the author has not observed any differential effects on the colonies due to position on the slide.

Culture solution

Although on the basis of the results described in the following chapter, a variety of completely suitable culture solutions may be devised, one in particular was established as the standard for these investigations. CCS5 (Cordylophora culture solution, 0.05 M NaCl) was made up with reagent grade salts in demineralized water, with the following composition:

salt	molarity
NaCl	0.05
KCl	0.001
$CaCl_2$	0.005
$MgCl_2$	0.005
$NaHCO_3$	0.001

The first 4 salts represent the requirements, and the bicarbonate buffers the solution at approximately pH 7.8. In many instances, $KHCO_3$ at 10^{-3} molar was substituted for the KCl and $NaHCO_3$.

The demineralized water was prepared in a Barnstead Bantam demineralizer and on the basis of its conductivity had less than 0.1 ppm. salts as NaCl.

CCS5 may be prepared in tap or distilled water if disodium ethylenediamine tetraacetate ("versenate") is added to sequester heavy metal ions

(cf. Loomis and Lenhoff, 1956). Cordylophora-versenated-tap or -distilled water (CVT or CVD) was used whenever precise definition of the aqueous environment was unnecessary.

These standard solutions have been prepared from paired 50 X stock solutions (Table 2).

Nutrition

Cordylophora is a carnivore, and it would be difficult to find a more suitable food source than washed larvae or nauplii of the brine shrimp, Artemia. Large quantities of dry, stable Artemia eggs may be purchased, and these eggs may be readily hatched under controlled conditions in virtually unlimited quantities. Nauplii of uniform size, age and physiological state are thus made available as a source of nutrients. Another crustacean, Daphnia, was the standard food for coelenterates (Hyman, 1937, p. 141) before Artemia was introduced by Crowell (1953), Hauenschild and Kanellis (1952), and Loomis (1953). No certain method of rearing Daphnia was available, and because culture contained Daphnia of assorted ages, the organisms provided a heterogeneous sourc of nutrients. The introduction of Artemia, more than any other factor, makes possible the development of controlled conditions.

Artemia were seeded daily and hatched at 21 ^{o}C on a 48-hour schedule following the methods described by Loomis and Lenhoff (1956). A modified salt solution was substituted because their solution gave a poor yield of nauplii, which was traced to heavy metals in the tap water (in both New York City and Woods Hole). A hatching solution was developed containing versenate and a buffer (A solution) which gave maximum yields of viable nauplii. A 30-fold concentrated stock A solution was prepared by adding, to 3200 ml. hot tap water, 345.6 gms. U.S.P. NaCl, 4.8 gms. disodium versenate, and 9.6 gms. $NaHCO_3$.

The hatched nauplii were collected and washed in a net as described by Loomis and Lenhoff (1956). The Cordylophora cultures were fed to repletion for about an hour, after which the medium was changed.

Handling cultures

The medium of the beaker-slide cultures was changed by lifting out the slide, pouring out the used medium, refilling the beaker with fresh culture solution, and returning the slide to the beaker.

When in active use, the cultures were fed daily, with a culture solution change one hour later and a second after about 6 hours. They were maintained in the dark in a constant temperature incubator at 22 ^{o}C.

TABLE 2

Stock solutions for CCS5 and derivatives

One liter lots of each 50x stock solution are prepared volumetrically, using demineralized water for the CCS5 stocks and ordinary distilled water for the CVD and CVT stocks.

		grams per liter	
CCS5 Stock II	$CaCl_2.2H_2O$	36.8	CVD or CVT Stock I
	$MgCl_2.6H_2O$	50.0	
CCS5 Stock I	NaCl	146.2	CVD or CVT Stock II
	$KHCO_3$	5.0	
- - - - -	Na_2 versenate	2.5	

When not in use, cultures survived extended periods without feeding, provided the culture solution was kept moderately clean, excessive evaporation avoided, and the temperature kept moderate (e.g., 15 to 25 °C). Young cultures (ca. 20 hydranths) have survived 4 months or more at room temperature in closed containers without medium change.

Starting new cultures

Since the Cordylophora used in this study have all been asexual, colonies were set up from pre-existing colonies. These new colonies may be designated, as Kinne (1956) has done, as secondary colonies. Fine silk thread was wound once around a slide, and held in position with a drop of molten wax. A single upright, usually with only 1 or 2 hydranths and 5 to 7 mm. of attached stem, was slipped between the thread and the slide, and the slide transferred to an appropriate container of culture solution. Cultures were started in groups of 9 in staining dishes with removable slide racks, which simplified handling.

For some purposes, suitable young cultures were provided simply by trimming away most of an older colony with a razor blade and permitting it to grow out again. To distinguish such colonies from secondary colonies, they are referred to as cut back older colonies. Unless otherwise stated, secondary colonies were used for the experiments described in this and the following chapter.

RESULTS

I. General pattern of growth

An upright tied to a slide soon sends out a stolon at its proximal end, which attaches to the slide and begins to grow in an essentially straight line along the substratum. This stolon branches at irregular intervals. At regular intervals along the stolon, uprights arise and soon bear a hydranth at their apex. The uprights lengthen and develop side branches which bear additional hydranths. At the same time the distal portion of the explanted upright continues to elongate and branch to produce a somewhat irregular growth, which usually does not attach to the slide (Figure 3).[2] This distal growth

[2]The distal growth of secondary colonies is relatively erratic, especially for studies of colony pattern. A more regular pattern may be obtained by cutting away the distal growth after the proximal, stolon growth has several hydranths; a second stolon will form at the new cut surface. The erratic pattern of distal growths does not, however, influence the regularity of growth curves, obtained as described below, and consequently secondary colonies were used for the experiments described in this and the following chapter.

shows essentially the same pattern as the growth of an upright in an older colony. Figures 2 to 4 illustrate colonies of various ages.

II. Exponential growth

The proper measurement of growth is a problem which has received a remarkable amount of attention, but little agreement seems to have been reached as to what constitutes growth or a valid measure thereof (cf. Weiss, 1955). But regardless of the issues involved, one needs a convenient and quantitative measure of what, in a general sense, constitutes increase with time.

The fact that Cordylophora is a colonial organism makes the situation somewhat different from that of hydra, where each organism can be considered a separate individual and can be treated in the same way as a microbial cell (Loomis, 1954). But when one examines a Cordylophora colony, it is clear that there is one unit which is reasonably correlated with the size of the colony and which can be conveniently measured with time (i.e., without sacrificing the colony) -- the hydranth. A count of increase in hydranth number with time should therefore yield a useful measure of colony growth.

With colonies growing under the conditions described, this measure of growth results in an essentially exponential curve (Figure 5). Such "log growth is exactly what is observed for the solitary hydranths of hydra (Loomis, 1953).

This result permits the determination of growth rate in a Cordylophora colony using the standard equations for exponential growth. If n represents the number of hydranths and t the time, the relative growth rate, k, remains constant as a function of hydranth number:

$$\frac{dn}{dt} = kn$$

This may be integrated to yield:

$$\ln n = \ln n_o + kt$$

where n_o equals the number of hydranths at $t = 0$.
If the time for the number of hydranths to double, T, is measured, this equation can be simplified (Loomis, 1954):

$$\ln (n/n_o) = kt$$

$$k = \ln 2/T = 0.693/T$$

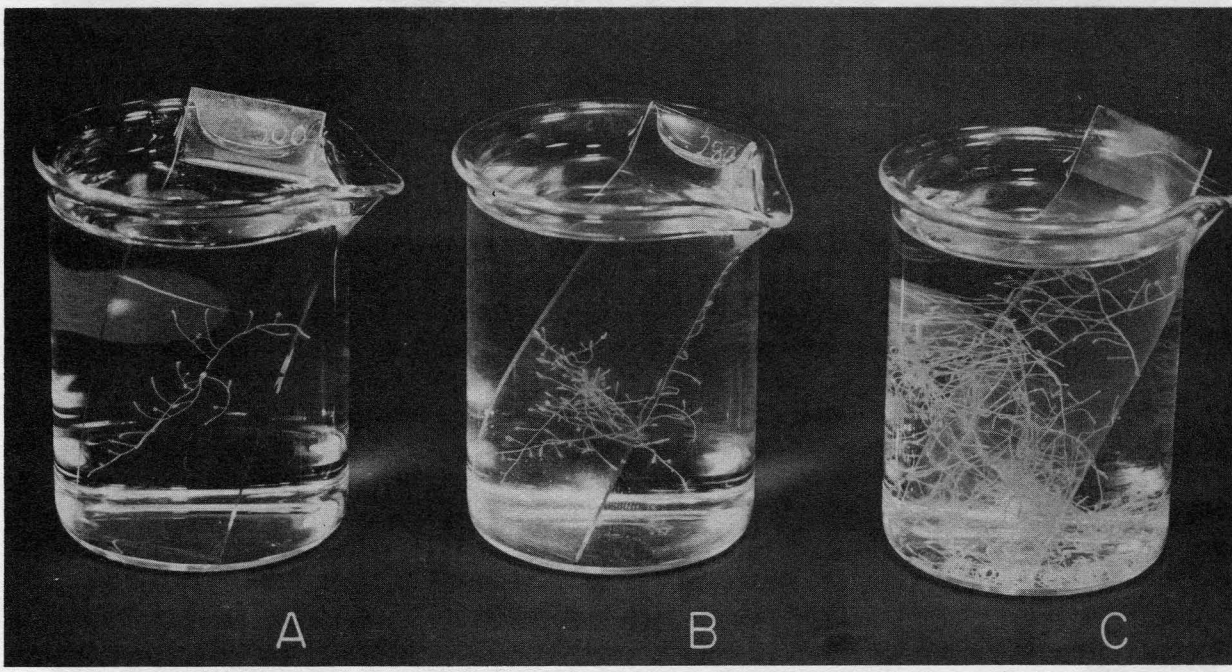
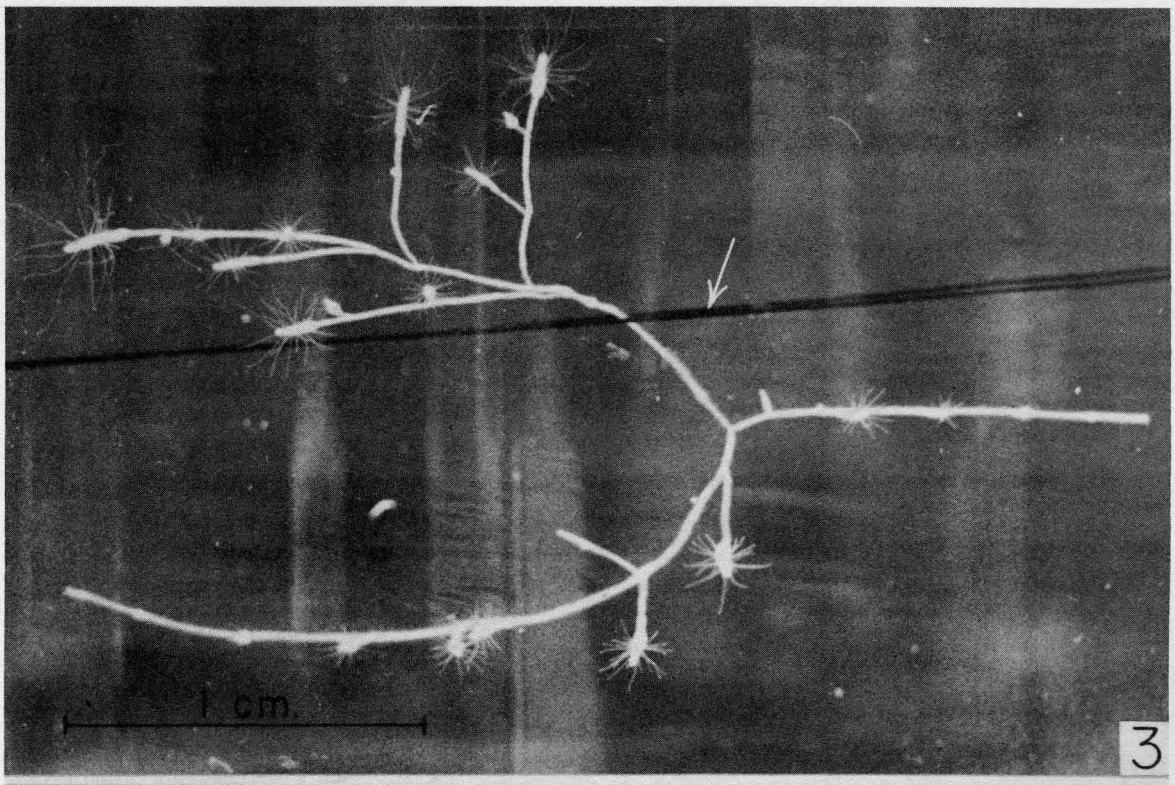


Figure 2. Three Cordylophora colonies growing under standard conditions on 1 x 3 inch microscope slides slanted in 100 ml. beakers. Colony A, which still has the thread attached with a drop of wax, was started from a single hydranth 13 days before the photograph was taken, and has 21 hydranths. Colony B is 25 and colony C 40 days old.



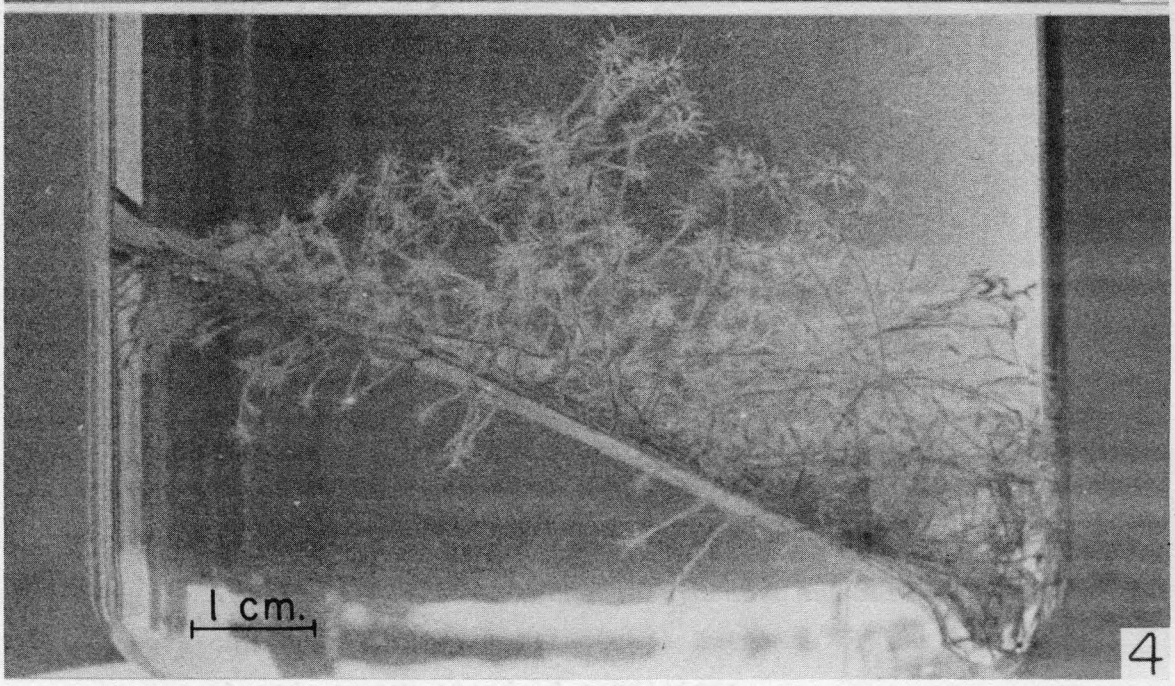


Figure 3. A young Cordylophora colony growing on a slide in CCS5. There are 23 hydranths. The portion above the thread is unattached, while the portion below, having two stolons, is attached. The thread is indicated by an arrow.

Figure 4. A colony approaching maximum density, transferred to a larger beaker for photography.

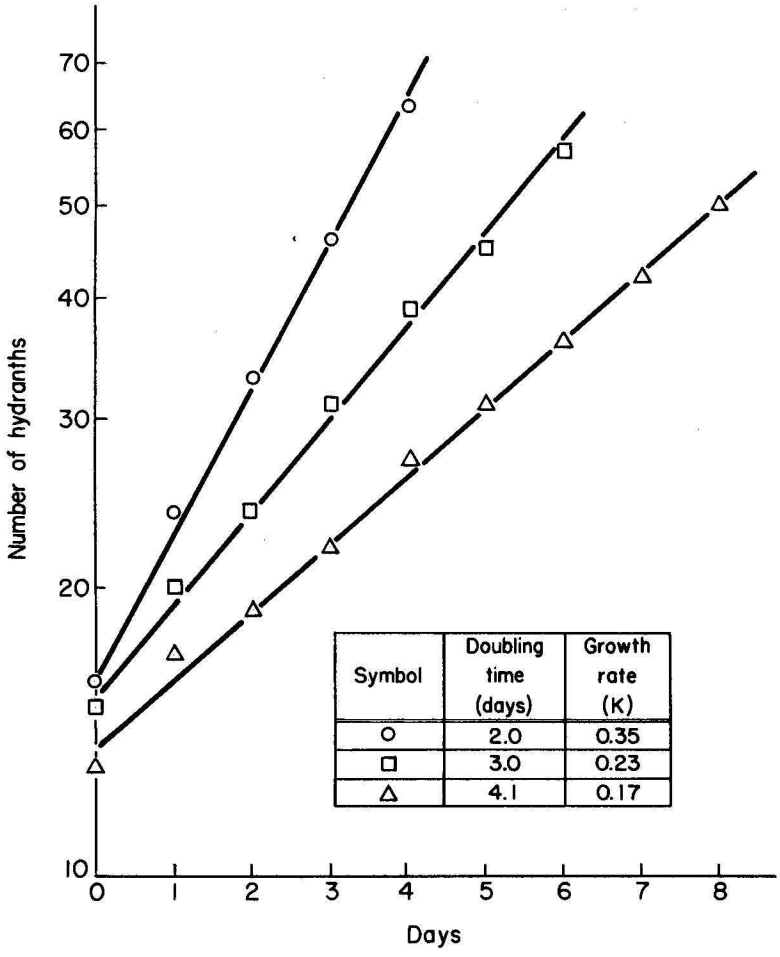


Figure 5. Exponential increase in hydranth number. Three growth curves selected to illustrate extremes of variation in growth rate under standard conditions.

In practice, the number of hydranths in a given colony was counted on a series of successive days. These data were plotted on semi-logarithmic paper, and the points interpolated to give a straight line, from which the doubling time T was determined to the nearest tenth of a day, and the growth rate calculated.[3]

Figure 5 shows three selected growth curves, and the doubling times and growth rates derived from them. Although all three of these growth curves were obtained under the standard conditions described, the growth rates vary by as much as a factor of two. Growth rates of 0.30 or above (i.e., a doubling time of less than 2.3 days) have been observed only rarely. Between the beginning of culture of clone A (August 28, 1957) and May 31, 1958, such rates were observed nine times out of a total of 97 cultures whose growth rates were followed. In 205 additional cultures followed before January 1960, only one case of growth above 0.30 was observed. In spite of a great deal of work on growth conditions, no insight has been gained into the conditions which erratically produce this high growth rate.

The phrase "essentially exponential" is qualified in Chapter VII. Because of the difficulty of counting accurately above 60 or 70 hydranths, growth generally has not been followed beyond that point. Further, because with fewer than 10 hydranths the growth curve is particularly erratic (due apparently only to the small numbers), growth experiments are not started until secondary colonies have grown to about 10 hydranths. Thus the maximum range of a growth curve is from about 10 to 70 hydranths, or two doublings. Furthermore, because of the small numbers involved, slight day to day variations in hydranth number from ideality can produce considerable deviations in the slope of the growth curve. All of these factors, in addition to biological variability, tend to reduce the precision of the growth rate.

To illustrate this, all of the data from experiments in which the growth rate of two or more replicate cultures was followed have been gathered (Table 3). Replicate cultures are here defined as cultures grown under standard conditions. The conditions within a group are not necessarily identical; such

---

[3]Other methods of measuring exponential growth rates are available, especially that used by Monod (1943, 1949), but the above method is used to permit direct comparison with the data of Loomis (1954).

variations as substratum or light which have not influenced growth rate (see below) are included as replicate cultures. A total of 22 experiments, with 78 cultures, are available. After the sample of cultures was taken, the doubling time of each culture was interpolated from the growth curve plotted on semi-logarithmic paper, and the growth rate determined. This growth rate was then compared with the growth rate determined for the same culture at the time of the experiment. The two independent determinations of growth rate provide a measure of latitude in visual determination of doubling time, and also of the deviation of growth curves from linearity. For the 78 growth curves, the mean growth rate in both determinations was 0.21, and the average deviation[4] in interpolation 0.013. This indicates an uncertainty of about a tenth of a day in interpolation of doubling time from the average growth curve.

As can be seen from the tables in this and the following chapter, the regularly observed growth rates result from a doubling time of 3 days, occasionally falling below 4 days. The major deviations in growth rate came between, rather than within, experiments, unless significant variations in conditions were introduced within an experiment. This is shown by the data in Table 3, and graphically in Figure 6. The average deviation from the mean growth rate ($k = 0.21$) of the 79 cultures is 0.030. If each of the 22 groups is calculated separately, the mean growth rate varies from 0.11 to 0.29, and the average deviation from 0.000 to 0.031. The "within group" average deviation has a mean of 0.013, or less than half the variability of the growth rates taken as a whole.

The range of growth rates within an experiment (Table 3) provides an estimate of the variability encountered in replicate cultures. In half of the 22 experiments, the range was 0.02 or less; in 21 of the 22 experiments, the range was less than 0.08; and in one of the experiments the range was 0.10. Thus it may be estimated that 95 per cent of the time a difference in growth rate of 0.08 or more between two cultures is significant.[5]

---

[4] Average deviation as used here is defined as the arithmetic mean of the difference between two sets of growth rates, i.e.,

$$\text{Average deviation} = \frac{\Sigma |k_2 - k_1|}{n}$$

where n equals the number of cases.

[5] A similar result follows if one assumes that the distribution of growth rates is normal and calculates the standard deviation and standard error of the mean. This is to be expected since range is an effective estimator of distribution with small samples (Snedecor, 1956, p. 38).

TABLE 3

Growth Rates of Replicate Cultures

Group no.	No. of cultures	Mean growth rate ($\bar{k}$)	Average deviation[1]	Range[2]
1	3	0.11	0.000	0.00
2	3	0.16	0.007	0.02
3	2	0.19	0.005	0.01
4	3	0.19	0.007	0.02
5	3	0.19	0.004	0.01
6	3	0.21	0.009	0.02
7	2	0.21	0.010	0.02
8	5	0.21	0.007	0.02
9	2	0.23	0.015	0.03
10	2	0.23	0.010	0.02
11	3	0.23	0.007	0.02
12	2	0.24	0.015	0.03
13	3	0.26	0.009	0.02
14	2	0.26	0.010	0.02
15	3	0.29	0.011	0.03
16	8	0.20	0.017	0.07
17	8	0.19	0.018	0.06
18	5	0.21	0.031	0.08
19	5	0.21	0.015	0.05
20	3	0.24	0.031	0.07
21	4	0.25	0.015	0.04
22	4	0.20	0.031	0.10
Mean values				
"Within group"	3.5	0.21	0.013	0.035
All cultures as a group	78	0.21	0.030	0.19

[1]Arithmetic mean of the absolute value of the deviation from the mean growth rate of the group.

[2]Maximum difference of any two growth rates in the group.

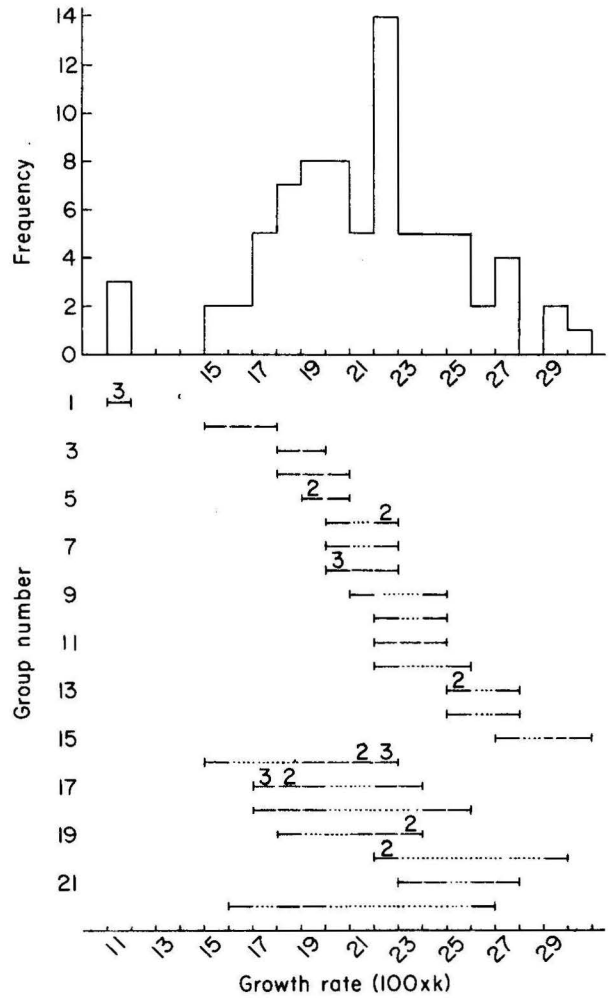


Figure 6. Range of variation of growth rate within and between experiments. The 78 cultures from 22 experiments are the same as those given in Table 3. The histogram shows the distribution of growth rates of the 78 cultures. In the lower portion of the figure, the solid horizontal lines indicate the growth rate of each replicate culture within a group while the solid vertical lines indicate the range of growth rates encompassed by a group.

In general, where a condition has been found to influence growth rate, it has been possible to show that extreme variations of the condition reduce the growth rate to zero. Where this has not been possible, the influence of the condition has been evaluated by repeated experiments. Consider the two cases tabulated below, which represent growth rates obtained under two pairs of conditions in repeated experiments.

	Condition I		Condition II	
	A	B	A	B
Growth rate	0.19	0.23	0.10	0.23
	0.22	0.21	0.14	0.25
	0.26	0.19	0.11	0.21

The growth rates for Condition I are considered similar or not significantly different; in other words variation of Condition I does not influence growth rate. In such cases, it is always possible that slight effects of the environmental factor on growth rate may be masked by the variability of k. Under Condition II, it is clear that the growth rate of cultures in group A is significantly lower than those in group B.

III. Methodological variables

Culture container and substratum

Colonies grown on the bottom of dishes rapidly become covered with debris, particularly undigested food and bacteria. In slanted cultures, undigested food falls to the bottom of the beaker from whence it is discarded with medium change, and while the slides in old beaker cultures tend to become covered with a thin film of bacteria (several unidentified species), it never becomes as pronounced as in cultures grown horizontally.

For short-term experiments, however, the horizontal cultures are often useful, as in the control of gaseous environment described in Chapter IV. During such experiments horizontal cultures are able to grow almost as rapidly as beaker cultures, as indicated by the experiment shown in Table 4. The growth rate of such horizontal cultures, however, falls off rapidly in comparison with the beaker cultures. On day 13, the colonies were dissected to obtain accurate counts of hydranth number, and while the beaker cultures had continued to increase at an essentially undiminished rate, the petri dish cultures had grown relatively little (Table 4). If continued, the horizontal cultures soon become necrotic, whereas beaker-slide cultures can be grown almos indefinitely (e.g., Figure 4).

TABLE 4

Comparison of growth in beaker vs. petri dish

Slide cultures were grown up as described, and transferred to either 100 ml. beakers or 20 x 100 mm. petri dishes containing 80 ml. CCS5. The cultures were fed daily and maintained at 22°C, with a second culture solution change.

Culture container	No. of hydranths on day: 1	2	3	4	5	6	7	8	9~13		Growth Rate (k)
Petri dish	10	13	14	18	23	27	32	36	47	66	0.19
	13	16	19	23	25	31	39	44	47	77	0.17
Beaker	12	16	22	26	29	38	49	54	68	118	0.23
	12	17	22	26	30	39	57	68	82	146	0.25

Exchange of gases between the beaker cultures and the atmosphere does not seem to be an important aspect of the method, since in three experiments cultures have grown at similar rates in open beakers and in beakers sealed with parafilm.

The substratum on which the cultures are grown is also not critical. Cultures are routinely grown on glass microscope slides which have not been pre-treated in any way, but they grow at similar rates on slides which have been pre-washed, etched in hydrofluoric acid or strong alkali, or on pieces of Lucite plastic.

Culture solution

CCS5, CVT and CVD have been used interchangeably in the maintenance of stock cultures and in growing up young colonies, and it has been found that the growth rate is not significantly influenced by the water used or the addition of versenate. If CCS5 is prepared in tap water without the addition of versenate, the colonies fail to grow and become necrotic. While the cause of this has not been traced, it is probably copper, as is the case with hydra (Chalkley and Park, 1947; Loomis and Lenhoff, 1956). If cupric chloride is added to CCS5 to a concentration of 10^{-6} molar, prey capture is much reduced and breaks develop in the coenosarc tissue. Curiously, while the coenosarc tissue becomes disrupted the hydranths remain healthy looking. The addition of citrate accentuates the effects of 10^{-6} M $CuCl_2$, but the addition of disodium versenate completely overcomes the toxicity of the cupric ion.

Although demineralized water is essentially free of ionic materials, it contains non-ionic substances and leachings from the resins, either of which might influence growth rate. The growth of Cordylophora has been found to be the same, however, whether the CCS5 is prepared with demineralized or Pyrex re-distilled water.

Nutrition

Because of the suitability of Artemia nauplii as a uniform source of food, it is impossible to make a really valid comparison with other organisms as a source of nutrients. The white worm, Enchytraeus, recommended as a nutrie for Cordylophora by Kinne (1956, 1958a, personal communication) has to be cut up into packets of the right size and hand fed individually to each hydranth. The growth rate of Cordylophora fed Enchytraeus daily was compared with cultures fed Artemia and found to be, if anything, somewhat slower.

Only two variables are introduced by the use of Artemia hatched under controlled conditions: the genetic variable introduced by varying batches of dried eggs, and the variable resulting from the growth of bacteria during the hatching of the nauplii. The influence of these two variables on growth has been investigated. Three different lots of Artemia eggs (probably representing different species, Dempster, 1953) have been compared and found to give similar growth rates. Artemia eggs were sterilized by the method of Provasoli, et al. (1959), and hatched in autoclaved A solution; the Cordylophora cultures fed sterile nauplii grew at a rate similar to that of control cultures.

Culture solution change

The result of changing the culture solution only once daily is similar to that obtained if the colonies are grown on the bottom of a dish. In a short-term experiment, growth rate is only slightly slower than if the medium is changed twice daily, but the cultures rapidly become dirty and the growth rate falls off sharply. In fact, if the second medium change is omitted in dense cultures, they rapidly become necrotic and begin to regress.

Since the method for Cordylophora involves growing these sessile organisms in a standing environment, it seemed possible that localized gradients might accumulate in the microenvironment around the colonies, and that such gradients might stimulate or inhibit growth. However, colonies agitated on an improvised shaker 15 times a minute grew at the same rate as standing cultures.

IV. Dense cultures

Although it is difficult to obtain accurate counts of hydranth number above 60 or 70 without dissecting the colonies, it is possible to grow cultures to considerably greater densities (Figures 2c, 4). One can obtain the weights of such colonies removed from their substratum, and as will be shown in Chapter VII, weight is a constant function of hydranth number. The wet weights of several cultures, all grown on 1 x 3 inch slides in 100 ml. beakers, are given in Table 5. For young cultures, the wet weight per hydranth is approximately 0.5 mg. Old colonies reached wet weights as high as 870 mg., or 1740 hydranth equivalents. Such colonies are still in good health.

It is possible to make an approximate calculation of the growth rate of such old cultures. Determinations for the cultures in Table 5 give doubling

TABLE 5

Wet weights of cultures as sacrificed

Colonies which had starved for 24 hours were scraped off slides with razor blades, the excess water removed with filter paper, and the tissue weighed (to ± 2 mg.).

Colony number	Age in days[a]	Wet weight in mg.	Calculated no. of hydranths[b]	Growth rate (k)[c]
261[d]	19	10	20	0.17
198	52	380	760	0.13
146	72	545	1090	0.09
239	75	430	860	0.09
179	80	660	1320	0.09
135	92	870	1740	0.07
217	98	810	1620	0.07

[a]Age in days from day colony started from 1-2 hydranths.

[b]Hydranths calculated on the basis of a wet weight of 0.5 mg. per hydranth.

[c]The growth rate which would have prevailed from the day the culture was started if the rate remained constant.

[d]One of six young cultures sacrificed as controls. Actual no. of hydranths was 21. The other young cultures gave similar values.

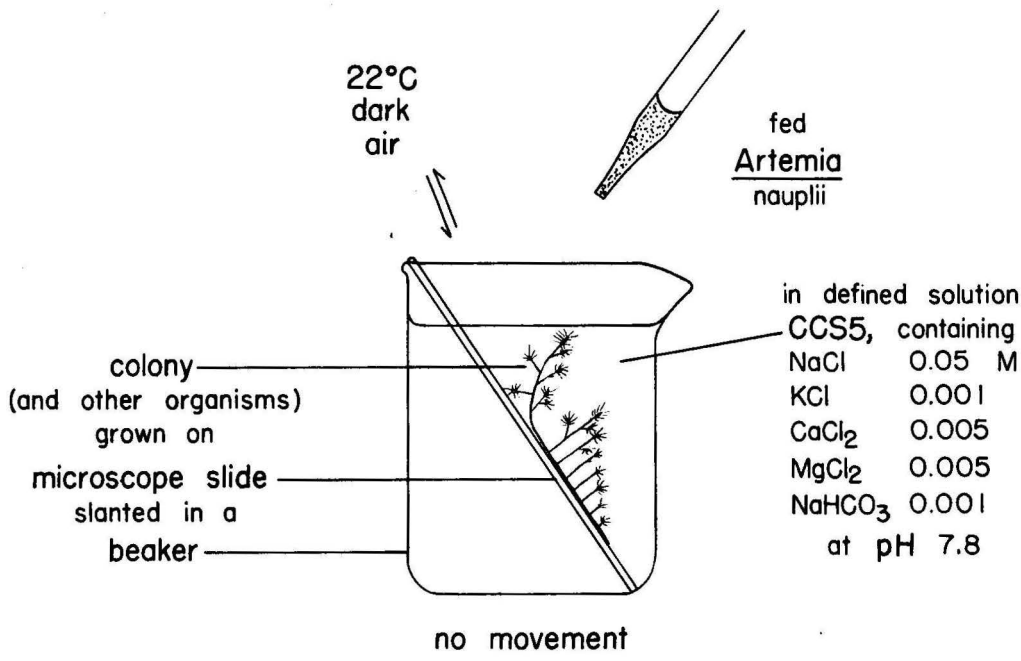
times as low as 10 days, or a k of 0.07; because of the nature of the calculation during the latter portion of the growth period the rate must be considerably lower.

In general, the denser cultures (greater than ca. 500 hydranths) cease to be useful. Without fastidious attention, and especially a second daily medium change, they quickly become necrotic and begin to regress. Even with a second medium change, the pattern of such colonies begins to become erratic. The most striking feature of the colonies is that the uprights often continue to elongate for considerable distances - as much as 4 cm. - without developing any branches.

DISCUSSION

The essential features of the culture method are diagrammed in Figure 7. Of the variables, the major advances of the method are (1) the growth of the colonies on slanted slides rather than on the bottom of dishes, (2) the development of a defined aqueous environment, and (3) the feeding of Artemia nauplii on a regular schedule. The method is analagous, in simplicity and reproducibility, to that developed for hydra by Loomis (1954).

The method may be compared with those used by the two workers who grew Cordylophora in the laboratory previously. Roch (1924, p. 366) indicates only that the cultures were "supplied regularly with food animals, and every 14 days the solutions renewed," and expresses his observations with such statements as "very good development." In the studies of Kinne (1956, 1958a), the colonies were grown on the bottom of dishes in sea water diluted with tap water. They were fed, at irregular intervals with Artemia, Daphnia, other copepods, and several species of worms -- apparently as available. The water was changed every few days. Kinne presents several growth curves, plotted linearly and apparently without realization that the increase in hydranth number with time was exponential and could be used to compute growth rate. Examination of his figures permits estimation of the growth rates he obtained. The three most rapid growth rates given in Kinne (1956) correspond to doubling times of 11-13 days, or a k of less than 0.06. In Kinne (1958a), the rates are similar, with the exception of one culture (Fig. 9, p. 425) which grew with a doubling time of 5 days giving a k of 0.14. Kinne's conditions were uncontrolled, and, on the basis of this author's observations, suboptimal.



Growth of Cordylophora under Controlled Conditions

Figure 7. A schematic illustration of the essential features of the culture method.

Although discussion of the significance of exponential increase in hydranth number in a colonial hydroid may best be deferred until more detailed data on the development of the colonies have been presented, certain aspects of the relative growth rate may be considered here. Using growth rate (k) as defined herein, Loomis (1954) found the maximum observed growth rate of Hydra littoralis to be 0.44, but under essentially similar conditions growth rates as low as 0.30 were observed. For Cordylophora lacustris, growth rates as high as 0.35 have been observed, but in general the rate is about 0.21-0.23 (Figure 6).

Monod (1949) emphasized that "the accuracy, the ease, the reproducibility of bacterial growth constants is remarkable, and probably unparalleled, as far as biological quantitative characteristics are concerned." In the same review, Monod considers exponential growth rates to represent a measure of the steady state rate of an increasing population. It should be noted, however, that these considerations depend on the fact that bacterial growth rates are usually determined over the course of many generations. In contrast, the growt rates of hydra and Cordylophora are determined from about two doublings, and thus must not be considered to always represent a long-term steady state rate. For example, Thimann (1955, p. 556) points out that while Escherichia coli has been found on occasion to grow with a generation time of as little as 17 minutes, over longer periods generation times of 30-40 minutes are observed. Or, in a recent study of the growth of Chlorella in synthetic medium, Sorokin and Krauss (1959) found that while steady state cultures grew exponentially with a minimum doubling time in weight of 2.5 hours, cultures synchronized by light could grow for a short time with a reduced doubling time of 1.5 hours.

The Cordylophora colonies which have grown with a k greater than 0.30 represent an enigma which has not yet been resolved. Such rapidly growing cultures appeared in approximately 10 per cent of the cultures followed during the first year, but in only about 0.5 per cent of the many cultures followed since. Including the studies to be detailed in the next chapter, many possible explanations for this phenomenon have been sought without success. Perhaps the rare high growth rates are due to the chance meeting of some peculiar set of conditions such as those which occasionally give rise to reduced doubling times in such organisms as E. coli, Chlorella or Hydra.

One distinct difference between studying the growth rate of most organisms and Cordylophora should be mentioned. With other organisms, if one wishes to perform a growth experiment, one simply removes replicate populations from a common pool and uses these aliquots for the experiment. In marked contrast, Cordylophora is a sessile colonial organism, so that for growth experiments one must set up the replicate cultures a week or more in advance of the experiment and use them after they have grown up. Thus some variability may be introduced into the growth rate of Cordylophora by microphysiological differences which develop during the development of replicate cultures.

With hydra, it was possible to develop mass culture methods, by which 10,000 or more hydra could be reared in a single dish (Loomis and Lenhoff, 1956 Cordylophora does not lend itself readily to such methods. Hydra, being solita and motile, will distribute itself throughout a culture, so the major limiting variable in the size of a given culture is the size of the container. In contrast, even if Cordylophora colonies were grown on large glass slides in large beakers, the older portions of the colonies would rapidly achieve a density inimical to rapid growth and normal development. If one wished to grow large quantities of Cordylophora, several alternative procedures could be developed. The most direct would be to grow many of the standard beaker cultures, each to yield several hundred hydranths. A variation of this method would be to attach many single uprights to a single large substratum simultaneously, so that the entire surface would quickly become covered with a colony of uniform density. An alternate procedure would be to grow Cordylophora in a continuously flowing culture solution. The critical necessity of a second daily medium change for dense cultures suggests that the gradual limitation of growth is due to the development of an unfavorable external milieu; if this hypothesis is valid the difficulty could be eliminated by continually changing the aqueous environment.

Now that Cordylophora can be grown under controlled conditions, it would be worthwhile to progress in the direction of defined conditions. The first step would be the rearing of colonies in the absence of other flora and fauna, with the exception of sterile Artemia nauplii as food (monoxenic culture; Dougherty, 1959). If this could be accomplished, the possibility of introducing artifacts due to associated organisms could be eliminated; but it has not been possible as yet. Enumeration of the bacteria present per hydranth in several colonies by triturating individual hydranths in saline followed by dilution and plating on Penassay agar have given a remarkably constant number

of about 3 x 10^3 bacteria per hydranth. At least 6 species are represented, based on colony and bacterial morphology. Growing the colonies in the presence of antibiotics has failed to erradicate this population, although cultures of the bacteria were themselves sensitive to the antibiotics used. Presumably these bacteria are protected in the coelenteron, since the addition, for example, of aureomycin (20 µg./ml.) to the culture solution dramatically reduces the external flora without destroying the internal flora or influencing growth rate. The elimination and possible role of this internal flora will require further study.

Chapter IV

Environmental Factors Influencing Growth Rate

There are at least three reasons for systematically studying the influences of various controllable factors on the growth of an organism. First, the results of such a study indicate which factors are essential and which are dispensable for growing the organism in the laboratory, permitting simplification of the conditions in some cases and rapid tracing of unexpected difficulties in others. The results also provide manipulatability, for with a knowledge of the effects on the organism of all the factors which can readily be varied, one is not bound to one set of conditions which work, but can devise modifications of the conditions to suit the needs of particular experiments. Finally, such a study can provide significant clues pertaining to the growth of the organism in nature, or to other biological aspects of the organism. For example, Eagle's studies of the requirements of cells in tissue culture led directly to insight into the metabolism of these cells (Eagle, 1959).

In the present study, the methods described in the previous chapter are used as a basis for study of the various parameters influencing growth of Cordylophora. The basic criterion for the influence of conditions is the growth rate, measured in terms of increase in hydranth number with time, but other observations made during the experiments are reported as well. The approach of the study is analagous to that used by Loomis (1954) for hydra; to facilitate comparison the results are tabulated in the manner used by Loomis.

Table 6 lists those factors which seem especially relevant. Those variables more or less fixed by the basic method of culture have been discussed in Chapter III. Of those factors which remain to be discussed, the only ones listed in the table which have not as yet been adequately studied are the intrinsic factors. Preliminary studies of the growth rate of various clones, and of the influence of the associated flora, has led to several complications, indicating that the intrinsic variables will require special study. Within the framework of the results reported here, however, there has been no indication that the variables of the organism would invalidate the results of the experiments in any manner.

METHODS

Unless a change in procedure is indicated for a given experiment, the cultures were grown under the following basic conditions. Secondary colonies of Cordylophora clone A were grown on 1 x 3 inch microscope slides slanted in

TABLE 6

Factors potentially influencing the growth rate of *Cordylophora* colonies under controlled conditions

I. Intrinsic factors
   - A. Genetic constitution of clone of *Cordylophora*
   - B. Organisms growing in association with *Cordylophora*
     1. Symbionts providing growth factors
     2. Parasites removing growth factors or producing toxic by-products
   - C. Colony pattern

II. Aqueous environment
   - A. Ionic requirements
   - B. Ionic interactions
   - C. Hydrogen ion concentration
   - *D. Toxicity of water or salts

*III. Physical conditions of culture
   - A. Substratum
   - B. Volume of culture solution
   - C. Movement or agitation

IV. Physical conditions of environment
   - A. Temperature
   - B. Light
   - C. Gaseous environment
     1. Oxygen
     2. Carbon dioxide

V. Nutrition
   - *A. Nature of food source
   - B. Amount of food

*Variables of method, Chapter III

100 ml. beakers as described in Chapter III. The culture solution was CCS5, made up with reagent chemicals and demineralized water containing less than 0.1 ppm salts (as NaCl). The cultures were fed to saturation for an hour once each day with Artemia nauplii, and the culture solution changed thereafter and again 6 to 8 hours later. Between feedings the cultures were maintained in the dark at a constant temperature of 22 °C.

In general, the results reported represent only the final experiments done to confirm the picture obtained in one or many preliminary experiments. Where data from more than one experiment are reported in a table, each experiment is reported separately because variation in growth rate is greater between experiments than within an experiment, as discussed in Chapter III.

Growth rate (k) is calculated from doubling time. For convenience, negative growth (decrease in number of hydranths) is recorded as $k = 0.00$. Where growth is positive, but at a rate too low to measure, it is recorded as $k < 0.1$. The day that the cultures were first transferred to experimental conditions is termed day zero, and is usually not reported in the tables.

Under decidedly unfavorable conditions, all of the hydranths of a colony are either resorbed or fall off, yielding a hydranth number of zero. In most cases, the coenosarc tissue of such colonies is able to regenerate new hydranths on return to favorable conditions.

RESULTS

I. Ionic requirements with CCS5 as base

Preliminary studies indicated that Na^+, K^+, Ca^{++}, Mg^{++}, and Cl^- were required for growth of Cordylophora, and suggested that CCS5 was a suitable combination of these ions.

Na^+ requirement

Sodium ions are an absolute requirement for the growth of Cordylophora (Table 7). In the absence of sodium ions, the ability of the hydranths to capture Artemia (i.e., release nematocysts) is abolished, the tentacles swell, and hydranths are gradually resorbed. High concentrations of sodium ions do not immediately inhibit prey capture, but result in a gradual contraction followed by dissociation of the hydranth tissue.

The requirement for sodium ions is probably not an osmotic requirement, since the colonies survive and grow in 1/10-1/50 the optimal concentration,

and since Cordylophora colonies are known to grow in fresh water.

In lowering the sodium concentration the concentration of chloride ions is simultaneously reduced, but that this is not responsible for the effects observed is shown below.

K^+ requirement

If potassium ions are omitted from the medium the hydranths also undergo gradual resorption (Table 8). However, the process is slower, so that even after a day or so in the absence of potassium the hydranths appear reasonably healthy and are able to capture prey and eat. Even 10^{-4} molar KCl is insufficient to allow continued maintenance of hydranths. It is curious that regression of hydranths in response to potassium deficiency is not completely random; in several cases the last hydranth to be resorbed has been the terminal hydranth on the upgrowth of the colony.

Excess potassium ion (0.1 molar) results in rapid resorption of hydranths.

Ca^{++} requirement

In the absence of sufficient calcium ions, the tissue gradually dissociates (Table 9). Low calcium (10^{-4} molar) permits survival, but not growth. An excess of calcium results in resorption of hydranths.

It is probable that in Cordylophora one of the functions of calcium ions is to bind the cells together, as has been suggested for other systems (cf. Steinberg, 1958). Calcium is the only required ion the absence of which results in dissociation rather than resorption of hydranths. It is significant that in most unicellular organisms the Ca^{++} requirement is low (e.g., Eagle, 1956), whereas the cells of all metazoan tissues known to the author tend to dissociate in the absence of calcium.

Mg^{++} requirement

The results from one experiment in which the concentration of magnesium ion was varied are presented in Table 10. It is clear that in the absence of added magnesium growth continues, but at about half the rate of cultures to which magnesium is added. This result has been obtained in five experiments.

It is not clear whether the requirement for magnesium is only partial under these conditions or cannot be demonstrated as absolute because of traces of Mg^{++} in the other salts (cf. Eagle, 1956). It would be possible in theory to remove these traces of Mg^{++} with sequestering agents, but a search of possibl

TABLE 7

Sodium requirement for growth of Cordylophora

All solutions contained $KHCO_3$. 0.001 M; $CaCl_2$, 0.005 M; and $MgCl_2$, 0.005 M.

NaCl M	No. hydranths on day: 0	1	2	3	4	5	Growth rate (k)
none	18	0					0.00
0.001	19	19	22	25	29	32	0.13
0.01	15	16	20	26	30	35	0.20
0.05	17	21	25	31	40	52	0.22
0.10	13	14	17	17	17	23	0.11
0.20	12	12	12	12	11	11	0.00
0.30	9	0					0.00
0.40	16	0					0.00

TABLE 8

Potassium requirement for growth of Cordylophora

All solutions contained NaCl, 0.05 M; $CaCl_2$, 0.005 M; $MgCl_2$, 0.005 M; $NaHCO_3$, 0.001 M.

KCl M	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
none	10	10	0				0.00
10^{-5}	14	14	0				0.00
10^{-4}	10	10	5	4	1	1	0.00
5×10^{-4}	11	13	14	18	19	24	0.15
10^{-3}	11	13	14	17	20	25	0.16
2×10^{-3}	14	16	19	22	27	31	0.16
10^{-2}	16	18	20	28	28	31	0.14
10^{-1}	0						0.00

TABLE 9

Calcium requirement for growth of Cordylophora

All solutions contained NaCl, 0.05 M; KCl, 0.001 M; $MgCl_2$, 0.005M; $NaHCO_3$, 0.001 M.

$CaCl_2$ M	No. hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
none	0							0.00
10^{-5}	15	0						0.00
10^{-4}	9	10	10	10	10	10		0.00
10^{-3}	14	17	20	27	30	37	45	0.20
2×10^{-3}	14	16	19	22	29	36	42	0.18
5×10^{-3}	12	15	15	18	21	26	34	0.17
10^{-2}	16	19	21	24	28	35	40	0.15
10^{-1}	7	0						0.00

TABLE 10

Magnesium requirement for growth of Cordylophora

All solutions contained NaCl, 0.05 M; KCl, 0.001 M; $CaCl_2$, 0.005 M; $NaHCO_3$, 0.001 M.

$MgCl_2$ M	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
none	12	12	12	14	16	20	$\leqslant$0.13
10^{-5}	12	15	19	21	24	28	0.17
10^{-4}	12	13	16	17	22	24	0.15
10^{-3}	12	16	23	25	29	37	0.23
5×10^{-3}	12	15	20	28	31	36	0.25
10^{-2}	14	15	15	24	31	35	0.23
10^{-1}	0						0.00

agents has revealed none which have a greater affinity for Mg^{++} than for Ca^{++} (cf. tables in Charberek and Martell, 1959). Disodium versenate added to the medium at concentrations up to 10^{-5} molar does not enhance the magnesium requirement; this is to be expected since versenate is known to selectively bind all Ca^{++} present before complexing with Mg^{++}.

Excess magnesium causes dissociation of the hydranth tissue.

Cl^- requirement

Determination of a chloride ion requirement for growth is difficult since the cations are normally supplied in the chloride form and other anions tend to be inimical to growth (cf. Eagle, 1956). It was possible, however, to compound suitable media for Cordylophora, and to demonstrate an absolute requirement for chloride (Table 11). In the first experiment illustrated, the culture solution is more dilute than CCS5, but adequate for growth. All relevant combinations of the ingredient anions are tested, and it is clear that except in the absence of chloride ions growth occurred. The second experiment, using a medium having the cationic composition of CCS5, gives the same result.

Although somewhat contracted, the hydranths remain essentially normal in appearance in the absence of chloride and are able to capture and eat Artemi larvae.

Conclusion

Figure 8 summarizes the ionic requirements of Cordylophora with CCS5 as base. Under the conditions described, in the absence of Na^+, K^+, or Ca^{++} the hydranths cannot survive, whereas in the absence of Cl^- the hydranths survive and feed but no growth ensues. In the absence of Mg^{++}, growth continues at a reduced rate. It may be noted that CCS5, which was developed by trial and error, contains an essentially optimal amount of each required ion.

Roch's medium

The work of Roch (1924), mentioned in Chapter III, includes the only previous study of the ionic requirements of Cordylophora. As part of this stud he prepared a dilute artificial sea water and similar solutions from which each of the salts were omitted. He reports that distilled water and pure salts were used. Roch found that if Na^+, K^+, or Cl^- were omitted, the colonies perished. Mg^{++} ions were found to be dispensible, and "calcium salts present a certain impediment for the development of Cordylophora" (p. 371). Since his methods of

TABLE 11

Chloride requirement for growth of Cordylophora

EXPERIMENT I

All solutions contained Na+ (as NaCl or Na_2SO_4) 0.01 M (except last culture which contained 0.02 M); K+ (as $KHCO_3$) 0.001 M; Ca++ (as $CaCl_2$ or $Ca(NO_3)_2$) 0.001 M; and Mg++ (as $MgSO_4$) 0.001 M.

Chloride conc.	Na+ as Cl^-	Na+ as $SO_4^=$	Ca++ as Cl^-	Ca++ as NO_3^-	No. hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
0.012 M	+		+		12	13	15	17	19	24	25	0.13
0.002 M		+	+		13	16	17	19	21	24	27	0.13
0.010 M	+			+	10	11	11	12	15	18	20	0.12
0.000 M		+		+	8	8	9	9	9	9	9	0.00
0.001 M	+	+		+	9	10	12	13	14	16	18	0.12
0.010 M	+	+		+	13	14	17	19	22	24	30	0.15

EXPERIMENT II

All solutions contained Na+ (as NaCl or Na_2SO_4) 0.05 M; K+ (as $KHCO_3$) 0.001 M; Ca++ (as $Ca(NO_3)_2$) 0.005 M; and Mg++ (as $MgSO_4$) 0.005 M.

Chloride conc.	Na+ as Cl^-	Na+ as $SO_4^=$	No. hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
0.000 M		+	18	19	16	16	16	16	16	0.00
0.001 M	+	+	13	14	17	16	17	17	17	<0.10
0.010 M	+	+	12	14	16	16	18	18	22	<0.10
0.050 M	+		13	18	21	26	34	37	45	0.25

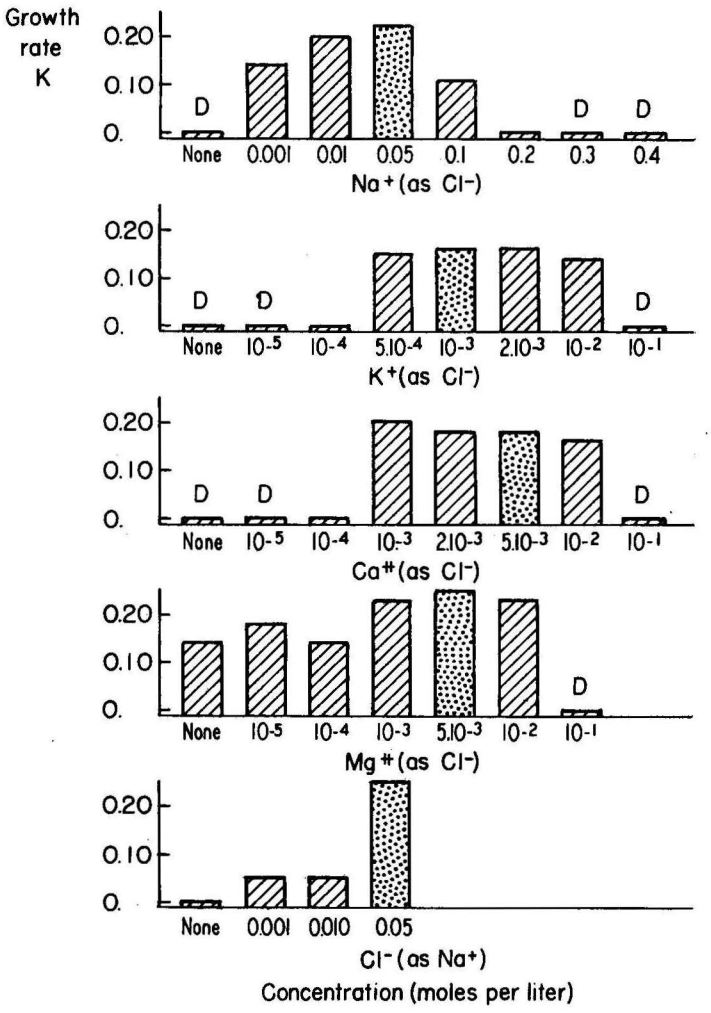


Figure 8. Influence of the ionic constituents of CCS5 on the growth rate of Cordylophora. The histograms represent the data contained in Tables 7-11. A "D" indicates that the hydranths regressed under the indicated conditions. The stippled blocks indicate the approximate ionic composition of CCS5.

observation were not quantitative, it is not surprising that no influence of magnesium ions was detected, but his observation that calcium ions, if anything were harmful, disagrees sharply with the observations reported here, as well as with the general observation that calcium is required for the maintenance of metazoan tissue integrity.

Because of the possibility that there was something special about Roch's medium which produced this phenomenon, the growth of colonies was followed in a calcium-free medium essentially identical to Roch's, and in aliquots to which various concentrations of $CaCl_2$ were added. The results of the experiment were unexpected (Table 12). In the complete absence of calcium, or in 10^{-5} molar calcium, the hydranths rapidly dissociated, as they do in calcium-deficient CCS5. However, as the concentration of calcium was increased to the level found in CCS5, the medium became increasingly inimical to survival. It is not known whether an intermediate concentration of calcium would permit growth in Roch's medium. If one assumes that enough calcium was present as an impurity in Roch's calcium-free medium to maintain tissue integrity, his results have been confirmed.

The explanation of this toxicity of calcium is not readily apparent. The amounts of Na^+, K^+, Mg^{++}, and Cl^- in Roch's medium should be adequate on the basis of the studies with CCS5 (cf. Table 12 and Fig. 8). Bromide ions are present in Roch's medium but at low concentration (3×10^{-5} M), and it is difficult to see how Br^- could make Ca^{++} toxic or vice versa. The absence of a buffer in Roch's medium is probably not responsible, since as will be described below colonies grow well in CCS5 without buffer. Understanding of this phenomenon requires further study.

II. Ionic interactions

Since the experiments reported have indicated requirements for 5 ions, there could be as many as 10 interactions of pairs of ionic species. Certain of the more likely of these have been selected for examination.

Na^+ vs. K^+

With CCS5 as base, growth ceases completely when the concentration of NaCl reaches 0.2 molar (Table 7). Yet it has been known for many years, both from observations in nature and in the laboratory (cf. Roch, 1924; Kinne, 1956) that Cordylophora will tolerate a wide range of salinities. This result has been confirmed for the clone of Cordylophora in use (Table 13). The NaCl concentration of 80 per cent sea water is 0.34 molar.

TABLE 12

Influence of calcium on Cordylophora in Roch's medium

All solutions contained NaCl, 0.035 M; $MgCl_2$, 0.0027 M; $MgSO_4$, 0.0009 M; K_2SO_4, 0.0003 M; and KBr, 0.00003 M. This solution is identical to Roch's Ca-free medium (1924, Tables 4-5), except that equimolar KBr is substituted for $MgBr_2$. When Roch added calcium, he added $CaSO_4$, 0.0008 M and $CaCO_3$, 0.00007 M.

$CaCl_2$ M	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
10^{-5}	1	0					0.00
10^{-4}	14	15	15	13	11	7	0.00
10^{-3}	15	17	17	15	10	4	0.00
5×10^{-3}*	14	14	10	7	4	0	0.00

*Concentration of $CaCl_2$ in CCS5.

TABLE 13

Growth in serial dilutions of artificial sea water

Artificial sea water was prepared according to the formula of the Marine Biological Laboratory (E.B. Harvey, 1956, p. 156) and diluted with demineralized water to give the indicated percentage (volume/volume) of sea water. The concentration of bicarbonate was adjusted so that all were 0.001 M $NaHCO_3$.

Per cent sea water	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
0.0	0						0.00
2.5	12	11	11	15	17	19	<0.12
5.0	13	14	15	21	26	33	0.21
10.0	12	14	20	21	25	31	0.19
20.0	14	16	19	22	27	35	0.18
40.0	12	13	15	21	30	39	0.23
80.0	10	11	13	16	21	24	0.17
20.0*	13	15	17	18	25	30	0.17

*Natural sea water taken from the running system of the Marine Biological Laboratory was filtered and diluted with demineralized water.

To determine which ionic constituent or constituents of the culture solution become limiting as NaCl is increased, a medium was prepared having the composition of CCS5 except for a five-fold increase in NaCl from 0.05 to 0.25 molar. Each other constituent of the medium was then varied, and it was found that potassium could reverse the effect of high sodium (Table 14), while none of the other ions was effective. If the concentration of Na^+ ions is increased five-fold, the concentration of K^+ must also be increased 5 to 10 fold to permit growth, indicating a definite antagonism between Na^+ and K^+ (cf. MacLeod and Snell, 1948).

Ca^{++} vs. Mg^{++}

Interactions between calcium and magnesium ions are encountered frequently, and since growth will continue in a medium without added magnesium (Table 10), it was of interest to see what effect such a medium would have on the Ca^{++} requirement. The results fail to indicate any interaction (Table 15, cf. Table 9), and also demonstrate that the magnesium requirement is not due to a toxicity of calcium ions in CCS5.

These studies of ionic interactions could profitably be extended, not only to specify the interactions which occur but also to use this information to develop rules by which suitable media varying in their salinity could be compounded. Such media could profitably be used in studying the development of pattern in Cordylophora colonies (cf. Chapter VII). The study has already yielded three such media: CCS5, which contains 0.05 M NaCl; a medium containing 0.01 M NaCl (Table 11, Expt. I); and one containing 0.25 M NaCl (Table 14). All three solutions permit growth at a similar rate.

III. Trace requirements

A wide variety of experiments have failed to indicate any stimulation of growth by ions other than the five already discussed. Bicarbonate ions, routinely incorporated into CCS5 as a buffer, may be removed or replaced without effect. Other anions, added at subinhibitory concentrations, have not influenced growth rate: iodide (Ham and Eakin, 1958, showed that traces of iodide enhanced the rate of hydra regeneration), bromide, borate, molybdate, nitrate, sulfate, phosphate. Mixtures of subtoxic concentrations of the metals commonly found as trace requirements (Mn^{++}, Fe^{+++}, Co^{++}, Ni^{++}, Cu^{++}, Zn^{++}) did not enhance growth, nor did a soil extract. Natural sea water, a rich source of trace ions, gives the same growth rate as artificial sea water

TABLE 14

Potassium requirement in the presence of high sodium

All solutions contained NaCl, 0.25 M; $CaCl_2$, 0.005 M; $MgCl_2$, 0.005 M; $NaHCO_3$, 0.001 M.

KCl M	No. hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
0.0001	0							0.00
0.001*	7	7	7	2	0	0	0	0.00
0.005	24	27	36	41	45	47	50	0.17
0.01	19	25	30	38	40	45	53	0.20
0.02	17	20	22	24	25	26	26	<0.1

*Concentration of KCl in CCS5.

TABLE 15

Calcium requirement in the absence of magnesium

All solutions contained NaCl, 0.05 M; KCl, 0.001 M; $NaHCO_3$, 0.001 M.

Molarity $CaCl_2$	Molarity $MgCl_2$	No hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
none	none	13	0						0.00
10^{-5}	"	12	6	0					0.00
10^{-4}	"	15	17	15	15	11	9	9	0.00
10^{-3}	"	14	16	16	17	18	18	21	<0.1
$5x10^{-3}$	"	14	15	17	19	21	22	23	<0.1
$5x10^{-3}$	$5x10^{-3}$	16	20	23	25	28	34	38	0.14

at the same concentration (Table 13). The addition of versenate to CCS5 does not reduce growth rate, although it undoubtedly sequesters traces of any heavy metals present.

Nevertheless, Cordylophora almost certainly requires traces of most of these ions, since all organisms for which the environment has been sufficiently purified have been found to require a considerable number of trace elements (e.g., Edsall and Wyman, 1958, p. 5). The situation is similar to that observed by Eagle (1956), where no trace requirements could be demonstrated for mammalian cells in culture in media containing 1 to 5 per cent dialyzed serum. Artemia nauplii must supply Cordylophora with an adequate source of trace ions, as well as other required, although completely unknown, nutrients.

IV. Hydrogen ion concentration

Study of the influence of pH on the growth of organisms is frequently difficult due to the scarcity of buffers in the physiological range which are neither toxic nor interact unfavorably with components of the medium (cf. Loomis 1954; Provasoli, et al., 1957). With CCS5, phosphate was used below pH 7 and tris (hydroxymethyl) aminomethane for pH 7 and above. Neither buffer exerted, in itself, any significant influence on growth rate (Table 16). As the table indicates, the growth of Cordylophora colonies is quite indifferent to the pH of the aqueous environment, the growth rate being virtually unaffected between pH 6.3 and 8.6. At pH 5.1 the colonies survived but failed to grow. Attempts to prepare stable buffers above pH 9 with carbonate resulted in precipitation.

It is of interest that in each of the solutions listed in Table 16, the action of Cordylophora was to drive the pH toward neutrality. Whether this is due to an alteration of the metabolism of Cordylophora under acidic or alkaline conditions, or to the excretion of neutral buffers by Cordylophora is unknown.

V. Physical factors influencing growth

Physical variables more directly pertinent to conditions of culture have been discussed in Chapter III. The major physical variables remaining are temperature, light, and gaseous environment.

Temperature

Kinne (1956) reported that Cordylophora colonies remain healthy from about 8 to 24 °C, and that the range tolerated is influenced by salinity (per cent sea water). The author finds that at temperatures below about 8 °C, the

TABLE 16

Influence of pH on growth of Cordylophora

All solutions contained NaCl, 0.05 M; KCl, 0.001 M; $CaCl_2$, 0.002 M; $MgCl_2$, 0.005 M. Buffers employed were: (1) 0.005 M NaH_2PO_4, brought to the desired pH with NaOH, and (2) 0.005 M tris (hydroxymethyl) aminomethane, brought to the desired pH with HCl. Readings of pH were taken regularly with a glass electrode, both before and after exposure of the solutions to the colonies, and maximum variations in pH are recorded below.

Buffer	pH	No. hydranths on day:					Growth rate
		1	2	3	4	5	(k)
PO_4	5.10 + 0.03	16	16	16	16	16	0.00
PO_4	5.80 + 0.05	25	30	37	41	47	0.16
PO_4	6.30 + 0.05	18	22	29	36	43	0.22
PO_4	6.90 + 0.00	16	20	25	32	43	0.24
Tris	7.32 - 0.10	15	17	28	35	47	0.27
Tris	8.00 - 0.07	16	20	26	32	38	0.24
Tris	8.80 - 0.22	18	20	27	31	44	0.24
Tris	9.45 - 0.55	10	12	13	16	19	0.15

hydranths are resorbed, although the coenosarc remains viable for extended periods and can regenerate hydranths on return to a favorable temperature. At 10 to 14 °C, the colonies remain healthy but grow very slowly (Table 17).

The most useful range for growth is 18 to 26 °C; unfortunately this is also the most difficult range to control due to its nearness to room temperature. A detailed experiment was done in this range using improvised temperature controls which, although they maintained the average temperature at the desired value, allowed considerable variation (Table 18). The results are interesting in that there is no striking difference in the growth rates obtained within this range of temperatures, a result usually interpreted to indicate the balance of an interaction between growth stimulating and growth inhibiting reactions, both of which increase in rate with temperature (cf. Thimann, 1955, p. 153).

Cordylophora clone A can grow slowly at 30°C, but at higher temperatures the hydranths regress (Table 17).

Light

Allman (1872 p. 254) considered Cordylophora to be a "light-shunning animal," and others, including Roch (1924) have shared this view, but nowhere have any data been presented to support the conclusion.

Several experiments with fairly intense light have failed to demonstrate any influence, positive or negative, on growth (e.g., Table 19). In the experiment tabulated, a pair of colonies was grown at room temperature (22-25 °C) about 4 inches from a 30 watt daylight fluorescent bulb, left on continuously. One culture, kept in a covered 2 liter glass beaker, was continually in the light; the other, in a stainless steel beaker of the same size, was in complete darkness except during feeding and medium change. The two cultures grew at a similar rate, and showed no differences.

Whether or not a culture can grow in continuous darkness has not been determined, and indeed would require special techniques for study. The question however, appears to have little immediate pertinence from either an experimental or ecological point of view.

Gaseous environment

The two gases which seem most pertinent for investigation are carbon dioxide and oxygen. The study of the influence of these on growth has been accomplished by adapting a method of Loomis (personal communication; 1959a, p. 281). Cordylophora colonies, growing on microscope slides, are placed on

TABLE 17

Influence of temperature on growth of Cordylophora

The results of two experiments are pooled in this table. The cultures were grown in CCS5, and temperatures maintained to ± 0.5°C.

Temperature °C	No. hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
10	11	13	13	14	14	14	14	<0.1
14	20	23	23	24	24	26		<0.1
22	12	16	22	29	34	41	52	0.26
30	8	11	12	14	14	15	16	0.11
34	0							0.00

TABLE 18

Influence of temperature (18 to 26°C) on growth of Cordylophora

Cultures were maintained in CCS5. During most of the experiment, temperatures were maintained within ± 0.5 °C, but occasional deviations were observed as indicated below:

Temp.	Method of maintaining	Max. variation	When observed
18°C	Refrigerated bath	+ 5 °C	Rose briefly to 23°C on day 4.
20°C	Flowing sea water	+ 2 °C	On hot days
22°C	Constant temperature box	- 2 °C	Extreme, due to evaporation
24°C	Water bath	+ 2 °C	On hot days
26°C	" "	+ 2 °C	On hot days

Cultures were in diffuse daylight except for those at 22°C. All were at room temperature for an hour during daily feeding.

Group A consists of cut back older colonies; Group B of secondary colonies.

Temp. °C	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
			GROUP A				
18	10	12	13	26	35	40	0.29
20	8	9	13	19	23	26	0.27
22	10	12	17	21	27	40	0.29
24	11	15	19	26	32	35	0.27
26	9	11	18	26	32	33	0.30
			GROUP B				
18	15	16	17	18	25	27	0.15
20	14	14	19	28	34	44	0.26
22	17	19	25	35	36	44	0.22
24	15	15	21	28	32	38	0.24
26	11	14	17	19	21	23	0.18

TABLE 19

Influence of light on growth of Cordylophora

The experimental procedure is described in the text.

	No. of hydranths on day:							Growth rate
	1	2	3	4	5	6	7	(k)
Light	17	19	26	31	33	43	54	0.22
Dark	12	15	17	21	27	32	38	0.19

the bottoms of 20 x 100 mm. petri dishes and covered with 50 ml. of CCS5, forming a layer about 1 cm. deep. These dishes are placed individually in vacuum desiccators (Corning no. 3118); 50 ml. of water or appropriate solution is placed in the bottom of the desiccators to maintain humidity or remove traces of gases. The desiccators are evacuated with a water aspirator attached to a mercury manometer, and refilled with appropriate gas mixtures to atmospheric pressure. To add small amounts of gases, syringes are used (the volume of these desiccators is approximately 2000 ml.), while the manometer is used for larger volumes. After refilling with gas mixtures the desiccators are placed on a rocker table attached to a synchronous motor which rocks the table once every 30 seconds, assuring constant exchange between the culture and the gaseous phase. Every 24 hours the dishes are removed for feeding, counting, and medium change, and then returned to the desiccators where the appropriate gas mixtures are re-established. This procedure is cumbersome, and permits handling only a few cultures per experiment, but it permits fairly accurate control of the gas phase without many separate cylinders of gas mixtures or other special apparatus.

Carbon dioxide

Undissociated carbon dioxide has been found to stimulate the growth rates of a variety of organisms, including bacteria (cf. Lwoff and Monod, 1947) and mammalian cells (cf. Geyer and Chang, 1958). With Cordylophora, three experiments were performed to ascertain the influence of removal of CO_2 from the atmosphere or slight increases in pCO_2 above atmospheric on growth rate (Table 20). In each experiment, the desiccators were evacuated, and CO_2-free air, prepared by passing air through an alkali train, reintroduced along with the indicated amount of CO_2. The CO_2-free atmospheres (0.00 per cent CO_2) were maintained with alkali in the bottom of the desiccators.

All three experiments indicate that growth rate is reduced in a CO_2-free environment, but fail to show any enhancement of growth rate attending increases of pCO_2 above the level found in air. Although growth rate is lower in the absence of CO_2, an absolute requirement for CO_2 has not been demonstrated. Perhaps the cells of Cordylophora have an absolute pCO_2 requirement, but because the organism is to a limited degree a closed system (multicellular, with much of the tissue surrounded by perisarc) enough interval CO_2 may accumulate to allow growth at a reduced rate even though external CO_2 is not available.

TABLE 20

Influence of pCO_2 on growth of Cordylophora

Cultures were grown in CCS5 at room temperature (21-25 °C) under the conditions detailed in the text. In Experiments I and II, the CCS5 was buffered with 0.001 M $NaHCO_3$, while in Experiment III 0.005 M phosphate buffer, pH 6.44, was substituted to eliminate possible retention or production of free CO_2 by the bicarbonate. CO_2-free atmospheres were maintained with alkali.

EXPERIMENT I

pCO_2 (per cent)	No. hydranths on day: 1	2	3	4	5	6	7	Growth rate (k)
0.00	13	15	17	20	25	29	31	0.17
0.03*	11	13	13	17	25	30	35	0.23
0.30	10	12	13	14	19	22	26	0.22

EXPERIMENT II

0.00	12	14	17	19	25	27	30	0.16
0.03*	14	19	22	28	35	41	48	0.23
0.30	12	17	19	28	35	36	36	0.24

EXPERIMENT III

0.00	12	12	15	19	19	21	21	0.11
0.03*	10	12	14	18	20	22	25	0.17
0.30	12	15	18	21	23	26	28	0.17

*Equivalent to the pCO_2 of air.

Even though attention is just beginning to be directed to pCO_2, it is clear that many biological processes are influenced by this variable (cf. Loomis, 1959b). It is curious that a CO_2 requirement has not been sought in hydra, where pCO_2 has been shown to induce sexual differentiation (Loomis, 1957) and to inhibit growth and differentiation at higher concentrations (Loomis, 1959c). In several systems, CO_2 is known to enter directly into metabolic pathways, many of which are energy-producing (Fruton and Simmonds, 1958, esp. p. 510 ff.). Lenhoff (1959) has shown that in hydra $C^{14}O_2$ is rapidly incorporated into many cell constituents, including acids of the citric acid cycle and several amino acids. Hammen and Osborne (1959) have demonstrated the incorporation of $C^{14}O_2$ into dicarboxylic acids in a diversity of marine invertebrates. Thus it is not surprising that the pCO_2 in air enhances the growth of Cordylophora.

Oxygen

In the first experiment with Cordylophora, the oxygen tension was varied simply by growing the colonies in mixtures of nitrogen and oxygen (Table 21). Growth was clearly reduced in the absence of added oxygen, but was continuous during the 6 days of the experiment. One per cent oxygen permitted growth at a slightly reduced rate, whereas 4 per cent oxygen (ca. 40 per cent saturation) gave maximal growth.

While this experiment indicated a low oxygen requirement, a second experiment had to be performed to determine if removal of all oxygen would reduce growth to zero. The oxygen absorbant selected, alkaline pyrogallol, has the disadvantage that it removes not only O_2 but also CO_2 from the atmosphere. If reduced O_2 concentration reduces the respiration of Cordylophora (cf. Lenhoff and Loomis, 1957, for hydra), then CO_2 production would also be reduced and CO_2 might become limiting under conditions of low O_2. Therefore the experimental design included 3 desiccators, containing (a) alkaline pyrogallol (Umbreit, et al., 1957, p. 70), (b) alkali (40 per cent KOH), and (c) water. After every feeding, each of these desiccators was evacuated and refilled with nitrogen (Ohio water-pumped), and sealed with silicone stopcock grease and bunsen valves (Umbreit, et al., 1957, p. 203).

The results of this experiment are presented in Table 22. In the complete absence of O_2 (and CO_2), some of the hydranths were either resorbed or fell off, particularly the younger ones. There was also some disorganization and regression of the coenosarc tissue, especially behind stolon tips.

TABLE 21

Influence of oxygen tension on growth of Cordylophora

Cultures were grown in CCS5 under the conditions described in the text.

Per cent O_2 added	pO_2 (mm. Hg)	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
0	0.0	13	19	20	21	22	24	<0.1
1	7.6	19	21	24	25	30	35	0.12
4	30.4	16	18	21	26	29	33	0.16
20*	152	15	20	22	25	29	35	0.17

*Approximately the oxygen tension of air.

TABLE 22

Oxygen requirement for growth of Cordylophora

Cultures were maintained in CCS5 under conditions described in the text. The atmosphere was nitrogen.

Solution in desiccator	Gases absorbed	No. hydranths on day: 0	1	2	3	4	5	6	7	Growth rate (k)
Pyrogallol	$O_2 + CO_2$	15	8	8	8	8	7	7	7	0.00
Alkali	CO_2	12	11	12	14	14	14	14	14	0.00
Water	none	10	12	13	15	15	16	17	17	<0.1

Those hydranths which survived the 7 days of anaerobiosis were peculiar. When first removed from the desiccator, these hydranths were highly contracted and looked unhealthy, but as oxygen re-entered the culture solution, they expanded and became able to capture and eat Artemia nauplii. There was no evidence of growth in this culture. With traces of O_2 present, but no CO_2, the colony remained healthy but showed almost no growth, whereas with traces of both O_2 and CO_2 present the colonies grew very slowly as in the previous experiment.

These two experiments indicate a distinct, but low, oxygen tension requirement for the growth as well as the maintenance of Cordylophora. A more sophisticated study would be required to determine the amount of oxygen required.

In many microorganisms, including several fungi (Cochrane, 1958, p. 23) growth will occur under nitrogen and an absolute oxygen requirement has only been demonstrated by growing them over alkaline pyrogallol. In view of the fact that the growth of such organisms may also be dependent on carbon dioxide (cf. Cochrane, p. 228), it is important to ascertain whether the critical variable being removed by alkaline pyrogallol is carbon dioxide or oxygen. In the case of Cordylophora, it is clear that while removal of CO_2 may further reduce growth under conditions of O_2 deficiency, it is not the removal of CO_2 which produces the effects of alkaline pyrogallol (Table 22).

VI. Nutrition

Due to the completely undefined nature of the source of nutrients - living prey - only two questions can be asked about the influence of nutrition on growth rate. The suitability of the food source, Artemia nauplii, has been considered in Chapter III. The influence of the amount of food on growth can be approached both by varying the length of time the cultures are left with food, and by varying the intervals between feeding. In both cases the cultures are fed to saturation with Artemia nauplii during each feeding period.

The length of time the cultures are left with food has little or no influence on growth rate (Table 23). Most hydranths capture a repletion level of Artemia (about 20-30 nauplii per hydranth) within the first few minutes after feeding.

Variation of the length of time between feedings gives a much more dramatic effect on growth rate, as presented in Table 24. In CCS5 at 22°C,

TABLE 23

Influence of time with food on growth of Cordylophora

Cultures were maintained in CCS5 at 22 °C. Each hydranth was fed to sturation with Artemia and the culture solution changed after the indicated interval.

Time with food	No. hydranths on day: 1	2	3	4	5	6	Growth rate (k)
15 min.	17	22	28	35	40	48	0.22
30 min.	10	14	16	20	25	28	0.23
60 min.	13	15	17	20	25	28	0.18
120 min.	12	15	16	18	23	28	0.17

TABLE 24

Influence of intervals between feedings on growth of Cordylophora

Cultures were grown in CVD at 22 °C, and fed to saturation for one hour at 9 a.m. and 9 p.m. as appropriate.

Interval between feedings	No. hydranths on day: 1	2	3	4	5	6	7	8	9	Growth rate (k)
hours										
12	12	13	16	25	28	40	48	60	70	0.23
24	17	19	22	27	31	36	42	50	64	0.18
48	15	17	17	18	19	22	24	26	28	<0.1
duration of expt.	13	15	15	16	14	13	11	11	11	0.00

1 feeding per day yields a growth rate approaching maximum, while with 0.5 feedings per day growth is much reduced. Starvation regularly results in a slight increase followed by a gradual decrease in hydranth number to a steady state level which is maintained for an extended period.

DISCUSSION

Each parameter of colony growth, with the exception of the intrinsic factors, has been systematically examined (cf. Table 6). Certain of the variables, such as light, have no measurable influence on the growth of colonies. Growth is quite independent of others, including temperature, oxygen tension, and pH. Relatively few variables profoundly influence growth rate, in particular the composition of the culture solution and the amount of food supplied.

It would now be possible to perform factorial experiments, in which, for example, the aqueous environment, temperature, and feeding rate are varied simultaneously. Kinne (1956, 1958a) has obtained evidence for an interaction between salinity and temperature in the growth of Cordylophora; this would warrant further study under controlled conditions. Since such treatments influence colony pattern as well as growth rate, the experiments have been deferred until the study of colony pattern under one set of conditions is completed (Chapter VII).

Several other observations warrant further study. The experiments described have been short-term, and have involved at most 2 or 3 doublings. In other studies, it has been found that deficiencies frequently only manifest themselves after several generations. For example, Lwoff and Monod (1947) found that under CO_2-deficiency the growth rate of bacteria continually decreased until it reached zero, suggesting gradual exhaustion of some essential cell constituent. Similar results might follow for Cordylophora if it were grown under Mg^{++} or CO_2-lack for many generations.

It has been observed that cut back older colonies consistently show a slightly faster growth rate than secondary colonies growing under similar conditions (cf. Table 18). This observation remains without explanation, but may in some way be correlated with the rapid growth rates (k greater than 0.30) described in Chapter III.

Under many adverse conditions, the hydranths regress, but the coenosarc tissue of such colonies often is capable of recovery. For example, the colonies which regressed in demineralized water (Table 13) or at 34 °C (Table 17) regenerated hydranths upon return to favorable conditions. Under such drastic conditions, the regression of hydranths is frequently accompanied by considerable disorganization of the coenosarc tissue, and the reorganization after such trauma warrants careful study.

A critical factor influencing Cordylophora colony growth is the aqueous environment. The ionic requirements for growth include all the major ions of sea water except sulfate and bicarbonate (H. W. Harvey, 1957). The similarity of these requirements to those of mouse fibroblasts or HeLa cells is striking. Cordylophora requires Na^+, K^+, Ca^{++}, Mg^{++}, and Cl^-; though in quantitatively different amounts the mammalian cells require exactly the same ions plus phosphate (Eagle, 1956).

The only similar organism (in a broad sense) to Cordylophora for which data on the growth parameters are available is Hydra littoralis (Loomis, 1954). A comparison of the conditions necessary for the two organisms is presented in Table 25. The only striking difference in the growth requirements is in the culture solution. Hydra littoralis requires significant amounts only of Ca^{++} for growth, though recently it has been found that Na^+ in trace amounts improves growth rate (Lenhoff and Bovaird, 1959a).[1] In addition, the requirement for Ca^{++} is about 10-fold higher in Cordylophora than in Hydra. It would appear that Hydra is able to retain enough of the K^+, Cl^-, Mg^{++}, and Na^+ obtained from Artemia to suffice for continued growth in their absence in the aqueous mileu, whereas Cordylophora, being a more open system with respect to these ions, must have them continually supplied in the aqueous environment.

The laboratory observations on the influence of environmental variables on growth may be examined from an ecological point of view. Cordylophora lacustris has been found throughout the world, in habitats varying in their salinity from fresh water to almost sea water (Roch, 1924; Hand and Gwilliam,

---

[1] Although no supporting data are presented, Ham, Fitzgerald and Eakin (1956) report that Pelmatohydra oligactis requires Na^+ and K^+ in addition to Ca^{++} for growth. These authors also found the H. littoralis requires Na^+ and K^+ for regeneration.

TABLE 25

Comparison of growth conditions for Hydra littoralis and Cordylophora lacustris (Data for Hydra from Loomis, 1954)

Factor compared	Hydra	Cordylophora
Organization	solitary	colonial
Growth rate (k)		
a. mean value[a]	0.37	0.23
b. maximum observed	0.44	0.35
Ionic requirements for growth		
a. absolute	Ca^{++}	Na^{+} K^{+} Ca^{++} Cl^{-}
b. less critical	Na^{+}[b]	Mg^{++}
c. Ca^{++} conc. required	10^{-4} M	10^{-3} M
Range for growth		
a. Hydrogen ion conc. (pH)	5.3 to 8.7	6.3 to 8.6
b. NaCl conc. (M)	none[b] to 0.03	<0.001 to >0.25[c]
c. Temperature (°C)	13 to 30	15 to 30
d. Oxygen tension (mg./l.)	>2	>2
Temperature interval where growth rate constant (°C)	20 to 27	18 to 26
Rate of feeding required for good growth (Artemia)	1 feeding/day	1 feeding/day

[a] From tables in Loomis (8 values) and present chapter (16 values). Highest value from each table averaged

[b] Lenhoff and Bovaird (1959a).

[c] If other ionic conditions suitable.

1951). The water of Nye Pond, from which the Cordylophora used in these studies was collected (Chapter III), has a very low salinity; analysis of the chloride ion of 3 different water samples gave values of 2, 7 and 4 milliequivalents Cl^- per liter.[2] No such solution has as yet been designed which gives a good growth rate in the laboratory, although cultures will grow slowly in the laboratory either in water from Nye Pond or in a solution based on the analysis of Nye Pond water. It would require a separate study to determine what factors are critical for the growth of Cordylophora in fresh water.

The present study indicates the composition of the aqueous environment to be the most critical variable in determining the ability of Cordylophora to live in a given body of water, not only in terms of salinity but also in terms of proportions of ions present. The tendencies of fresh water to contain high calcium and carbonate but very little sodium and chloride (Hutchinson, 1957, p. 555) may be a major factor in restricting Cordylophora to relatively few fresh water localities. In brackish water, in addition to the required presence of adequate amounts of the five required ions, the most critical limiting factor would appear to be the Na^+/K^+ ratio (cf. Table 14). Cordylophora cannot grow in sea water, because the total salt concentration is simply too great.

The typical habitat for Cordylophora appears to be that given by Allman (1853) in his original description of the species: "In aquis dulcibus, quietris, corpora varia submersa obducens, et locos obscuros amans." But Cordylophora has been found in strikingly different habitats, such as that described by Clarke (1878) in Baltimore, Maryland, where the hydroid grows "in the channel where the sunlight is strongest, ... where the current is

---

[2] A complete analysis of one sample of Nye Pond water has been made. Sodium and potassium were analyzed by flame spectrophotometry through the courtesy of Dr. James W. Green of Rutgers University, while the other ions were determined by titration. The results, in milliequivalents per liter, are:

Na^+	1.56	Cl^-	3.96
K^+	0.123	SO_4^{--}	0.65
Ca^{++}	0.20	CO_3^{--}	0.00
Mg^{++}	0.83	HCO_3^-	0.16

The anions are 21 per cent in excess, indicating some quantitative errors in the analysis. The results are atypical of fresh water in that sodium is the major cation and chloride the major anion.

most rapid ..., and changes in surrounding conditions must be greatest." This latter environment is similar to that in which Cordylophora grows in Nye Pond, where it forms a thick mat along the edges of a culvert running from a pond to a salt-water marsh.

Almost any habitat in which the aqueous environment is suitable should be able to support the development of Cordylophora colonies, since the organism is remarkably insensitive to temperature, pH, oxygen tension, light, etc., and should readily tolerate the range of variation of these parameters found in most bodies of water (Hutchinson, 1957). The only other frequent limiting factor would be the amount of prey organisms available as food. The cosmopolitan nature of Cordylophora lacustris would support the idea that many bodies of water meet the necessary requirements.

Chapter V

Imino Acid Control of the Feeding Reaction of *Cordylophora*

A diversity of organisms make use of chemicals emanating from their specific food either to seek it out or to select it from among other edible objects (Prosser, 1950, Chap. 12). Coelenterates, either as swimming medusae or sessile polyps, do not seek out their food, but wait passively until a potential prey collides with their tentacles. If the prey has suitable properties (Zick, 1932), batteries of nematocysts are released from the tentacles which puncture the cuticle or epidermis of the prey, simultaneously holding and poisoning it. Whether or not captured prey are eaten depends on a second reaction (Ewer, 1947). If the prey elicits a suitable response, the tentacles holding it are contracted to the mouth, the mouth opens, and the object is swallowed into the coelenteron. It is this second reaction which is known as the feeding reaction.

Coelenterates of all major groups have been shown to give a feeding reaction in response to tissue juices in the complete absence of any solid prey. Meat juice, added to water containing sea anemones, class Anthozoa, can produce tentacle waving and mouth opening (Pantin and Pantin, 1943), while reef-building corals are sensitive to meat juices diluted 1000-fold or more (Abe, 1938). Jellyfish, class Scyphozoa, also react to meat juices (Henschel, 1935).

The most useful studies have been made with organisms of the class Hydrozoa, and in particular with hydra.[1] Hydra will eat only living prey, and will not feed on dead specimens of the same species. Beutler (1924) found that hydra will swallow bits of gelatin, but only after they have been soaked in fresh tissue juice. Similar observations were made for other hydroid (Beutler, 1926). Ewer (1947) found that fresh tissue juice could produce a feeding reaction in hydra even though no particulate matter was present.

In 1955 Loomis demonstrated that the feeding reaction of Hydra littorali is mediated by reduced glutathione (GSH) released in the body fluids of captured prey. GSH induces the feeding reaction at concentrations as low as 10^{-4} molar, and the feeding reaction is extremely specific.[2] In the presence of GSH, Hydra occasionally become cannibals.

---

[1] As used here, the term hydra refers to any species while Hydra refers to H. littoralis as studied by Loomis.

[2] Loomis (1955) found no other compounds, not even aspartathione, able to induce the feeding reaction. Cliffe and Waley (1958) found that replacement of the -SH group of glutathione by $-CH_3$ resulted in a more active compound than GSH, while replacement with -H resulted in a less active compound.

From the point of view of comparative biochemistry, it is of interest to know if GSH controls the feeding reaction of other coelenterates. Lenhoff and Schneiderman (1959) found that the feeding reaction of both the large, polymorphic Portugese Man-of-war, Physalia and the calyptoblastic hydroid Campanularia are also controlled by GSH. Since the hydroids responding to GSH represent three very different groups in the class Hydrozoa (Table 1), it appeared that chemoreception of prey through GSH was a primitive hydrozoan characteristic.

Therefore it was surprising to find that Cordylophora -- a closer relative of Hydra than either Physalia or Campanularia -- gave no response to GSH. Investigation showed that the feeding reaction of Cordylophora is controlled by the α-imino acid proline.

MATERIALS AND METHODS

The Cordylophora used in these experiments were grown in CCS5 or CVD as described in Chapter III. Unless otherwise indicated, the colonies had been starved for about 24 hours when used in an experiment. For bioassays, full-grown hydranths were removed from colonies with a short length of attached stem.

Bioassays were performed in Pyrex spot plates having 3 or 9 depressions. To 1 ml. of test solution, a drop of CVD having 2 or more hydranths was added, and the reaction scored using a dissecting microscope. For most purposes, serial dilutions of a test material were performed directly in the depressions of the spot plates.

At first the bioassay environment was CVD or CCS5, but for reasons described below another assay solution, Phos-CCS5, was developed. As this solution precipitated overnight, it was prepared fresh each day. Five X stock CCS5 was prepared volumetrically in demineralized water (per 500 ml., add NaCl, 7.30 gms.; KCl, 0.186 gms.; $CaCl_2.2H_2O$, 1.84 gms.; $MgSO_4.7H_2O$, 3.08 gms.). An 0.066 M phosphate buffer having a pH of 6.8 was also prepared (per 100 ml., add KH_2PO_4, 0.46 gms.; K_2HPO_4, 0.57 gms.). Phos-CCS5 was compounded by mixing 2 volumes stock CCS5 and 1 volume phosphate buffer in 7 volumes of demineralized water; the solution had a pH of about 6.6.

Hydra littoralis were grown in BVT following the methods of Loomis and Lenhoff (1956). The assays were performed on 24-hour starved individuals in BVT.

Artemia extract was prepared by homogenizing dense suspensions of nauplii (either fresh or frozen were suitable) in demineralized water in a Pyrex tissue grinder. Insoluble material was centrifuged out and discarded. Trichloroacetic acid (TCA) was added to a final concentration of 0.3 molar, and the solution chilled and centrifuged. The supernatant was extracted 5 times with 2 volumes of ethyl ether to remove the TCA and extractable lipids, and after removal of the ether in vacuo or with heating the solution was ready for use.

Fresh yeast extract was prepared by boiling a pound of baker's yeast suspended in a liter of demineralized water for 10 minutes, centrifuging out the insoluble material, and precipitating with TCA and extracting with ether as described above.

For ion exchange, Dowex 1-x8 and Dowex 50W-x8 resins were used (both 100-200 mesh). To prepare them for use, they were washed on a Buchner funnel with water, 2N HCl, water, 2N NaOH, water, 2N HCl, and demineralized water. The columns were made of 20 mm. Pyrex tubing. Material was allowed to flow through by gravity and the effluent collected manually in test tubes. The active effluent was concentrated in a rotary evaporator.

Paper chromatography was accomplished on Whatman no. 1 paper. As a control for R_f values a mixture of four amino acids was used, containing 2 mg. per ml. each of L-glycine, L-leucine, L-lysine.HCl, and L-proline. An aliquot of 0.002 ml. of this mixture and 0.004 ml. of the unknown mixture were spotted. One known and 3 unknown spots were placed in a row on an 11 x 30 cm. strip of paper. The ascending chromatograms were run in 6 x 12 inch Pyrex battery jars with 200 ml. solvent; the jars were covered with aluminum foil and sealed as described by Roberts, et al. (1953, p. 32). The solvents were prepared as described by Smith (1958, p. 61), and equilibrated in the jars for 24 hours before the chromatograms were run.

The papers were dried in air, and a strip containing one known and one unknown application cut off and treated with ninhydrin in acetone-pyridine (Smith, 1958, p. 66). The other unknown strips were cut up and eluted for bioassay with Phos-CCS5 in an elution apparatus similar to that described by Roberts, et al. (1953, p. 32). For satisfactory bioassays it was necessary (a) to adjust pH of eluants from butanol-acetic acid chromatograms, and (b) to extract residual phenol from the phenol-ammonia chromatograms with slightly acidified ether.

The photometric assay for proline was performed with reagents and color development as described by Chinard (1952), except that after heating the total volume of glacial acetic acid was added to the tubes immediately. Readings of optical densities were made with a Bausch and Lomb Spectronic with 13 mm. tubes; all were read against a reagent blank. Control assays with reagent proline indicated that the optical density at 515 mμ was a linear function of the concentration of proline from 2×10^{-5} to 1×10^{-4} molar, as reported by Chinard (1952), and this was used to construct a calibration curve from which the concentration of proline in the test samples was calculated. Sample blanks of either proline or Artemia extract at highest concentrations tested failed to develop significant color.

RESULTS

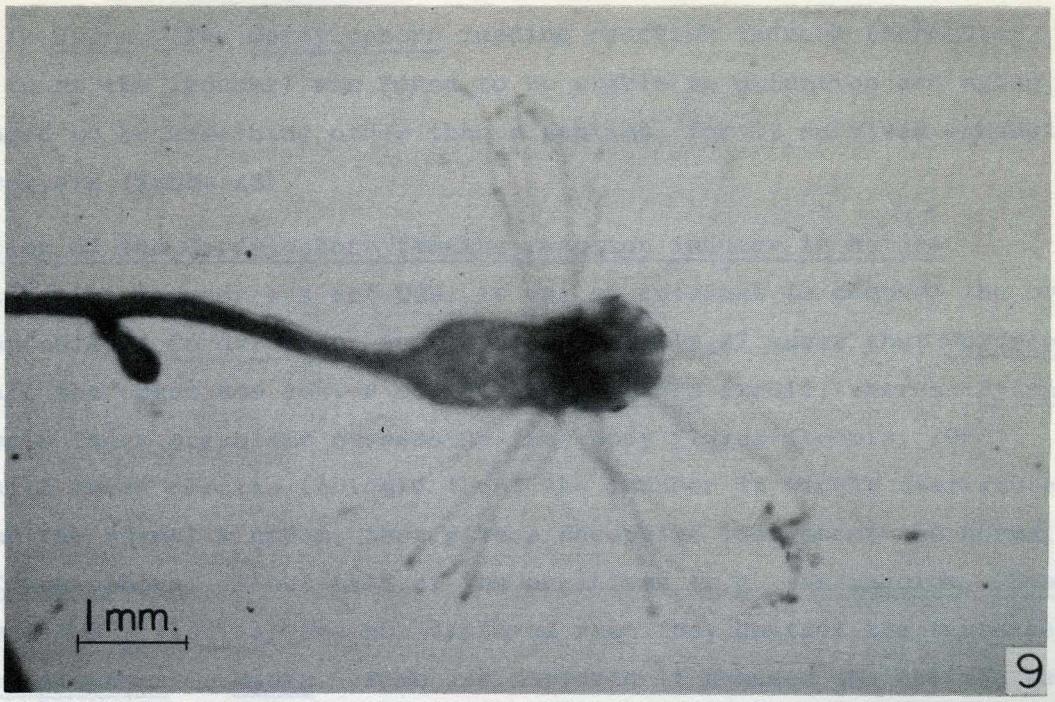
I. Characterization of the feeding reaction inducer

When Cordylophora are fed lightly with Artemia nauplii, the feeding reaction can be closely observed. A captured nauplius is quickly paralyzed, and the tentacle holding it curled in towards the mouth. The oral cone bends toward the nauplius. On contact, the mouth spreads around the prey and swallows it. This response is very similar to that observed with Hydra. Cordylophora tentacles often capture miscellaneous objects, such as bits of dried agar or hairs. These are held by the tentacles for a time and then dropped without having elicited any further reaction.

Many attempts were made to obtain a feeding reaction to GSH at a variety of concentrations and with hydranths which had starved for up to a week. In no case could a definite response be elicited, though higher concentrations frequently induced some waving of tentacles. The GSH solutions were tested with Hydra and found to induce a feeding reaction.

It was found, however, that fresh Artemia juice induced a feeding reaction similar in all respects to that observed in hydranths fed living nauplii, even when diluted 10,000 times. Hydranths were even induced to attempt to eat the glass walls of the container (Figure 9) or to become cannibals (Figure 10).

It was thus clear that Cordylophora had a chemically mediated feeding reaction, mediated by some compound other than GSH. Cordylophora was also found to eat hamburger, which does not contain GSH and which elicits no



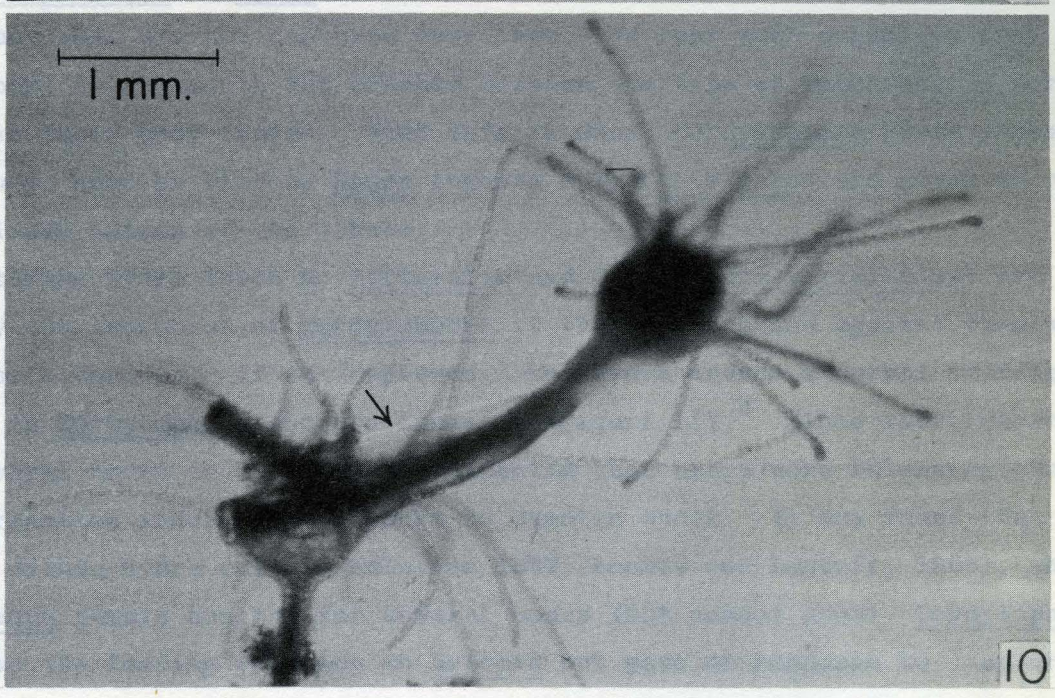


Figure 9. A Cordylophora hydranth attempting to eat the glass wall of its container in response to Artemia extract at a 1/10,000 dilution.

Figure 10. Attempted cannibalism induced by Artemia extract. The lower hydranth is attempting to ingest the upper one. Note the stretching of the mouth (indicated by an arrow).

response in Hydra. The Cordylophora feeding reaction inducer (hereafter referred to as the inducer) was found to be stable to oxidation and aging, and appeared to be something other than a peptide, for it survived exhaustive acid hydrolysis (Table 26).

Distribution of the Cordylophora feeding reaction inducer in nature

Since the inducer was not GSH, it was of interest to compare the organisms acceptable to Cordylophora and to Hydra. Table 27 shows that Cordylophora ingests all the organisms tested except Cordylophora itself, whereas Hydra only ingests those organisms possessing free body fluids (Loomis, 1955).

While these results indicate theat the inducer is widely distributed throughout the animal kingdom, they give a deceptive idea about the normal diet of Cordylophora. About half of the organisms (e.g., Paramecium, Chaos, Stenostomum and Philodina) are not captured when they contact the tentacles of either Cordylophora or Hydra. Some are captured if pressed against the tentacles, but some are not captured even then. To test such organisms they must be held next to the mouth and crushed between the tips of watchmaker's forceps to release their body fluids. When this is done, Cordylophora gives a response to all these species whereas Hydra ingests only Chaos chaos and gives no respon to the tissue juices of the others.

Starved hydra (both H. littoralis and Chlorohydra viridissima) are caught by the tentacles of Cordylophora if they are pressed against them with watchmaker's forceps. If so "captured," the hydra induce a normal feeding reaction in Cordylophora and are ingested (Figure 11).[3] Since hydra dissociate after several hours in CVD, it was suspected that hydra were releasing the feeding reaction inducer in response to osmotic shock. It was found that in the more dilute hydra culture solution (BVT, Loomis and Lenhoff, 1956), where Cordylophora remain healthy for several hours (but cannot grow), Cordylophora would give the feeding reaction to Artemia but gave no response to "captured" hydra. Thus it would appear that Cordylophora eat hydra only if the inducer is released in response to osmotic shock. It was not possible, however, to induce Cordylophora to become cannibals by such a procedure.

---

[3] Hydra so ingested are ejected several hours later in a semi-dissociated state (similar to that obtained if hydra are simply left in CVD). In preliminary trials, it has not been possible to infect Cordylophora with the Chlorella of Chlorohydra by feeding Cordylophora the latter.

TABLE 26

Demonstration that the feeding reaction of Cordylophora is induced by something other than glutathione

Test Material	Cordylophora	Hydra
Reduced glutathione	-	++*
Fresh Artemia juice	++	++*
Fresh Artemia juice, treated with 3% H_2O_2	++	-*
Aged Artemia juice	++	-*
Artemia juice, hydrolysed in 6N HCl, 48 hours, 110 ^{o}C		
Hamburger	++	-

*confirming Loomis (1955)

TABLE 27

Organisms inducing the feeding reaction of Cordylophora

Tests were made in CVD using 24-hour starved Cordylophora hydranths. In part, the comparative results for Hydra littoralis were taken from Table 7 of Loomis (1955); all negative results were confirmed by the author. Organisms not available in the laboratory were obtained from Carolina Biological Supply Co., Elon College, North Carolina.

Species	Feeding reaction induced in Cordylophora	in Hydra
Protozoa		
Paramecium multimicronucleatum	+	-
Chaos chaos	+	+
Cnidaria		
Chlorohydra viridissima	+	-
Hydra littoralis	+	-
Cordylophora lacustris	-	-
Platyhelminthes		
Planaria dorotocephala	+	-
Stenostomum	+	-
Rotifera		
Philodina	+	-
Nemathelminthes		
Cephalobus	+	+
Chiloplacus	+	+
Annelida		
Enchytraeus	+	+
Stylaria	+	+
Arthropoda		
Daphnia	+	+
Alonella	+	+
Artemia	+	+
Chordata		
Rana pipiens tadpoles	+	+
Calf liver	+	-

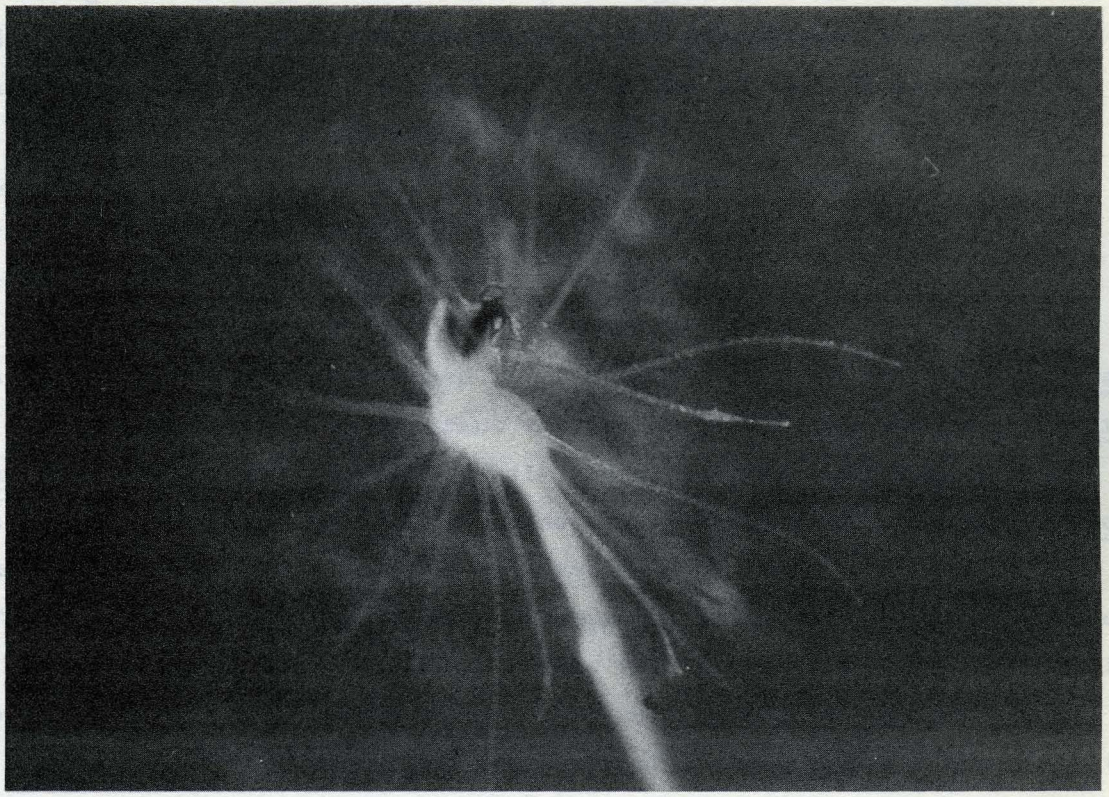


Figure 11. A Cordylophora hydranth eating a starved green hydra (Chlorohydra) in CVD.

In general, the remaining organisms listed in Table 27 were captured and eaten without difficulty. Exceptions to this are Planaria, which has a thick slime in which the hydranths become enmeshed, and frog tadpoles, which are too large to be ingested.

From these considerations, it is clear that while Cordylophora will give a feeding reaction to a wider variety of organisms than will Hydra, the normal diets of the two hydroids are probably not significantly different. The great bulk of the normal diet of both organisms appears to be composed of small aquatic arthropods and segmented worms.

Loomis (1955) has pointed out that "hydra require prey that contain vascular, coelomic, or pseudocoelomic fluids enclosed within a thin enough body wall so that nematocyst puncture results in the release of sufficient quantities of GSH to activate the 'feeding reaction.'" Since all the organisms listed may reasonably be assumed to have GSH as well as the Cordylophora inducer, it is not immediately apparent why Cordylophora responds to Paramecium, Planaria, Stenostomum, and Philodina while Hydra does not. Perhaps either there is more Cordylophora inducer in these organisms than GSH, or Cordylophora may be more sensitive to its inducer than Hydra is to GSH.

The eating of fragments of meat (hamburger, beef liver and kidney were tested) by Cordylophora is informative. They will eat such fragments only if they are fed as soon as put under water. If the fluid which leaches out of them is allowed to diffuse away, the hydranths will capture the fragments but fail to manifest any further reaction to them. Hydra will not eat such meat, though they will eat fresh mammalian tissue (Lenhoff, 1958). Cordylophora also gives a feeding reaction to various juices, including yeast extract (prepared as described above), raw eggs, or meat extract.

Bioassay of Cordylophora feeding reaction inducer

Bioassays of inducer activity performed in CVD gave satisfactory results with Artemia juice, but as characterization of the inducer was attempted, the results of bioassays became erratic. Therefore the bioassay conditions were studied, using both Artemia and yeast extract, which had similar activity and gave similar results. Dilutions were performed in assay solution, and hydranths added to score activity. A complete feeding reaction, which proceeded to mouth opening, was recorded as ++; a reaction which involved tentacle and oral cone searching but not mouth opening as +; waving tentacles only as ±;

and no response as -. Experience with Cordylophora permits scoring + and particularly ++ feeding reactions unequivocally and without confusing them with mouth opening or tentacle waving produced by unfavorable conditions.

The influence of pH on the feeding reaction was first investigated (Table 28). It was clear that phosphate buffer, pH below 7, gave substantially better activity than tris buffer, pH above 7. The activity in CCS5 having the usual bicarbonate buffer was ++ at 1/1000 but not at 1/10,000, or 10 fold lower than in phosphate buffer.

Further investigation showed, however, that the phosphate buffer was itself exerting an effect. Substitution of 0.005 M histidine buffer, pH 6.4, brought the activity back down to the level of tris-buffered CCS5. Furthermore, dilution of the phosphate buffer 1/4 reduced the activity 10 to 50 fold although the pH during the assay procedure remained constant at pH 6.59.

It seemed possible that part of the enhancement of inducer activity by phosphate buffer might be due to complexing of magnesium or calcium ions. It was found, indeed, that bioassays in regular (bicarbonate buffered) CCS5 containing 0.005 M $MgCl_2$ showed only about one-tenth the activity obtained in CCS5 without magnesium ions. The activity of phosphate-buffered CCS5 was not measurably increased by the removal of magnesium ions.

It thus appeared that magnesium inhibits the feeding reaction in response to inducer, and that in phosphate-buffered solutions this inhibition is abolished. These observations produced a bioassay solution (Phos-CCS5) which gave an equivalent feeding reaction with 10-fold less Artemia or yeast extract, and greatly increased the reproducibility of the assay. Without this assay solution, identification of the inducer might well have been impossible.

Characterization of the inducer

Most of the characterization studies were done prior to the development of the improved bioassay, and need not be described in detail since their primary interest is that they led to the identification of the inducer.

While the inducer did not survive ignition, it was otherwise very stable. In addition to its stability to acid hydrolysis, it was found to be readily dialyzable and not precipitated by TCA. It could not be distilled or extracted into non-polar solvents such as ether, benzene, or chloroform.

The inducer was loaded on small ion-exchange columns, and it was found that the activity could be washed off the anion-exchange resin Dowex 1

TABLE 28

Influence of buffer on the feeding response to Artemia extract

Unbuffered stock CCS5 was diluted in potassium phosphate or tris (hydroxymethyl) amino methane, so that the final buffer strengths were 6.6×10^{-3} M phosphate and 5×10^{-3} M tris. Artemia extract was diluted in these solutions, and the activity bioassayed with 24-hour starved Cordylophora. The pH was determined with a glass electrode.

Dilution of Artemia extract	pH				
	Phosphate			Tris	
	5.80	6.58	7.38	8.05	8.70
1/100	++	++	++	+	+
1/1000	++	++	+	±	-
1/10,000	++	++	±	-	-
1/∞	-	-	-	-	-

(chloride form) with water but had to be eluted from the cation-exchange resin Dowex 50 (hydrogen form) with acid.

When paper chromatograms were run in isopropanol-formate or butanol-formate (Roberts, et al., 1953, p. 33) the inducer moved on the paper and gave one active spot, suggesting that a single substance in Artemia extract has biological activity. No insight into the nature of the molecule could be gained from the chromatograms, however, because of the number of ninhydrin-positive substances present and because of salt effects.

II. Isolation and identification of the inducer

These experiments indicate that the feeding reaction inducer is probably a single molecule of low molecular weight, manipulatable on ion exchange resins and paper chromatograms. Assuming that the inducer is as active on a molar basis as GSH is for Hydra, it seemed that the isolation of enough material to manipulate by conventional techniques of organic chemistry might be a major task. Therefore the techniques of chromatography were used in such a way that it would be conceivable to identify the inducer without ever isolating enough to see. Ion-exchange columns were used to desalt and further purify an Artemia extract. This material was then spotted on paper, and the R_f of the inducer determined in a variety of solvents by bioassay. From these R_f values the compound was provisionally identified, and this identification was tested directly with the compound in question.

Three ml. of an Artemia extract, which had activity at a 1/10,000 dilution, was loaded on a 30 ml. column of Dowex 1 (chloride form), and the activity washed through with water. Ten ml. aliquots of the effluent were collected, and the activity came off within the first 50 ml. At least half the activity was collected in 30 ml. and these three tubes were pooled and concentrated by evaporation.

This fraction was loaded on a 25 ml. Dowex 50W (hydrogen form) column, and washed with 100 ml. of water and 240 ml. 0.1 N HCl, during which time no measurable activity came off. Then elution was begun with 1.5 N HCl, and 15 ml. aliquots were collected. The activity began to come off quickly, and most was concentrated in five tubes, containing a total of 75 ml. These tubes were pooled, concentrated, and brought to dryness twice over NaOH flakes to remove HCl. The residue was dissolved in 0.3 ml. of water.

The concentrate was spotted on paper along with a mixture of known substances containing glycine, leucine, lysine and proline. These knowns were selected to provide a check on the accuracy of the R_f determinations, and the selection was based on examination of the R_f tables in Smith (1958) for ninhydrin-positive compounds which would distribute themselves adequately. Ascending chromatograms were run in 3 solvents, after which the papers were dried and residual solvent extracted. A strip containing a known and an unknown was treated with ninhydrin, and other unknown strips were cut into segments which were eluted and bioassayed in Phos-CCS5.

A tracing of the 3 chromatograms treated with ninhydrin is given in Figure 12. The four knowns gave R_f values corresponding closely to those in Smith (1958). There were 5 to 6 ninhydrin-positive spots on the chromatograms of the unknown; one of these correlated perfectly with inducer activity. That spot had the R_f value of proline and gave the yellow ninhydrin color characteristic of proline. Accordingly, reagent L-proline was tested for inducer activity, and was found to induce a ++ feeding reaction at concentrations as low as 10^{-5} molar.

The remaining 5 ninhydrin spots on the chromatograms have been tentatively identified. One, the most conspicuous, has the R_f value of alanine in each solvent. The other spots did not differ sufficiently from one another to be unequivocally compared in different solvents, but provisional identifications were made as glycine or threonine, valine, leucine and/or isoleucine, and serine. These conclusions are supported by the observations of Hirs, et al. (1954), who found that none of these amino acids are held on Dowex 1 (Cl), and in elution from Dowex 50 (H) with 1 to 4 normal HCl, the amino acids not held on Dowex 1 come off in the following order: serine, threonine, glycine, alanine, valine, proline, methionine, isoleucine, leucine, etc. On this basis the only amino acid which should have been present in the inducer peak which was not found is methionine. Small amounts of methionine might be confused with the valine spots. Cystine, the next amino acid to be eluted on Dowex 50, is certainly not present in significant amounts, since its R_f would have been distinct.

This simple procedure thus led to the tentative identification of the Cordylophora feeding reaction inducer as proline, an identification supported by the high biological activity of reagent proline.

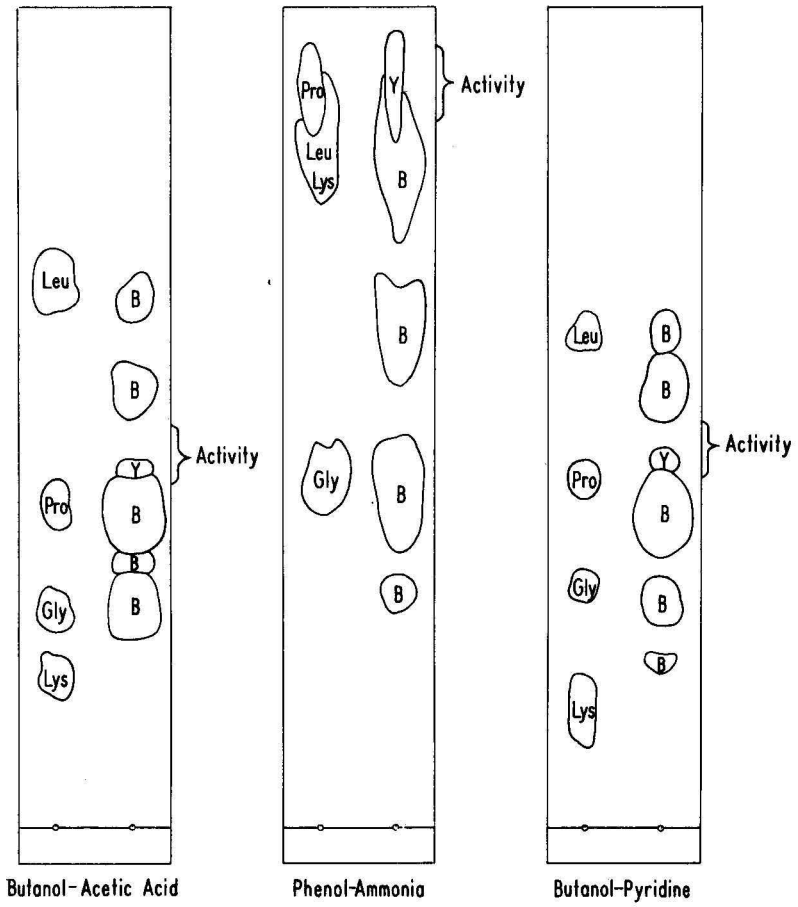


Figure 12. Tracings of the ninhydrin spots on the three ascending chromatograms described in the text. On each chromatogram, the strip to the left represents the known amino acids, that to the right the unknown sample. The brackets indicate the areas on adjacent unknown strips from which inducer activity could be eluted. The solvents used are described in Smith (1958); the actual size of the paper strips shown is 4 x 22 cm. Abbreviations: Gly, glycine; Leu, leucine; Lys, lysine HCl; Pro, proline; B, blue; Y, yellow.

III. Semi-quantitative comparison of the inducer and proline

To strengthen the hypothesis that the inducer and proline are equivalent, it seemed desirable to obtain some idea of the relationship between the amount of proline in Artemia extract and the biological activity of that extract. Aliquots of an extract were diluted in Phos-CCS5 and their activity on bioassay compared with the activity of dilutions of a 0.01 molar solution of L-proline. In all, five comparative assays were performed, from which it was concluded that proline had, on a dilution basis, roughly 5 to 10 times the activity of Artemia extract. If this was correct, the stock Artemia extract should have between 0.001 and 0.002 molar proline if proline and the inducer are equivalent.

Assays for proline were performed by the photometric method of Chinard (1952). Three separate comparisons of aliquots of Artemia extract and reagent proline indicated that the stock extract on dilution had an optical density equivalent to 1.25×10^{-3} molar proline.[4] To see if any of the compounds present in Artemia extract were interfering with color development, mixtures of proline and Artemia extract were assayed. Such experiments indicated no interference in reading the color developed by known aliquots of proline added to Artemia extract. For example, if proline giving an optical density reading of 0.095 (2×10^{-5} molar) and Artemia extract giving a reading of 0.07 (a 1/10 dilution of the stock) were combined, the reading was 0.170, the sum of the two readings.

The absorption spectrum of the ninhydrin-proline reaction product (Figure 13) is identical with that given by Chinard (1952). The curve for the ninhydrin-extract color, also shown in Figure 13, differs slightly but shows the same peak. The difference could be due to such compounds as cysteine and lysine (Chinard, 1952), and suggests that at least a small portion of the Artemia extract peak might be due to such contaminants. It may be calculated however, on the basis of the differences between the two curves, that no more than 1/10 of the proline peak can represent such contamination.

---

[4] This value represents the concentration of proline in the Artemia extract, which was considerably more dilute than the fluids present in Artemia nauplii.

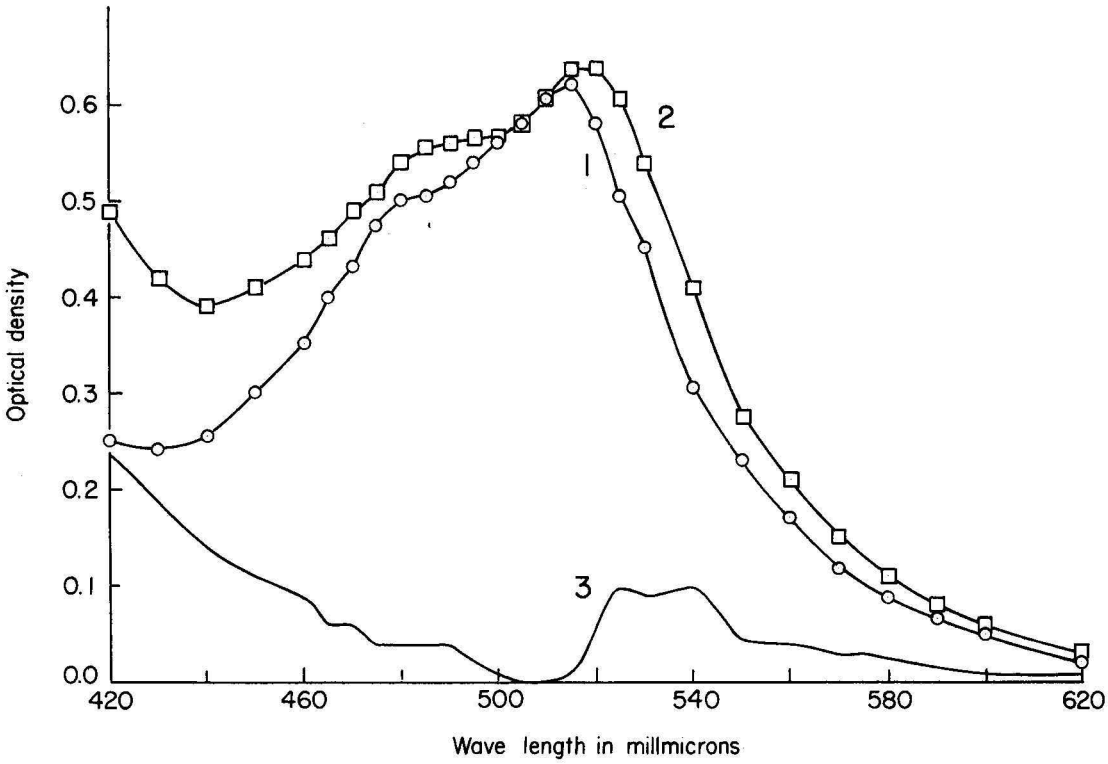


Figure 13. Absorption spectra of the Chinard ninhydrin reaction products of (1) reagent proline and (2) Artemia extract. Curve (3) represents the difference between curves (2) and (1). The spectra were determined in a Bausch and Lomb Spectronic with 13 mm. tubes.

On the basis of the bioassay procedures, it was estimated that there should be approximately 0.001 to 0.002 molar proline in Artemia extract, and on the basis of the Chinard reaction 0.001 molar proline was found. Ornithine also produces significant color in the region of the proline peak with the Chinard reaction, but unless ornithine is a major component of Artemi extract, the proline present is sufficient to account for most if not all of the biological activity of the extract. This is supported by the observation that only one active spot was found in bioassay of chromatograms of crude Artemia extract.

IV. Specificity of proline as the Cordylophora feeding reaction inducer

A general test for activity of amino acids revealed that while alanine and several others induced some tentacle waving at higher concentrations (e.g., 10^{-3} M), only proline had the ability to induce a complete feeding reaction. One such test is given Table 29. Ornithine was found devoid of activity. Proline in CVD induced feeding reactions erratically at various concentrations, and only in Phos-CCS5 was it equivalent to Artemia extract.

A more critical test of the role of proline in inducing the normal feeding reaction could be made if an antimetabolite could be found which would suppress the feeding reaction both to proline and to Artemia nauplii. In search for such an antimetabolite, and also to determine if structural analogues of proline have inducer activity, a variety of compounds have been bioassayed. Serial dilutions of 0.01 molar stock solutions were made in Phos-CCS5 (range 10^{-3} - 10^{-5} M). If the compound showed activity, the relativ activity was compared with that of proline. If inactive, the analogue was mixed with proline in various proportions; the greatest excess of analogue was 100-fold (10^{-3} M analogue with 10^{-5} M proline). The compounds examined are listed in Table 30, and the structures of some illustrated in Figure 14.

Among the active compounds, none are more active than proline. It is of interest that the recently discovered proline analogue with a 4-membered ring, azetidine-2-carboxylic acid (Fowden, 1956), has as much or slightly less activity than proline.[5] The 6-membered analogue, pipecolic acid, appears about 10 fold less active than proline. Other substitutions in the upper

[5]The author wishes to thank Dr. L. Fowden for providing a generous sample of the isolated compound.

TABLE 29

Factorial test for inducer activity of amino acids

The metabolite pools were prepared as used in the Bacterial Genetics course at Cold Spring Harbor.' Each compound was present at 2 mg. per ml. except thiamine (0.5 mg. per ml.). Ten-fold dilutions were made in Phos-CCS5; at 1/100 all assay solutions were between pH 6.3 and 6.5.

Pool No.	6	7	8	9	
1	Adenine	Histidine	Phenylalanine	Glutamic acid	-
2	Guanine	Leucine	Tyrosine	Serine	-
3	Cystine	isoLeucine	Tryptophan	Alanine	-
4	Methionine	Valine	Threonine	Aspartic acid	±
5	Thiamine	Lysine	Proline	Arginine	++*
	±	-	++*	-	Activity at 1/100

*Still ++ at 1/1000

TABLE 30

Proline analogues tested for inducer activity

All tests were made with 24-hour starved Cordylophora hydranths in Phos-CCS5. Results are described in the text.

Compound	Source
I. ACTIVE	
L-Proline (Pyrrolidine-2-carboxylic acid)	1
L-Azetidine-2-carboxylic acid	2
DL-Pipecolic acid.HCl (Piperidine-2-carboxylic acid)	1
4-Hydroxy-L-proline	1
Thioproline (4-Thiazolidinecarboxylic acid)	3
II. INACTIVE	
Pyrrolidine.HCl (Prolamine.HCl)	1
Pyrrole-2-carboxylic acid	4
2-Pyrrolidone-5-carboxylic acid (L-Pyroglutamic acid)	1
N-Acetyl-DL-proline	5
Glycyl-L-proline	5
L-Prolylglycine.H_2O	5
Sarcosine.HCl (N-Methylglycine.HCl)	1
Benzoylglycyl-L-proline	6
Benzoylglycyl-L-proline-methylester	6
Acetyl-DL-phenylalanyl-L-proline	6
L-Ornithine.HCl	1
Glutathione, reduced (γ-L-Glutamyl-L-cysteinylglycine)	7
L-Histidine.HCl.H_2O	1

Sources

1. California Corporation for Biochemical Research
2. courtesy of Dr. L. Fowden, University College, London
3. Nutritional Biochemicals Corporation
4. Aldrich Chemical Company, Inc.
5. Mann Research Laboratories, Inc.
6. courtesy of Dr. S. Moore, The Rockefeller Institute
7. Schwartz Laboratories, Inc.

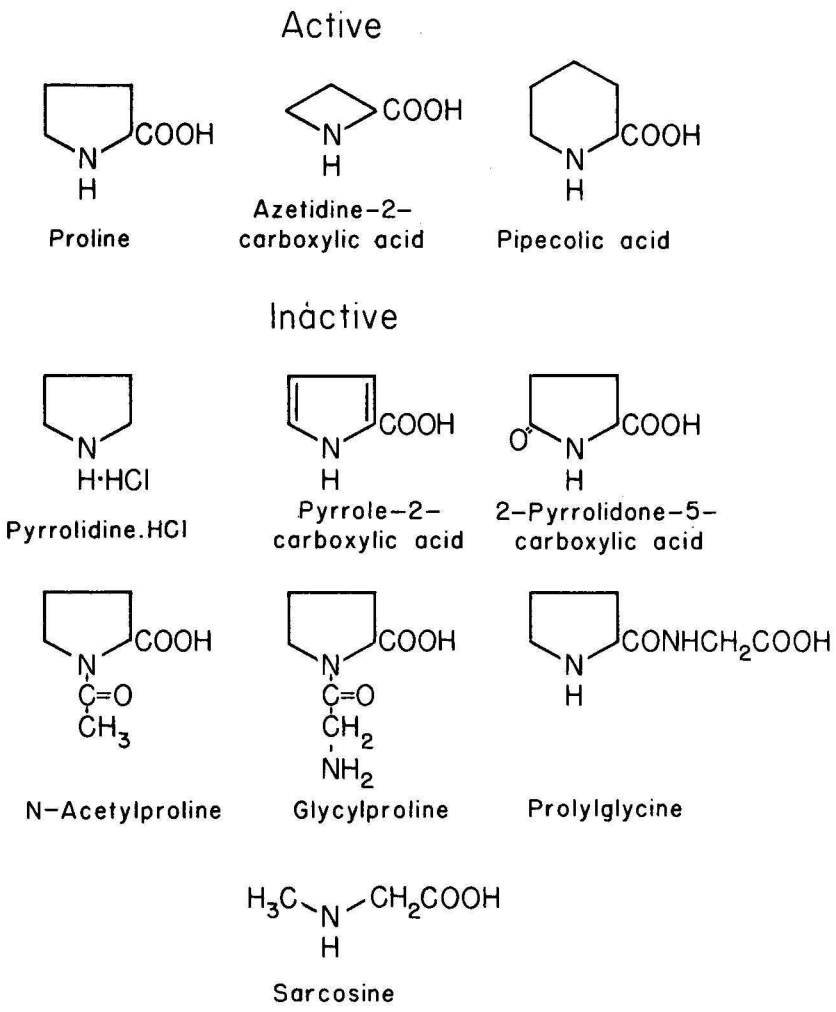


Figure 14. Formulae of some of the proline analogues tested for inducer activity.

part of the ring (hydroxyproline and thioproline) reduced the activity significantly but apparently did not abolish it.[6]

The other proline analogues tested were inactive up to molar concentrations more than 100 fold greater than that needed for activity of proline. Removal (pyrrolidine) or substitution (prolylglycine) on the carboxyl group destroyed activity, as did substitutions on the imino group (acetylproline, glycylproline). Substitution of either pyrrole or pyrrolidone rings for the pyrrolidine ring led to inactive analogues. Finally, sarcosine, a non-cyclic α-imino acid, had no activity.

Of these inactive compounds, none acted as an antimetabolite of proline, even at 100-fold molar excess. It is possible that if substantially higher concentrations did not induce side effects, antimetabolite activity could be demonstrated, since inhibition indices of 100 or greater are not uncommon (Woolley, 1952). No really effective proline antimetabolite is known for other systems, though hydroxyproline in excess inhibits the growth of some fungi (Meister, 1957, p. 118).

Several other derivatives, kindly provided by Dr. Stanford Moore, faile to show any activity or to antagonize proline: benzoylglycyl-L-proline, benzoylglycyl-L-proline-methylester, N-acetyl-DL-phenylalanyl-L-proline. Reduced glutathione, histidine, and ornithine, all inactive, were also unable to antagonize proline.

Three of the active proline analogues tested have been found in nature: azetidine-2-carboxylic acid and pipecolic acid, both found only in plant tissues so far (Fowden, 1956), and free hydroxyproline, in a variety of invertebrates (Simpson, et al., 1959). Several of the inactive compounds have also been found in nature. Proline peptides are probably not a source of inducer activity, as indicated by the experiments with prolylglycine and glycylproline

DISCUSSION

A conceptual picture of the feeding reaction of Cordylophora may be developed from these studies. Nematocysts puncture the body wall of a prey organism, leading to the release of body fluid containing the feeding reaction inducer, proline. In response to the proline, tentacles are brought to the mouth, and the mouth opens and swallows the prey. Under appropriate condition

[6] Hydroxyproline and thioproline are about 100 fold less active than proline, which could be accounted for by contamination. This possibility is under study

the same reaction may be induced artificially by immersion of a hydranth in a proline solution.

The response to prey organisms or proline is undoubtedly the result of coordinated contraction of the muscle fibers in a Cordylophora hydranth. These muscle fibers have been carefully described by Pauly (1902), and appear to be similar to those found in Hydra (Mueller, 1950; Slautterback and Fawcett, 1959). Fibers are found at the base of ectodermal cells which run parallel to the long axis of the hydranth, and other fibers at the base of endodermal cells which form a ring around the hydranth. The feeding reaction would appear to involve contraction of the longitudinal fibers of the ectoderm.

It is of interest that even the artificial condition of total exposure to Artemia extract or proline in Cordylophora or to GSH in Hydra does not result in total contraction of tentacles or hydranths. The response to such total exposure remains as coordinated as the response to gradients of feeding reaction inducer pouring out of prey organisms. Thus the feeding reaction is coordinated by some factors other than localized exposure to the inducer. Little is known, however, about the nervous system and coordination of hydroids (cf. Chapter VI).

For Cordylophora, and also for Hydra (Lenhoff and Bovaird, 1959b), the aqueous environment has been found to be important in determining the response to feeding reaction inducer. In Cordylophora, at least magnesium and possibly phosphate ions have been implicated. The response to proline in the standard culture solutions is negligible compared to that in Phos-CCS5. Yet the same hydranths will gorge themselves on Artemia or other suitable prey in CCS5 or CVD. The explanation of this paradox is probably to be found in the fact that with captured prey, proline is being released as a component of the body fluid, containing a host of other substances, including phosphate and other substances which can sequester magnesium ions. An untold number of these substances may provide suitable conditions for the induction of the feeding reaction by proline.[7]

The evidence which led to the conclusion that the normal feeding reaction of Cordylophora is caused by proline may be summarized as follows.

---

[7] Many other factors may also influence the feeding reaction, including the mechanical factor of prey capture, and the factor of localized exposure to relatively high concentrations of body fluid.

Cordylophora hydranths will capture inert objects, but give no further response to them. If they capture a piece of meat, they will give a feeding reaction only if the fluid which diffuses out of the meat has not been washed away. Extracts of Artemia nauplii and other organisms elicit a feeding reaction, and studies indicated that a small and stable molecule is involved in this response. Paper chromatograms of tissue extracts yielded only a single spot capable of inducing the feeding reaction. Isolation of the feeding reaction inducer from Artemia extract led to its identification as proline, and reagent proline induces the feeding reaction at low concentrations. There is sufficient proline in Artemia extract to account for the feeding reaction. Examination of the specificity of the reaction indicates that only certain imino acids are capable of eliciting the feeding reaction; the feeding reaction obtained in response to these imino acids is in all respects similar to that obtained with tissue extracts.

A number of determinations of the amount of free proline in body fluids of organisms are available in the literature, and several are given in Table 31. There is ample free proline in these tissue fluids to induce the feeding reaction if other conditions are suitable.

The specificity of proline has been found not to be absolute, and certain other imino acids which have been found in nature (azetidine-2-carboxylic acid, pipecolic acid and hydroxyproline) may complement proline in inducing the feeding reaction to certain organisms. On the basis of the limited survey of proline analogues reported above, it would appear that the requirements for biological activity are relatively simple: a heterocyclic α-imino acid which is not substituted or unsaturated in such a way as to influence the recognition of the imino acid group. The inducer activity of 4, 5 and 6-membered rings and probably of hydroxyproline and thioproline suggests that the chemoreceptor, whatever it may be, recognizes primarily the imino acid region.

Perhaps the most interesting aspect of this study at its present stage is from the point of view of comparative biochemistry and evolution. As mentioned above, the feeding reactions of three Hydrozoa -- Hydra, Physalia and Campanularia -- have been found to be induced by reduced glutathione (GSH). While there is little valuable discussion of evolution within the Hydrozoa available, it would probably be agreed by many that the scheme given in Figure 15 represents a reasonable deduction (cf. Hyman, 1940, pp. 634-636). As

TABLE 31

Free proline in tissue fluids

Organism	Fluid	Value given	Molar concentration	Reference
Insects				
Anacridium	hemo-lymph	0.17-0.42 milli-moles per 100 ml.	$2-4 \times 10^{-3}$	a
Cossus larvae	"	123 mg %	1×10^{-2}	b
2 saturnids	"	9-10 mg %	1×10^{-3}	b
Mammals				
Cat liver	extract	2.6 mg./100 gm. wet tissue	2×10^{-4}	c
Human	blood plasma	2.3-2.4 mg./ 100 ml.	2×10^{-4}	d

[a]Benassi, Colombo, and Peretti (1959)

[b]Duchâteau and Florkin (1953)

[c]Tallan, Moore, and Stein (1954)

[d]Stein and Moore (1954).

indicated in Chapter II (Table I), Cordylophora and Hydra are both gymnoblastic hydroids. They are similar in many respects, and are the two major hydroids which have established themselves in fresh water, suggesting that they may represent a common offshoot. In contrast, the calyptoblastic hydroid Campanularia belongs to a different sub-order, and Physalia belongs to a different order altogether. One could create alternate schemes to that of Figure 15, but on any scheme, present day evidence (i.e., based on present day structure, with no useful fossil record) would imply that Cordylophora and Hydra are much more closely related to one another than to Physalia or Campanularia.

If this is correct, the available data, scanty though they may be, would support the conjecture that Cordylophora represents an offshoot from the main line of organisms utilizing GSH - a deviationist. The validity of this conjecture, and determination of its significance, can only follow from a comparative study of the feeding reactions of Hydrozoa. Such a study is planned.

There is little in the written structure of GSH to suggest any possible similarity to proline (Figure 16). It has been suggested, however, that GSH exists in solution in several cyclized forms (Calvin, 1954; Wieland, 1954), one of which if correct introduces a heterocyclic α-imino acid into the molecule (Figure 16). While Cordylophora probably could not recognize such a molecule because of the substituents (cf. 2-pyrrolidone-5-carboxylic acid, Figure 14), it is conceivable that some evolutionary relationship may lie in this observation.

As Schneiderman and Gilbert (1958) have emphasized, the essential requirement for a chemical control system of any sort is not so much a requirement for the innovation of a new molecule but rather the development of a chemoreceptor system capable of recognizing a suitable molecule already available. For a hydroid, the only requirement is that it be able to select suitabl food. The GSH mechanism, as emphasized by Loomis (1955), restricts Hydra to living prey, but ecological considerations make it unlikely that the diet of Cordylophora is significantly altered by the utilization of proline rather than GSH. Due to size, availability, and other considerations, the major natur food of both Hydra and Cordylophora is probably small arthropod larvae and to a lesser extent small worms. Either proline or GSH or a variety of other

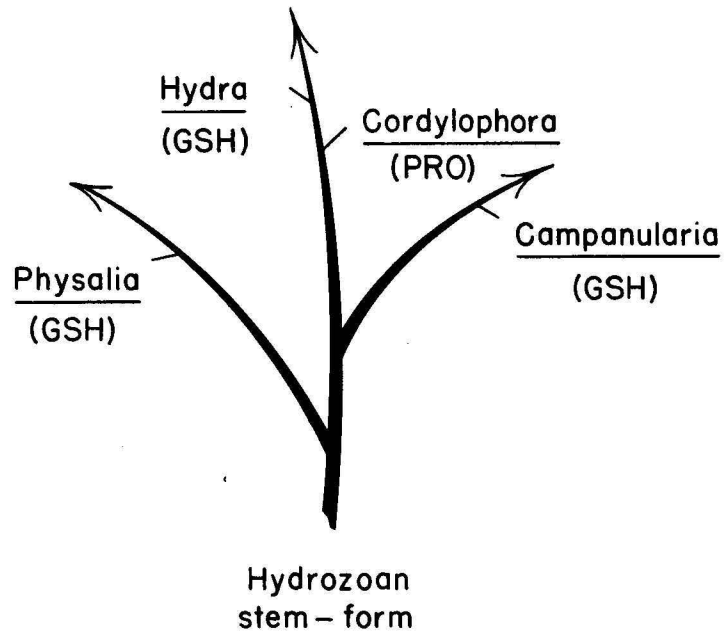
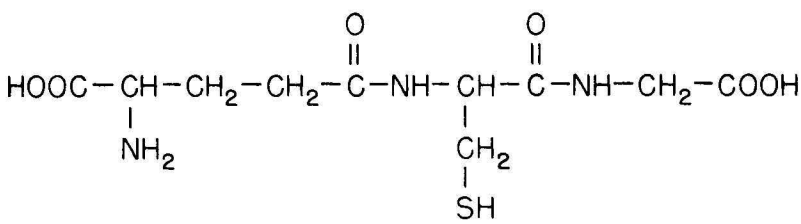
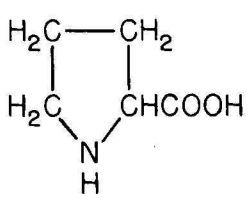


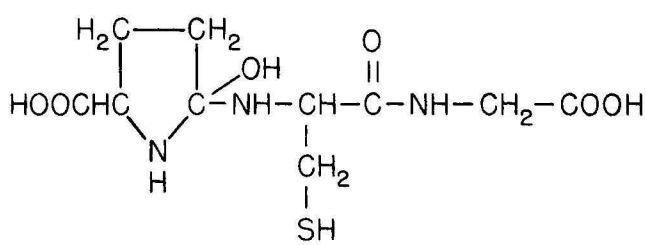
Figure 15. A diagram of the probable evolutionary relationships of the four Hydrozoa whose feeding reaction inducers have been identified.



Reduced glutathione (GSH)



Proline



Hydroxy-pyrrolidone form of GSH
(Hypothetical)

Figure 16. Formulae for glutathione and proline, and for one of the possible cyclized forms of glutathione.

molecules would provide suitably for recognition of these food sources, and the major question which might be asked is, if GSH is the primitive mechanism for the Hydrozoa, why was the proline feeding reaction ever selected for in the evolution of Cordylophora?

Chapter VI

Rhythmic Movements in *Cordylophora*

Although the question was settled to the satisfaction of all in the mid-eighteenth century, many who have looked at colonial hydroids find it difficult to believe that the exquisitely beautiful, brightly-colored flowers which line the sea coasts are in reality animals. In fact, the term hydranth is derived from the Greek for "hydra-flower" (Allman, 1871).

One of the reasons leading the casual observer to confuse colonial hydroids with aquatic plants is the fact that colonial hydroids move but little and what movements there are are confined to the hydranths. In the absence of prey, the hydranths of a Cordylophora colony are quiet, with tentacles greatly extended (Figure 17A). Upon prey capture (17B), the tentacles are brought to the mouth and the captured organisms rapidly ingested (17C). After feeding, the hydranths are again quiet, though they become bloated as they digest their food (17D) and then return to their resting appearance (17E). The movements observed are readily explained by the muscle fibers found in the hydranths of Cordylophora (Schulze, 1871; Pauly, 1902).

As part of a study of the asexual development of Cordylophora colonies, time-lapse movies were taken which revealed organized rhythmic movements in both hydranth and coenosarc tissue. Other movies, taken to study the reconstitution of hydranths from bits of coenosarc tissue, revealed a second series of movements. A description of these movements is presented here.

METHODS

Colonies

Cordylophora was grown in CVD or CCS5 as described in Chapter III. The movies were taken in CCS5 at room temperature (22 to 26°C).

Most of the movies of colonies or portions of colonies were made with a special horizontal apparatus (Figure 18). The Kodak Cine-Special camera was controlled by a timer and camera drive manufactured by Rolab Inc., Sandy Hook, Conn. A Kodak Cine-Ektar f 1.4 25 mm. lens was used with extension tubes.

The colonies, growing on microscope slides, were transferred to a special chamber constructed of a piece of Plexiglas tubing, with the two parallel walls of $3\frac{1}{4}$ x $4\frac{1}{4}$ in. lantern slide cover glasses held on with wax. Provision was made for continuous flow of CCS5 through the chamber.

The entire apparatus was mounted on a heavy aluminum plate. The culture chamber was held on a small jack to facilitate adjustment of position for

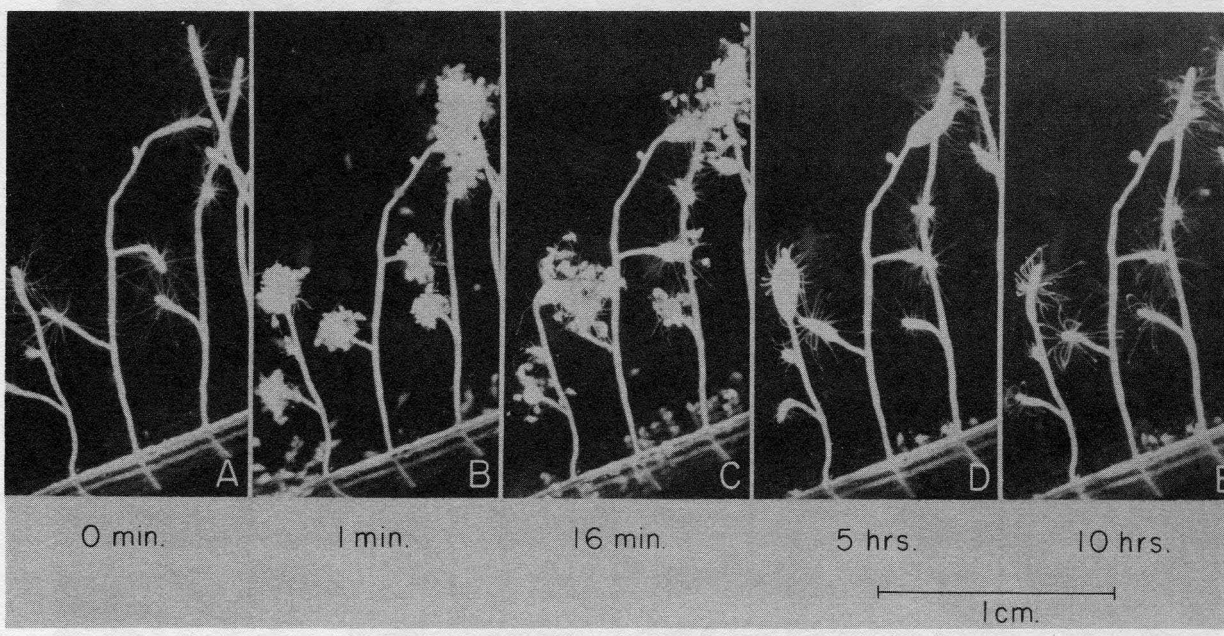


Figure 17. The daily cycle of Cordylophora. A portion of a resting colony (A) is shown immediately after capture of Artemia nauplii (B). Most of the nauplii have been eaten within fifteen minutes (C), and the hydranths soon become bloated (D). Within about ten hours, the hydranths have returned to a resting state (E). This sequence is taken from a time-lapse movie.

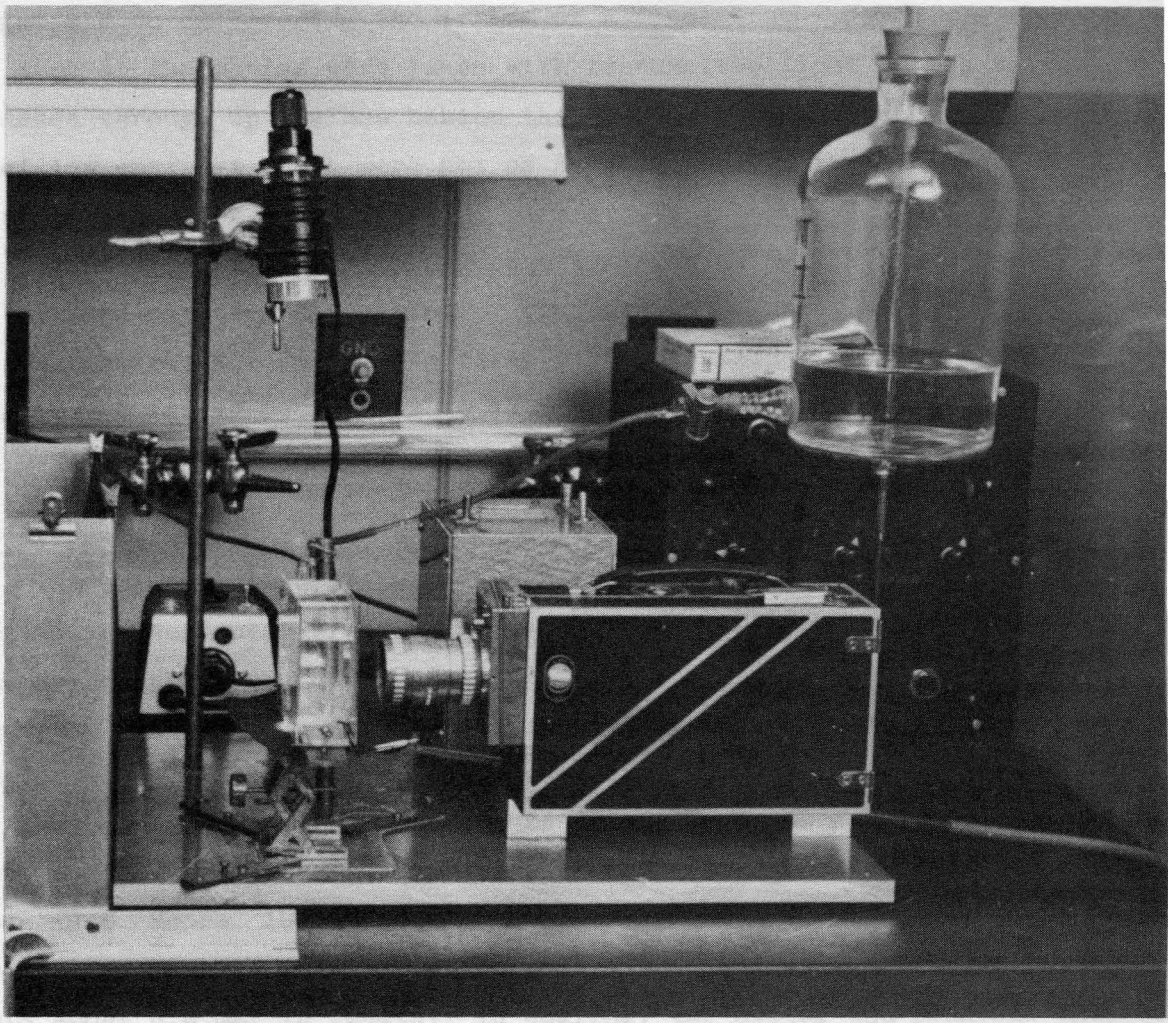


Figure 18. Apparatus used in photographing portions of Cordylophora colonies. In the foreground, from left to right, the stand for a black paper background, the culture chamber (mounted on a small jack), and the Kodak Cine-special lens and camera may be seen. In the background, the light source, camera drive, reservoir of culture solution and camera timer are visible.

photography. A black paper background was placed behind the chamber, and the portion of a colony being photographed was lighted with a bright spot of light. The light was on only during the taking of a frame.

Some of the movies were taken with transmitted light from a 25 watt bulb passed through opal glass behind the chamber. This arrangement produced films of low contrast which were not as satisfactory as those taken with direct light against a black background.

Movies at higher magnifications were taken using the stand provided by Rolab Inc. and either a dissecting or compound microscope.

In general, the films were taken at one frame per minute, though rates varying from 8 frames per minute to 12 frames per hour were employed. Kodak Plus-X negative 16 mm. film was used.

Tissue fragments

Stem segments were removed from colonies growing in CCS5 or CVD; for uniformity the segments were usually taken about 1 mm. below terminal hydranths (hydranths at the apices of uprights). The coenosarc tissue was extruded from the perisarc and cut up with knives ground from surgical needles. Two types of tissue fragments were prepared, and gave indistinguishable results. In a few cases, the coenosarc was cut into minute fragments a few hundredths of a millimeter in diameter. These fragments were piled together and allowed to coalesce (J.S. Moore, 1952a). In most cases, two-layered coenosarc tube was cut in half in the longitudinal direction. These halves were then cut into segments about 0.6 mm. in length. In healing, the distal and proximal ends of such a strip roll together so that the endoderm is returned to the inside and the ectoderm left on the outside. This results in a disruption of any polarization which may have existed in the tissue fragment on cutting. After healin the fragments had a diameter of about 0.2 mm.

Some of the movies of reconstitution were taken with the apparatus described above, but most were taken with equipment provided to the author in the summer of 1958 through the generosity of Dr. Nelson Spratt. They were taken with a 10 x objective and a 10 x ocular, with filming at 30 second intervals (in some cases 15 or 60 second intervals were also used).

Reconstitution was accomplished at room temperature in small petri dishes of culture solution. Demineralized water was added to compensate for evaporation.

Analysis

A variety of methods were employed in the analysis of the film, including frame by frame examination with a dissecting microscope, enlarger, or film editor. However, most of the information comes from projection of the films with a Bell and Howell 16 mm. projector. A film was projected at a constant speed, and the complete sequence under study timed. Then the number of times a given event occurred in a unit interval of viewing time (usually 10 seconds) was counted, and from this the rate of actual occurrence of the event computed. For example, if 12 events occurred during each 10 second interval, and if each 10 seconds represented 3 hours of filming, then the event occurred 4 times per hour. This method gave highly reproducible results.

RESULTS

I. Peristaltic waves in hydranths

Examination of the movies taken to follow the development of Cordylophor colonies revealed a wealth of peristaltic movements. On analysis, these movements proved to be polarized, rhythmic, and at least to some extent synchronize throughout a colony.

Proximally-oriented waves of contraction begin at the tips of hydranths and continue down the stems. Such peristaltic waves are the standard device used by nature for moving fluids in tubes (cf. Ramsay, 1952, Chap. 2). These waves show up clearly in projection of the time-lapse movies, but are not sufficiently distinct to be seen readily in prints of single frames from the movie (Figure 19). A diagram illustrates the course of a wave down a hydranth (Figur 20).

These peristaltic waves are separate from the muscular movements of the hydranths, which appear on the film at irregular intervals as abrupt contrac tions.

The peristalsis is highly rhythmic, so that the "beat" of a hydranth is steady and continuous with time. A wave of contraction travels fairly rapidly down a hydranth, and there is a period of rest before the next beat is initiate In resting colonies, the time interval between beats is 20 to 25 minutes, givir a rate of 2 to 3 beats per hour.

When a colony is fed Artemia nauplii (Figure 17 B and C), the frequency of the peristaltic waves increases dramatically, although the individual waves

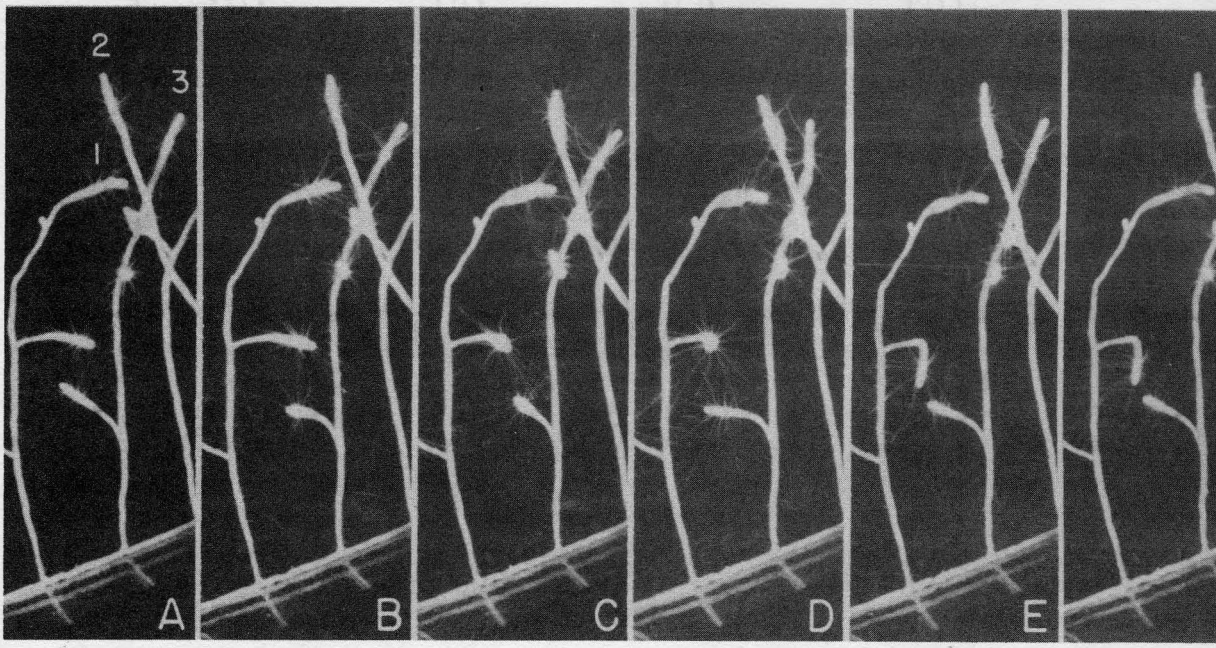


Figure 19. Peristalsis in Cordylophora. The six frames shown represent three minute intervals, and are from a film taken at one frame per minute. The same film is used in Figure 17, and for the analysis shown in Figure 21; the hydranth numbers are the same as those used in Figure 21. For description see text.

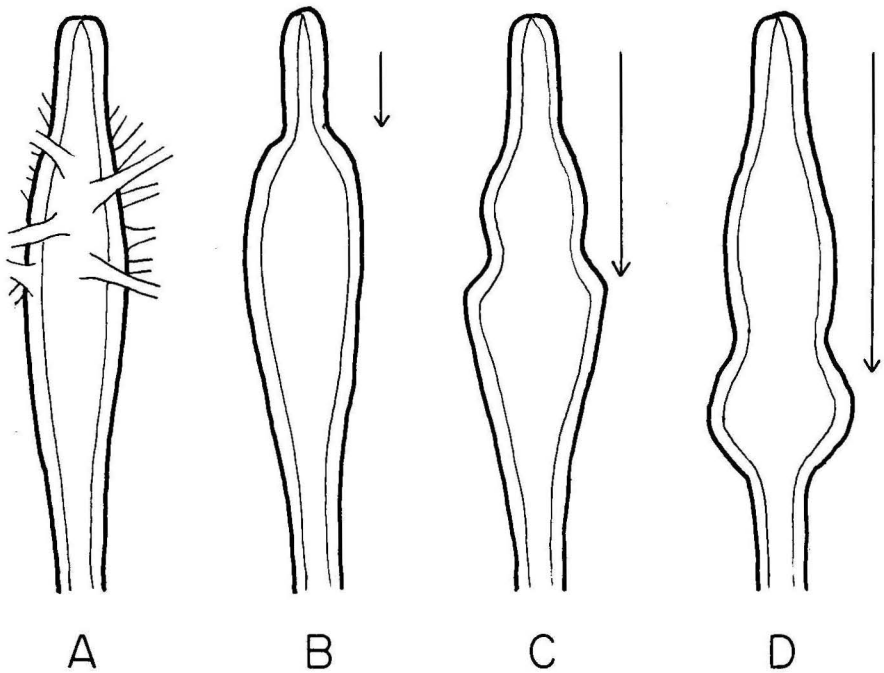


Figure 20. Diagram of a peristaltic wave in a Cordylophora hydranth. Compare Figure 19. The tentacles are indicated only in A.

become harder to see clearly because the hydranth becomes bloated. After the frequency increases, it gradually decreases to the resting frequency. An analysis of one sequence is given in Figure 21. The beat frequency increased 2 to 3 fold immediately after feeding, and fell over the course of the ensuing 8 to 10 hours to the resting rate of 2 to 3 waves per hour.

The most striking feature of the rhythmic peristalsis is that it is relatively synchronous throughout a colony. Several terminal hydranths in a field, separated from one another by a centimeter or more of coenosarc, all appear to beat simultaneously. This has been true of all sequences studied. To some extent this synchrony can be seen in the photographs shown in Figure 19 (especially D and E corresponding to Figure 20C and D).

The beat frequency of the 3 terminal hydranths shown in Figure 19 have been followed with time (Figure 21), and while the frequencies for each hydrant are similar, they are not identical as would be expected if they beat synchronously. This apparently is due to difficulty in accurately counting the waves, especially after feeding. These 3 hydranths have been followed many times without detecting any deviation from simultaneous beating.

The most striking evidence for synchrony was obtained when it was abolished. This has been accomplished in 3 experiments by ligaturing one or more of the uprights in a field of view. In time, the ligatured uprights began to beat asynchronously with the rest of the colony, though their beat remained rhythmic. This asynchrony is readily observed. Those parts of the colony whic are still connected by a common coelenteron continue to beat synchronously.

While the relative synchronization of beating is clear, the degree of precision of integration of beating time is unknown. A slight difference in the moment at which a beat occurs relative to another beat probably would go undetected in the films. Furthermore, in order to accurately see the peristalt waves, only a small portion of a colony, such as that illustrated in Figure 17, can be followed. Thus it is not known whether the synchrony extends over a whole colony, or only over parts of a colony. It is clear, however, that synchrony is maintained in terminal hydranths separated by several centimeters of coenosarc, as hydranths 1 and 2 of Figure 19.

The mechanism by which synchrony is achieved is not known. It has been observed, however, that if the stem of an upright is crushed with fine forceps, the hydranths of that upright and usually also all the other hydranths of the

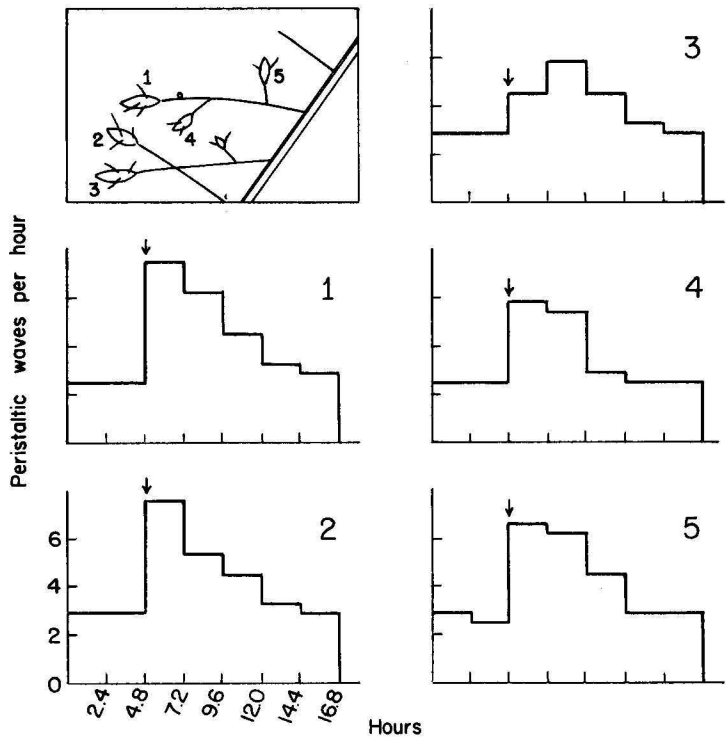


Figure 21. The rate of peristalsis and the influence of feeding on the rate. Five hydranths in one sequence were followed individually, counting the number of peristaltic waves per 10 second interval in repeated viewings of the film at constant speed. Each interval represented 2.4 hours. The arrows indicate the time of feeding.

colony contract. This indicates that a stimulus can be propagated through a Cordylophora colony. The transmission of such a stimulus is rapid, and as yet it has not been possible to follow a wave of contraction passing from the crushed upright through the colony.

II. Movements of stem and stolon tissue

In the movies described above, it was possible to see that the waves which began at the tips of the hydranths were propagated through the coenosarc right to the tips of the stolons, but it was not possible to see the orientation and nature of the coenosarc movements clearly. At higher magnifications the movements within the coenosarc could be followed. The relationship between these movements and those of the hydranths as seen at lower magnification has not been completely elucidated as yet.

Peristalsis in stems is proximally-oriented, although the orientation is not as striking as in the hydranths. Peristalsis continues undiminished in isolated stem segments (ca. 3 mm. in length), indicating that neither the motivating force or the necessary contractile tissue is localized in the hydranth. The polarity of cut stem segments may be permanently indicated by making one of the cuts through the perisarc at an angle. The peristaltic waves of isolated stem segments begin by being proximally oriented, resulting in an accumulation of a bulb of tissue at the proximal end of the stem. The orientation then seems to disappear (or reverse?), and a second bulb appears at the distal end. Peristalsis continues, and one or usually both ends regenerate a hydranth.

The peristalsis at the tips of the stolons has also been followed at higher magnifications. Stolon tips act as blind sacs at the end of the line, filling up and emptying as shown in Figure 22. It is difficult to detect any orientation in the filling-emptying cycle. In the sequence illustrated, the stolon completed one cycle an average of once every 10 minutes. The cycles came in pairs one right after the other, however, with an interval of time in between. Thus one might suggest that there are a pair of cycles every 20 minutes, bringing the rate into line with the rate in the hydranths. Further study is required to determine the coordination of these processes in a colony.

III. Reconstitution of tissue fragments

Bits of stem tissue placed in dishes of culture solution reconstitute hydranths in from 24 to 36 hours, depending on precise conditions. Static

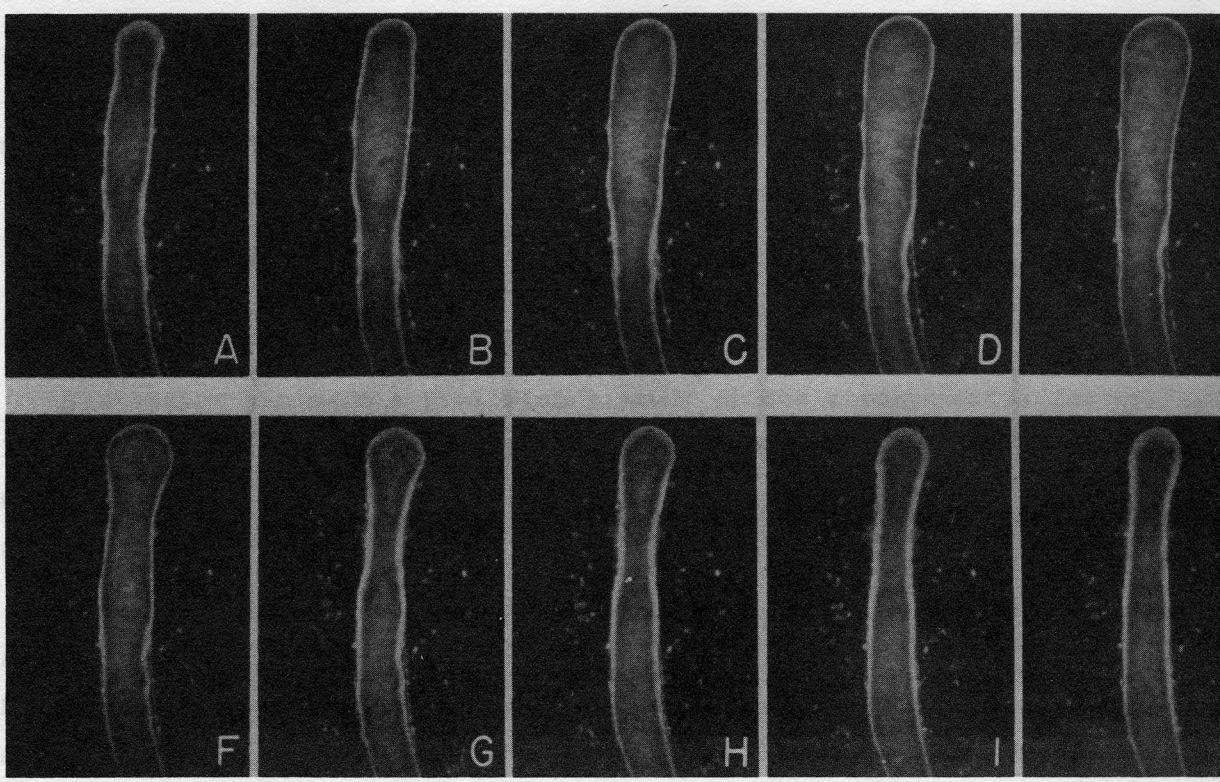


Figure 22. One cycle of filling and emptying of a stolon tip. The frames shown represent one minute intervals.

observations indicated that the process of reconstitution could be divided into five stages (Figure 23). (1) The cut fragment (2) heals to form a solid sphere, with ectoderm on the outside and endoderm on the inside. This sphere then becomes (3) hollow, and then (4) elongates. At the tip of the elongated ball, in the region referred to as the outgrowth, a (5) hydranth develops with tentacles and a mouth.

Reconstitution offers many aspects for study. Beadle and Booth (1938), and J.S. Moore (1952a) have found that bits of oral cone tissue implanted into stem tissue fragments induce the development of hydranths at the position of the implant. Moore (1952b) has made a careful histological study of this process. The only other major study is that of Zwilling (personal communicati who has shown that the ectoderm alone will reconstitute first a new endoderm and then, in some cases, a complete hydranth.

The author has made a preliminary study of the environmental factors influencing reconstitution, and so far has found no difference in conditions from those influencing growth rate (Chapter IV), with the exception that reconstitution is significantly more rapid at 26°C than at 22°C.

When this process of reconstitution is followed with time-lapse, a dynamic picture emerges which is rather different from the static picture described above. The cut fragment rounds up rapidly into a ball, with most of this healing being accomplished within the first hour (A to C of Figure 24). There is little change in the solid ball for about 6 to 10 hours, and no visib movement (D). Then it begins to hollow (E), and as it does so one sees consid able deformation in the surface of the ball. It bulges out on one side, and then another. There is no sign of polarization in these early multipolar movements.

By about the fifteenth hour, the first signs of polarization are eviden The movements become rhythmic and deform the hollow ball from a sphere to an ovoid and back again repeatedly (F to H) -- these are the bipolar movements. A single cycle, from sphere back to sphere again, takes 20 to 30 minutes. Of this, the bulk of the time is taken in the transformation from sphere to ovoid the return to a sphere is more rapid (Table 32).

By the twentieth hour the movements have developed a definite unipolar tendency (I to L). The same transformation as that described above takes place, but continues in that after elongation one end of the ovoid swells.

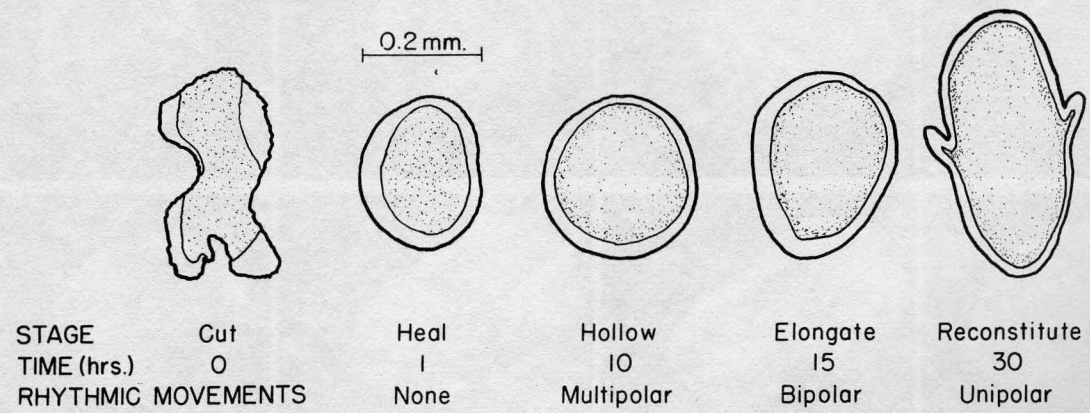


Figure 23. Diagram of the major stages in the reconstitution of Cordylophora. The tissue fragments are drawn to scale from the movie illustrated in Figure 24. The times to reach a given stage are approximate.

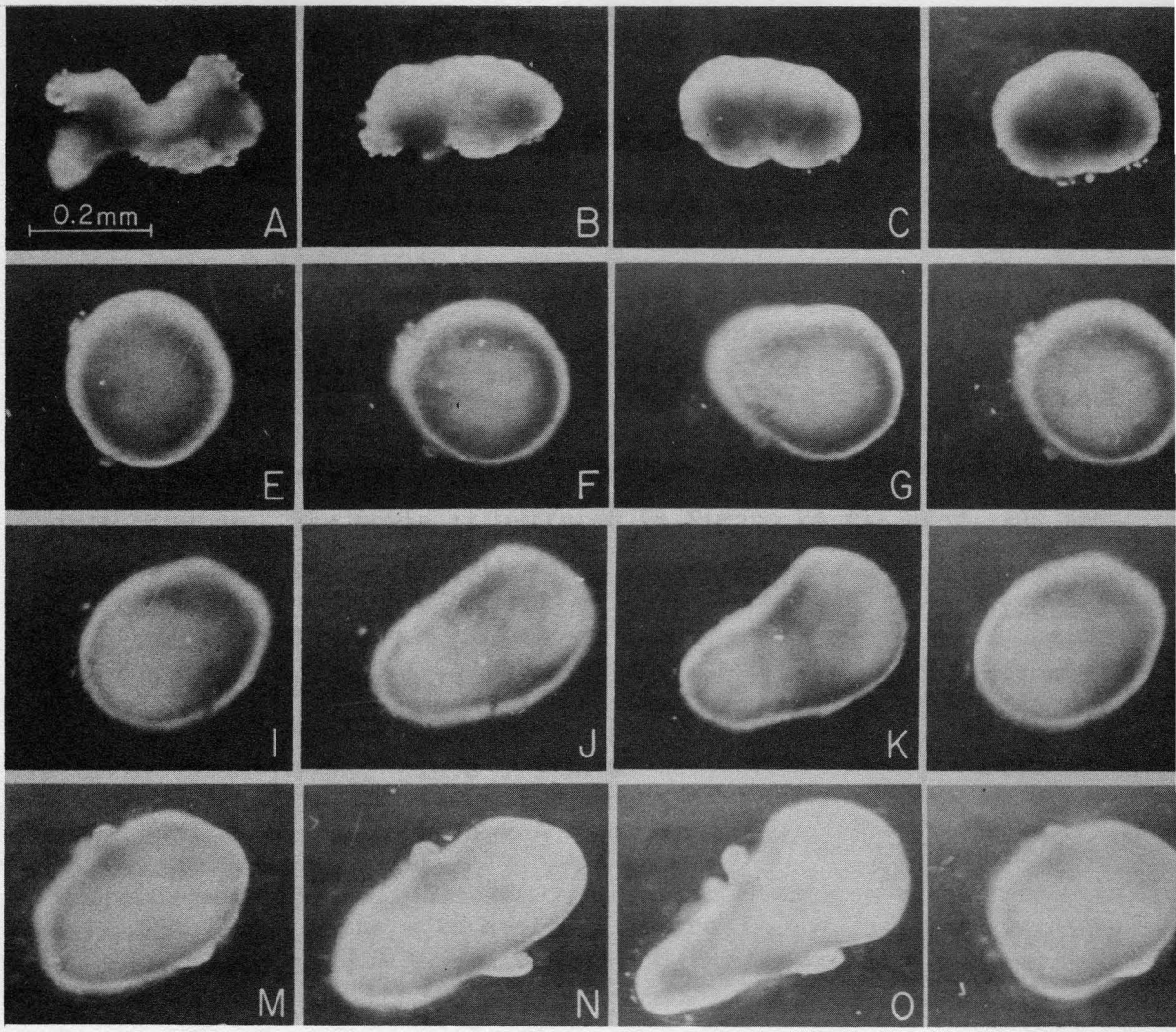


Figure 24. Reconstitution of Cordylophora. A series of frames taken from a single time-lapse movie to illustrate the tissue movements. Healing (A to D); hollowing (E, multipolar movements do not show clearly in still photographs); bipolar movements (F to H), unipolar movements (I to L), and unipolar movements after the tentacle buds have formed (M to P).

Time from start of filming (hours, minutes):

A	0,0	B	0,30	C	1,30	D	4,0
E	12,30	F	16,0	G	16,27	H	16,38
I	20,30	J	20,38	K	20,46	L	20,54
M	30,0	N	30,6	O	30,15	P	30,27

TABLE 32

Timing of rhythmic movements of reconstitution

The values were obtained by counting the number of frames occupied by the change from one form to another, and converting this value into minutes. The movie illustrated in Figure 24 was used, and the letters refer to stages as shown in that figure.

Bipolar movements			
	Sphere to ovoid to sphere		Total time for cycle (minutes
	F	G	H	
	17.5	8.5	26.0
	14.0	12.5	26.5
	18.5	8.5	27.0
	16.5	9.0	25.5
Unipolar movements			
	Sphere to ovoid to sphere		
	M	O	P	
	16.5	10.5	27.0
	17.0	10.5	27.5

This end will become the hydranth. By about 30 hours, the tentacle buds have made their appearance. Simultaneously with the appearance of tentacle buds, a new movement can be seen -- the erratic jerky contractions produced by the rapid movement of hydranth and tentacles. This jerky movement is superimposed on the slow rhythmic contractions of the tissue fragment, which continue to be unipolar (M to P) for as long as the reconstituted hydranths have been watched. The tentacles grow in length and number, a mouth breaks through (cannot be seen on films), and if fed such a little reconstitute develops a stolon and thence a colony.

Thus superimposed on the static picture of reconstitution there is a dramatic picture of movements, which progress from being completely disoriented to being oriented toward the end which will develop a hydranth. As in the case of the peristalsis of intact colonies, these movements are rhythmic, occurring two to three times per hour (Table 32).

The precise relationship of these movements to the peristaltic waves observed in the colonies is not immediately clear. One can readily duplicate the movements observed during reconstitution with a spherical balloon, and it is difficult to imagine how such a structure could manifest peristalsis, since almost by definition peristalsis requires a tube. If one indiscriminately pushed in one side of the balloon and then another, causing deformation of other parts, one would have the multipolar movements. Randomly oriented contractile elements, contracting independently, would produce the same result. If on the other hand, a spherical balloon is squeezed around one axis, it is deformed into an ovoid. Done rhythmically, one has the rhythmic bipolar movements of the tissue fragments, which could be produced by a battery of contractile elements as a broad belt oriented perpendicular to the direction of extension of the sphere, and contracting synchronously. The unipolar movements could be produced by the same mechanism as the bipolar movements, followed by a peristaltic wave running from the proximal to the distal end of the ovoid, forcing a bulge at the distal end.

This description suggests that peristalsis may be involved in the last of the movements, but if so it is oriented in a direction opposite to that which prevails in adult hydranths in a colony.

It was noted above that isolated stem segments retain and manifest the ability to produce peristaltic waves. It is likely, then, that the motivating

force behind the movements observed in reconstitution is the same. Apparently no movements can be seen, however, until the tissue fragment has become hollow (deformable?).

In the reconstitution of tissue fragments under these conditions, all polarity in the stem is destroyed on cutting and healing, and must reform afresh. The mechanism by which this occurs would be of utmost interest to study. One cannot help wondering if the gradual transition from multipolar to bipolar to unipolar rhythmic movements is perhaps a manifestation of the develo ment of a new polarity in the reconstitute.

DISCUSSION

There are no known mechanisms to account for any of the observed phenome -- for the slow movements themselves, for their rhythmicity, polarization, increase in rate after feeding, or the relative synchrony with which the peristaltic waves begin at the tips of each hydranth.

Contractility is said to be a general property of living systems, and there are many situations, particularly in the movements of cells, such as amoebae, where no fibers can be demonstrated with the microscope. This is also the case for Cordylophora. While a system of muscle fibers exists in the hydranth, and undoubtedly accounts for the rapid contractions manifested by that structure, no sign of muscle fibers can be found in the coenosarc of stems and stolons (Schulze, 1871; Pauly, 1902). It is possible that fibers might be seen in these systems with the electron microscope, or that contraction of glycerinated preparations could be induced by ATP (Hoffmann-Berling, 1959). The slow contractions probably do not require a very sturdy contractile element.

The rhythmicity and polarization remain a mystery, as indeed they do for most systems. The synchronization of peristalsis in colonies is also not understood, but perhaps is more amenable to analysis. The ligature experiments indicate that the synchrony is transmitted through the coelenteron or coenosarc both of which are blocked by ligatures. Transmission of stimuli through a colony has been demonstrated. But the mechanism of transmission is not clear. Nerve cells have been reported for Cordylophora, but they are apparently sparse if they occur at all. The nervous system of hydra has been more extensively studied. Hadzi (1909) and McConnell (1932) described and drew complex nerve nets for hydra; these form the standard textbook picture of the nervous system

of hydroids (e.g., Buchsbaum, 1948, p. 79). Recent workers such as Mueller (1950) have been able to find scattered nerve cells in the ectoderm of hydra, but no nerve net. The extreme is reached by Slautterback and Fawcett (1959), who could find "no cells identifiable as neural elements." In their studies of Cordylophora, Morgenstern (1901) and Pauly (1902) have drawn isolated minute cells at the base of the ectoderm or in the mesoglea which they refer to as Ganglienzellen. The author has observed minute cells in dissociated cell preparations of Cordylophora; these cells have two or more long processes and look much like the nerve cell figured by Pauly (1902, Figure 9b), but as yet no such cells have been found in sectioned material.

From this brief summary it should be clear that the presence or absence of an organized nervous system or even of scattered nerve cells in hydroids is an unsettled problem. But the question of a structural basis for the synchronized peristalsis, while of interest, assumes less importance since many systems are known where contraction is synchronized or stimuli transmitted without the use of nerve cells. A striking instance of this, perhaps analagous to that in Cordylophora, has been described by Cavanaugh (1955). The hearts of chick embr were dissociated with trypsin and the cells placed in tissue culture. Isolated atrium cells beat at a rate of 1.6 pulsations per second; ventricle cells at 0.9 pulsations per second. Each cell pulsed independently. However, when two or more cells came together they began to pulse synchronously with differences in rate averaged out. Such cells are without innervation or a pacemaker, and can tell one another about their rate of beating and can cooperate to produce a synchronous beat.

Certain movements analagous to those described here have been observed previously, although entirely in calyptoblastic hydroids. Huxley and de Beer (1923), using Campanularia, were apparently the first to notice these movements They draw the tip of a new growth, and show that while at one moment the tissue fills the perisarc, at another it is slightly withdrawn (cf. Figure 22). Saint Hilaire (1930) describes the same phenomenon in another calyptoblast, Clytia. The most important study was made Walp and Hammett, who filmed a Campanularia hydranth from bud to senescence with material collected from nature (Hammett, 1943; Hauschka, 1944; Crowell, 1957). As Hauschka (1944) reports, "the motion picture record reveals rhythmic contractions of the coenosarc throughout the hydrocaulus (stem) as well as in the developing zooids (hydranths). ... The coenosarc contracts in a peristaltic manner, and each wave of contractions exerts pressure on the liquid contents (hydroplasm) of the gastro-vascular tube This internal force causes outbulgings at the growing point..." It seems clear

that this phenomenon parallels that observed in Cordylophora.[1]

No such information is available for the gymnoblastic hydroids. M. Steinberg (personal communication) has prepared an excellent time-lapse movie of the regeneration of stem segments from Tubularia.[1] In these stem segments, one sees a movement of the tissue toward the distal end of the stems, but no evidence of peristalsis; nor is peristalsis evident in the hydranths which form. This suggests that peristaltic waves may not be universal in the hydroids.

Other types of rhythmic movements have been observed in the hydroids. Hyman (1940, p. 493) describes the feeding behavior of a solitary tubularian hydroid, Corymorpha. "The stalk bends over, mouth and distal tentacles are touched to the mud, the stalk then straightens, and food material adhering to the tentacles is conveyed to the mouth. Decapitated stalks will carry on this rhythmic bowing." The bowing is said to be repeated in quiet water about 20 times an hour. Hydrozoan medusae swim by rhythmic contractions, but these are rapid and usually a burst of such pulsations is followed by a period of rest (Hyman, 1940, p. 494).

One wonders about the function of peristalsis in an intact Cordylophora colony. Allman (1871, p. 130 ff.) believed most of the circulation in a hydroid colony to be carried out by the flagella which line the coelenteron, and this view is generally held today (Buchsbaum, 1948; Hyman, 1940). The flagella are active in Cordylophora, and can be seen to tumble solid debris about in the coelenteron. However, there can be little doubt that the directed peristalsis is also a profound factor in moving things about; perhaps its effects are greater than those of the flagella in moving the coelenteron fluid over long distances. If charcoal (embedded in gelatin and fed to hydranths with proline as adjuvant) is fed to a single hydranth of a small colony, the charcoal can be found in the endoderm cells of all the hydranths of the colony within two to four hours. As a Tubularia (Cohen, 1952), charcoal is taken up by the endodermal cells of the hydranth but not of the coenosarc. It was impossible to ascertain, however, whether the charcoal was distributed by

---

[1] Sears Crowell has kindly shown the author Walp and Hammett's film, which confirms the similarity of the peristalsis in Campanularia to that in Cordylophora. Malcolm Steinberg was also kind enough to show the author his film of Tubularia regeneration repeatedly.

peristalsis or flagellar motion or, most likely, by both.

The presence of peristalsis in Cordylophora but apparently not in the larger Tubularia may find its explanation in the construction of the coenosarc tube in the two organisms. In Cordylophora, the tube is a hollow cylinder, whereas in Tubularia one or more endodermal ridges or septa tend to partition the coelenteron. In Tubularia the motion of the flagella carries the coelenteron fluid up one of the canals formed by the ridges and down another (Allman, 1871, p. 131). This results in an organized circulation of the fluid throughout the colony. In contrast, in Cordylophora there is but a single channel, and it is possible that the proximally-directed peristalsis may to some extent be counteracted by a distally-directed beating of the flagella.

The role of the rhythmic movements in reconstitution is unknown. As has been suggested the movements may be similar to the peristalsis in intact colonies, and the gradual polarization may be a manifestation of the development of polarity in the tissue fragments. Hauschka (1944) used such tissue fragments in Campanularia on the belief that the peristalsis which occurs in the stems did not occur in these, and claimed that this showed that the development of a hydranth did not require these movements. However, it remains to be determined whether or not his tissue fragments did develop movements such as those observed in Cordylophora.

Chapter VII

Asexual Development of a Hydroid Colony

Medawar (1945) has emphasized that of the three parameters of growth -- size, shape and age -- only size and age can be expressed numerically. "We shall never be able to say that the shape of a fish is 'six' or 'seven' units; shape can only be expressed as a relationship between numbers.

This chapter represents a developmental analysis of shape in a Cordylophora colony, and forms a prelude to a more analytical study of colony formation. The chapter is divided into four parts: (1) a description of the shape of colonies; (2) a study of the relative rates of growth of parts of colonies; (3) a formalization of the development of a colony; and (4) an examination of colony development based on the formalization. Many aspects of this study are as yet incomplete.

METHODS

In general, only a single set of conditions was used in these experiments, to avoid the possible influences on colony pattern of variations in environment. These conditions have been detailed in Chapter III. Cordylophor clone A was grown in CCS5 or CVD on microscope slides slanted in 100 ml. beakers. The cultures were fed once daily to repletion, with a second culture solution change several hours later. Where possible, the cultures were maintained in the dark at 22°C, although of necessity the time-lapse studies were done at room temperature (22 to 26°C) in the light. As has been shown (Chapter IV), this change has little or no influence on the growth of the colonies.

The time-lapse movies of growth were taken with the horizontal apparatu described in Chapter VI. Since it was desirable not to move the cultures during the several days while their growth was being photographed, the culture solution was changed by flow through the chamber.

The rate of increase in length of a part with time was determined from the movies by projecting them on graph paper at a constant speed, and plotting the length of the part at regular intervals (usually every 10 seconds). From these plots, the actual dimensions and time intervals were calculated.

The measurements of parts of colonies were made with a dissecting microscope equipped with an ocular micrometer. Some of the more useful measurements referred to below are indicated in Figure 25. All measurements have bee converted to millimeters, and are accurate to the nearest 0.1 mm.

It was found that the dye trypan blue (diamine blue "B") stained the

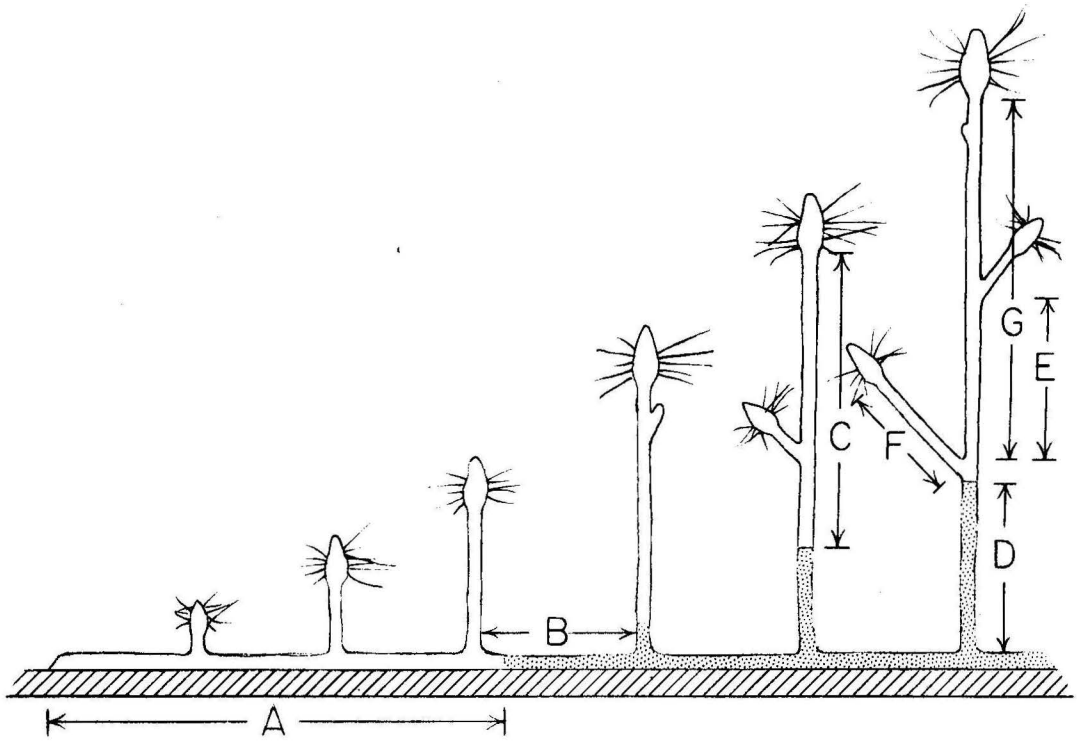


Figure 25. Diagram of a portion of a Cordylophora colony indicating the basic pattern and the kinds of measurements possible. The stippled area represents the portion of the colony marked with trypan blue. The distances indicated are:

A Length of new stolon; growth of stolon
B Interupright distance
C Length of new upright; growth of upright
D Length of old upright; distance to first branch
E Interbranch distance
F Length of side branch
G Length of upright above side branch F

perisarc dark blue without staining the tissue. Colonies grew in trypan blue at an undiminished rate. This provided a very convenient measure for following the growth of colonies; an entire colony could be permanently marked by staining with trypan blue, and then allowed to continue to grow, and the new growth readily separated from the old (Figure 26). To mark colonies, they were exposed to trypan blue in CCS5 for about 12 hours at a concentration of 5 µg. per ml.

Time-lapse movies and trypan blue marking provide excellent alternate procedures for following the development of colonies, but they are not interchageable. With time-lapse, one can accurately determine rates of growth, but the work progresses very slowly (i.e., only about one growth rate can be determined from a movie), not all of the movies are suitable for measurement (the part being followed must grow in a plane parallel to the film), and only a small part of a colony can be followed at a time. The trypan blue marking suffers from none of these drawbacks -- several colonies can be followed simultaneously, and the relative growth of all parts of a colony can be determined. But the marking does not lend itself to accurate determination of rates, and it has not been established that exposure to trypan blue does not slightly alter the ensuing developmental sequence.

RESULTS

I. The pattern of Cordylophora colonies

These observations are in part the result of detailed study of a few colonies, but in larger part represent the cumulative observations made while following the growth of several hundred colonies. The observations are an attempt to elucidate the regular and the erratic aspects of colony pattern. Many of the observations pose questions for future experimental attack.

Stolons

Stolons tend to grow in a more or less straight line along the substratum. As indicated by trypan blue marking and other observations, growth takes place at the tip in the sense that no new perisarc is added behind that region. The distance between two uprights, once they have formed, also remains constant. The stolon is not perfectly cylindrical, but flattened someon the side of the substratum. The area near the growing tip is always of a

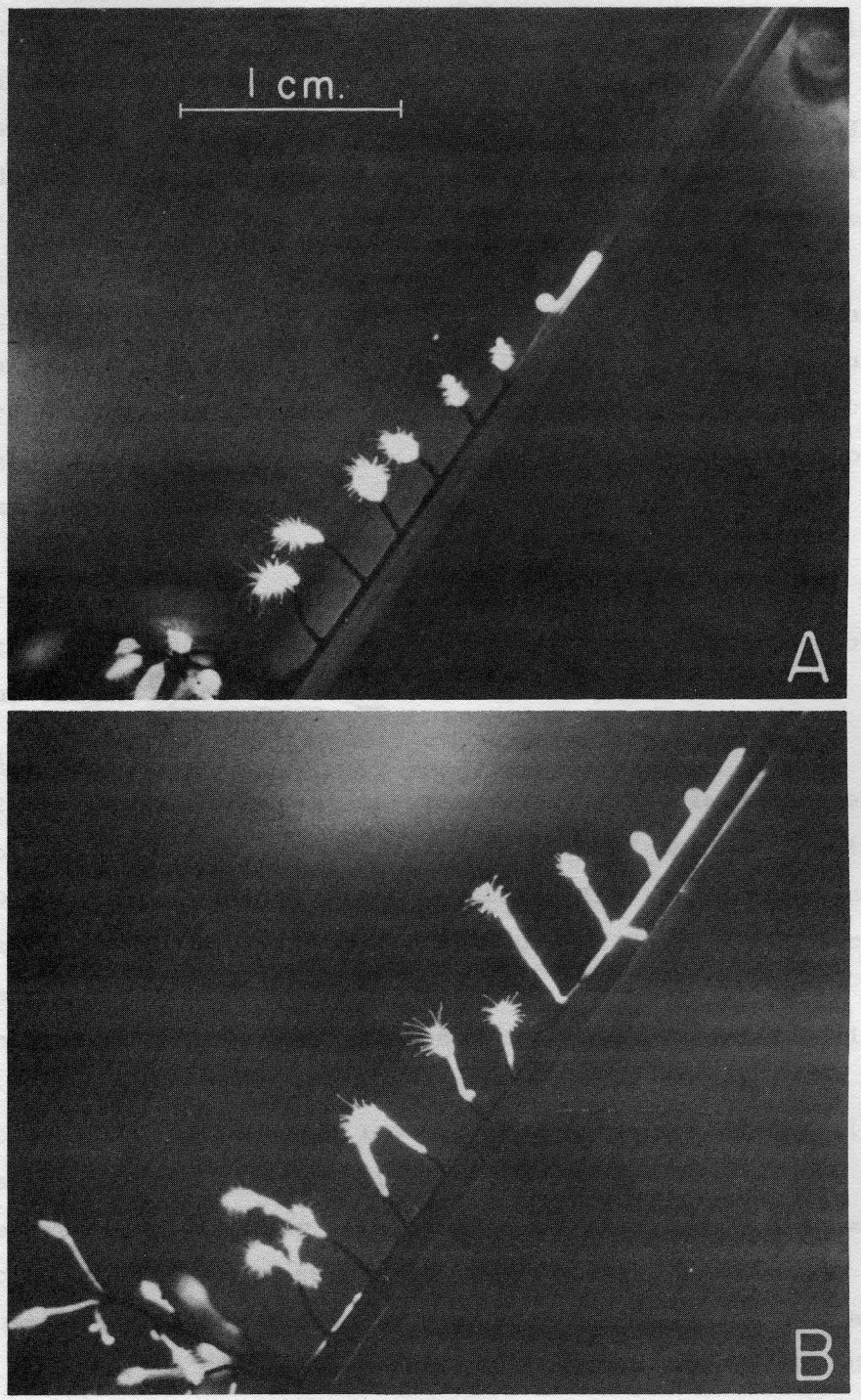


Figure 26. Portion of a Cordylophora colony marked with trypan blue. The colony is shown at the time of marking (A), and again after two days of growth (B).

slightly larger diameter than the older parts of the stolon, indicating that the perisarc shrinks as it ages.

As a stolon increases in length, uprights appear at fairly regular intervals, as discussed below. The bulge in the stolon which will give rise to an upright first makes its appearance after the stolon tip has advanced about one millimeter beyond that point.

In addition to uprights, stolons also produce secondary stolons. These develop at erratic distances along a stolon, usually well behind the tip. They represent the only asexual development observed in these studies where the new growth appears away from a growing tip, i.e., through old perisarc. In many cases, secondary stolons make their appearance at the base of uprights.

Both uprights and secondary stolons tend to leave a stolon at a right angle. On contact, stolons never fuse, but simply cross over one another, unlike the situation in Hydractinia (cf. Hauenschild, 1954).

Interupright distance

The distance between adjacent uprights on a stolon is quite regular, though not a constant. The average distance is about 3 mm., with extremes from 1.0 to 6.0 mm. having been observed. Schulze (1871), who made a careful study of the pattern of colonies growing in nature at Rostock, Germany, reported an average interupright distance of 3 mm.

The interupright distance appears not to be particularly sensitive to the conditions of culture. The only condition observed to result in significant alterations of the distance is low oxygen tension (cf. Table 21). Under low oxygen tension, the interupright distance averaged 4.7 mm., with extremes to 6.9 mm. Whether this bears any relationship to the factors determining the normal spacing of uprights requires further study.

It frequently happens that two or more stolons come together and grow in contact and parallel to one another for considerable distances. Under these circumstances, the distance between uprights is not affected, and the uprights on the separate stolons make their appearance independently. This observation indicates that if internally produced factors influence interupright spacing, these factors do not pour out into the surrounding environment in sufficient concentration to influence the development of uprights on immediately adjacent stolons.

Uprights

As mentioned above, uprights arise perpendicular to the stolons, just behind the stolon tip. This angle is not in any way influenced by the angle of the culture, but appears to be determined internally by forces acting upon the upright as it leaves the stolon.

Very soon after the appearance of an upright bud, it develops a hydrant at its apex. This terminal hydranth grows in size, and the upright begins to increase in length. As in the case of stolons, new perisarc is added only directly behind the hydranths.

As the upright grows, it develops side branches. These branches leave the upright at about a 45 degree angle, away from the stolon, as observed by Schulze (1871). Once they leave the uprights, the developmental sequence of a side branch follows that of an upright -- it develops a side hydranth, grows in length, and develops secondary or tertiary branches at 45 degree angles.

The side branches first make their appearance directly behind the hydranth, and as in the case of the development of uprights from stolons, new branches never make their appearance behind this region.

The distance between branches is less regular than the interupright distance. In particular, the length above a stolon where the first branch leaves an upright is irregular. Further branches from an upright tend to be spaced about 3 mm. apart. Branches tend to appear on alternate sides of an upright, but this is not always the case.

Integrated view

During the study of environmental factors influencing the growth rate of Cordylophora colonies, the patterns of colonies growing under many differer conditions were examined. In most cases the environmental factors were found not to significantly influence the pattern of the colonies. For example, consider the observations made during a temperature experiment (Table 33). The range of temperatures employed (18 to 26°C) did not significantly influenc growth rate. The number of stolons present was erratic, and not a function of any other measurable parameter of the colony (cf. stolon to growth rate ratios in Table 33). The interupright distance varied around a mean value of 3.2 mm. (based on 60 measurements), and was not significantly altered by the temperature.

One particular useful index is the hydranth to upright ration (H/U). This ratio is an index of the amount of branching in a colony. If H/U equals

TABLE 33

Parameters of Cordylophora colony growth and pattern with temperature as the major variable

Data from the experiment reported in Table 18 of Chapter IV.

Temp. °C	Final no. of			Growth rate (k)	Ratios		Mean IUD in mm.
	H	U	S		H/U	S/k	
			Group A: secondary colonies				
18	27	11	1	0.15	2.4	7	3.5(1)
20	44	22	7	0.26	2.0	26	3.2(6)
22	44	17	2	0.22	2.6	9	3.2(6)
24	38	11	1	0.24	3.5	4	(0)
26	23	16	2	0.18	1.4	10	3.3(9)
			Group B: cut back older colonies				
18	40	27	6	0.29	1.5	20	3.6(8)
20	26	17	2	0.27	1.5	7	3.2(9)
22	40	21	6	0.29	1.9	21	3.4(6)
24	35	19	5	0.27	1.8	18	3.2(7)
26	33	18	3	0.30	1.8	10	2.6(8)

Abbreviations: H = hydranth S = stolon
U = upright k = growth rate
IUD = interupright distance

one, then there are no side branches; each upright bears only a single, terminal hydranth. As indicated by the data in Table 33, the H/U ratio tends to be a fairly constant value under a given set of experimental conditions. Further, if the interupright and interbranch distances are not affected by the conditions, then the H/U ratio is also a measure of the relative amounts of upright and stolon tissue in a colony. As stolon length increases relative to upright length the H/U ratio approaches a value of one.

As mentioned in Chapter IV, cut back older colonies tend to grow at a slightly greater rate than secondary colonies. In addition to this difference, it has been observed (Table 33) that the cut back older colonies also tend to have a lower H/U ratio and more stolon tissue than secondary colonies. Cut back older colonies have been used in many of the experiments to be described below.

Conclusions

The shape of a Cordylophora colony may be considered to be the result of the patterning of a series of tubes. The tubes are of two types: stem tubes, which bear hydranths at their apices, and stolon tubes. Stolon tubes develop side tubes of two types: secondary stolon tubes and stem tubes (uprights), both of which leave the primary tube at right angles (but at planes perpendicular to one another). The uprights bear secondary stem tubes, or side branches, which leave at about a 45 degree angle. Tubes which bear hydranths always develop directly behind tips, whereas stolon tubes develop at variable intervals away from tips. The spacing of stem tubes is fairly regu being about 3 mm., whereas the spacing of secondary stolon tubes is erratic.

Without undue oversimplification, one may view the pattern of an asexual Cordylophora colony as the result of the (1) relative growth, (2) spacing, and (3) angles of two types of tubes of uniform diameter.

II. Relative growth rates

In view of this, it is important to know the relative rates of growth of the various parts of a colony. Since the tubes of interest (stems and stolons) are of essentially constant diameter, growth rate may be determined by plotting increase in length with time. The information for such plots has been obtained from time-lapse movies.

Stolons

Time-lapse studies have shown that stolons increase in length at a

constant rate (Figure 27). The growth rate of the stolon shown, which may be calculated from the slope of the curve, is 0.12 mm. per hour. Determination of the slope for each pair of points gives extreme values of 0.07 and 0.18 mm. per hour, with a mean of 0.123 mm. per hour. Thus the curve shows almost no tendency to leave linearity. There is a slight tendency on three of the four days for the rate of stolon growth to increase slightly after feeding, and to gradually decrease in rate until the next feeding, but further study is required to determine the significance of this observation.

Uprights

Uprights also increase in length at a constant rate, though usually more slowly than stolons (Figure 28). In the case shown, the mean rate of growth is 0.038 mm. per hour. All determinations of the growth rate of uprights have been made after the uprights reached a height of at least 1.5 mm.; for reasons to be described below it has become important to determine the growth rate of newly developed uprights.

The growth rate of branches has not yet been determined, and it would be difficult to obtain accurate values. For the purposes of the discussion to follow, however, side branches will also be assumed to increase in length at a constant rate.

Growth zones

For these studies of asexual colony development, it is important to know in what parts of a colony growth takes place. Are there localized region of cell division (meristematic regions) as in plants, or is cell division occurring throughout the tissue? There is no indication that cell enlargement plays a significant role in the increase in length of stolons or stems, so that in seeking growth zones one is seeking regions of cell division.

Attempts have been made to visualize cell division in Cordylophora with staining and autoradiography, but as yet these attempts have been unsuccessful Mitoses have been seen in Cordylophora embryos by Morgenstern (1901) and in adult hydra by McConnell (1933), but this author has not been able to detect mitoses in adult Cordylophora cytologically, even with Feulgen stain. Autoradiography was done using tritiated thymidine (Fitzgerald, et al., 1953; Cronkite, et al., 1959), with exposure of growing colonies to 5 μc. per ml. for up to 12 hours. The Vorticella and other organisms attached to the perisa of Cordylophora incorporated the isotope and gave intense autoradiograms, wher

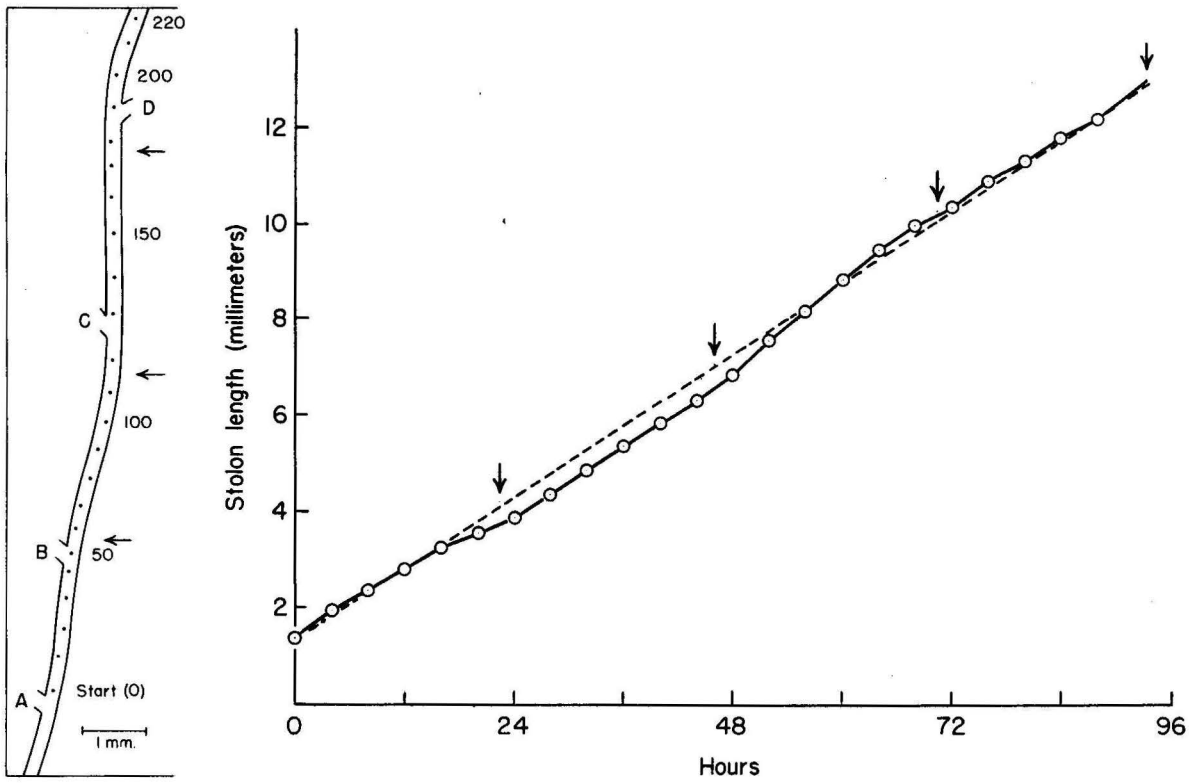


Figure 27. Linear growth of a stolon. On the left the plot made from the time-lapse movie is depicted; each dot indicates the tip of the stolon at 10 second intervals. The letters indicate the positions along the stolon at which uprights developed. On the right the length of the stolon is plotted against time. The arrows indicate the time of feeding.

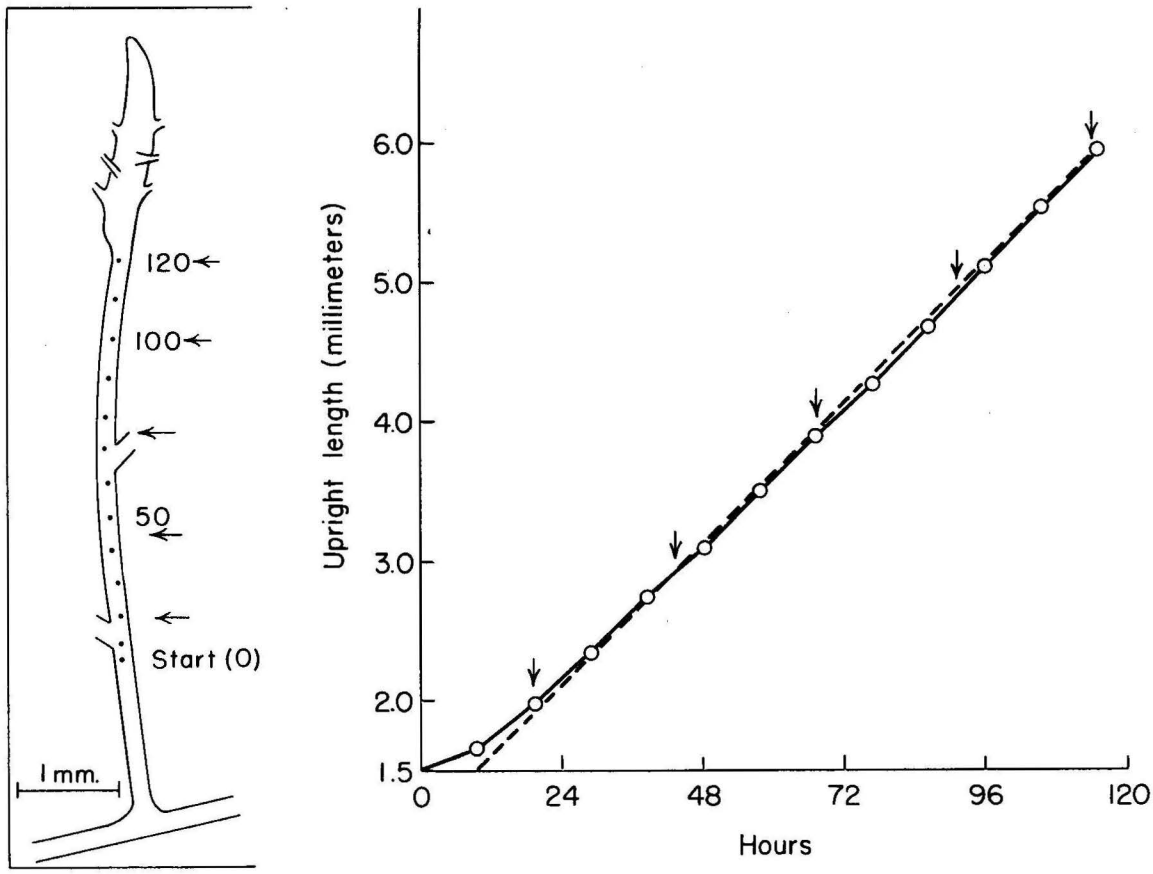


Figure 28. Linear growth of an upright. On the left the plot made from the time-lapse movie is depicted, with each dot on the stem indicating the position of the base of the hydranth at 10 second intervals. The side branches which developed are indicated. On the left the length of the upright is plotted against time. The arrows indicate the time of feeding.

no silver grains developed in the emulsion above the Cordylophora tissue. During the twelve hours of exposure to isotope, stolons should have increased in length about one mm. and uprights about 0.4 mm. There are two possible explanations of the lack of evident incorporation of the tritiated thymidine into Cordylophora nuclei. The organism may not incorporate thymidine, but this possibility is rendered less likely by the fact that H^3-thymidine has been used with a diversity of organisms under many conditions, and has always been found to be incorporated into dividing cells. The other possibility is that the nuclei of Cordylophora have relatively little deoxyribosenucleic acid per unit area. This is supported by the observations that Amoeba (Plaut and Sagan, 1958), Acetabularia (Brachet, 1959), and Spirogyra (Stocking and Gifford, 1959) all incorporate tritiated thymidine but fail to give nuclear autoradiograms. In these organisms it is also difficult to demonstrate Feulgen positive material in the nucleus.

There is little information on growth zones in the literature on hydroids. Kühn (1909) made a comparative study of the pattern of hydroid colonies, and from this study deduced where the growth zones would be, but contrary to the statements and figures of Bonner (1952) and Hyman (1940), he provided no evidence that his deductions were correct. Berrill (1949 et seq.) and Crowell (1957) also discuss growth zones as deduced from colony pattern. The only evidence for a meristematic region comes from the work of Brien (1953 and more recently Burnett (1959) and Burnett and Garofalo (1960), who provide evidence that new tissue develops in the region of the hydranth of hydra and moves proximally. This evidence, based on staining and grafting experiments fails to give critical indication of actual meristems or zones of cell divisio

In Cordylophora, the linear growth of stolons and stems would favor a localized region of cell division of unchanging size. It is possible, however to conceive of linear growth resulting from non-localized cell proliferation which continually decreases in rate as the structure increases in length. But the solution of this problem must await further study.

Growth of entire colonies

The demonstration that the tubes comprising a colony grow at a constant rate poses a problem, since it has already been shown (Chapter III) that the number of hydranths in a colony increases at an essentially exponential rate. This exponential growth has been used to determine growth rate under a wide variety of environmental conditions (Chapter IV), and has shown itself to be a powerful tool in such analyses.

The hyphae of fungi also increase in length only at their tips and at a constant rate (Smith, 1923). Thus the growth of a fungal colony is a function not of the total number of cells but rather of the number of hyphal tips and the rate at which nutrients are supplied to these tips, and for this reason fungal colonies tend to increase in size at a more or less constant rate (Cochrane, 1958). The rate of extension of Neurospora in a tube is perfectly linear (Ryan, Beadle and Tatum, 1943), and on an agar surface the radius of a colony increases at a constant rate. In liquid culture, the radius of a spherical Neurospora colony also increases at a constant rate, and because of this, as Emerson (1950) has shown, the cube root of the dry weight also increases at a constant rate with time.

In view of such considerations, it is important to determine if the exponential increase in hydranth number in a Cordylophora colony represents an exponential increase in the mass of the colonies, or if instead it represents an exponential subdivision of the mass of the colonies while the matter increases at some other rate (e.g., a constant rate). The mass is usually measured as dry weight, which of course can only be measured once for a given colony. Because of variability in the growth rate (increase in hydranth number with time), serial sacrifice of separate colonies for dry weight determinations might give erroneous results. But if exponential increase in hydranth number is paralleled by exponential increase in dry weight, then the ratio of dry weight per hydranth should be a constant for colonies of varying hydranth number. Therefore a number of colonies were sacrificed, the hydranth number determined (by dissection in denser cultures), and the dry weight of each colon measured. The results are presented in Table 34. The dry weight per hydranth remains constant at about 50 µg. per hydranth over a range in hydranth number of almost 50 fold, from 12 to 554 hydranths. Thus the dry weight of the colonies also increases exponentially with time.

III. Formalization of colony development

The resolution of linear growth of parts into exponential growth of the whole proved to be relatively straightforward. A first approximation to the model, shown in Figure 29, is for purposes of simplicity based on the growth of a single stolon, and assumes no stolon branching. During each unit of time (ut), the stolon increases in length one unit, and produces one upright The upright grows at the same constant rate, and produces one side branch per

TABLE 34

Relationship of hydranth number to dry weight

Colonies growing under standard conditions were starved for 24 hours, the number of hydranths counted (in dense colonies by dissection), and the entire colony sacrificed, dried at 105 °C for 2 hours in a tared shell, and weighed. The dry weights are accurate to ± 0.02 mg.

Colony no.	No. of hydranths	Dry weight (mg.)	Micrograms per hydranth
290	12	0.49	41
297	18	0.89	49
288	19	0.76	40
298	20	1.07	53
271	66	2.98	45
269	75	3.84	51
275	77	4.54	59
282	77	3.76	49
276	118	7.73	66
272	146	7.95	54
241	554	38.06	61

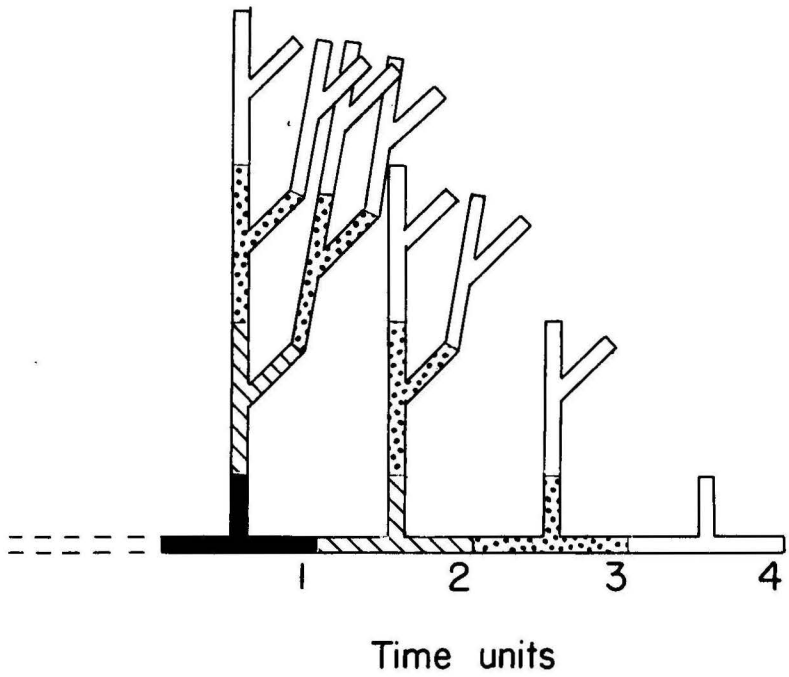


Figure 29. Model A. For description see text.

unit time. Similarly the branch grows one unit and produces one sub-branch per unit time. It follows from this model, in which each of the component tubes increases in length at a constant rate, that the number of growing tips increases exponentially at a rate 2^{ut}. Since one of these growing tips represents the stolon tip, which bears no hydranth, the number of hydranths increases exponentially at a rate $2^{ut} - 1$. Thus this model resolves the linear increase in length of tubes into exponential increase in hydranth number.

This model, which we shall call Model A, has two consequences for the pattern of Cordylophora colonies which are not supported by the observations. In the first place, the model is based on the assumption that stolons and uprights and branches all grow at the same constant rate, an assumption which is not supported by the time-lapse movies. One result of this equal growth rate of the different tubes is that the angle formed by the stolon and the tips of uprights, intersecting at the stolon tip, is a 45 degree angle (length of any upright equal to the length of the stolon beyond that upright; the tangent of the angle therefore equals one). The angles observed in colonies growing under standard conditions are considerably more acute.

A second consequence of the model is considerable branching, with tertiary branches on the oldest upright by the fourth unit of time (Figure 29). This branching is considerably more than is observed in the actual colonies.

Therefore Model A was modified to bring it more into line with the pattern of laboratory colonies; the result -- Model B -- is shown in Figure 30. Model B assumes that the relative growth rates of the different tubes have different values, but that the tubes still branch at constant distances from one another. These assumptions are borne out by the observations of colonies reported above. During each unit of time, the stolons are envisioned to increase in length four units and to produce one upright. Uprights grow two units per unit time, and produce side branches at the same space intervals as uprights are produced on the stolon, i.e., branches are produced linearly at $\frac{1}{2}$ per unit time. Finally, the branches themselves grow one unit per unit time and produce sub-branches linearly at $\frac{1}{4}$ per unit time. The consequences of Model B in terms of hydranth number are depicted in Figure 31 (Curve A), from which it may be seen that the increase in hydranth number with time continually falls away from exponential.

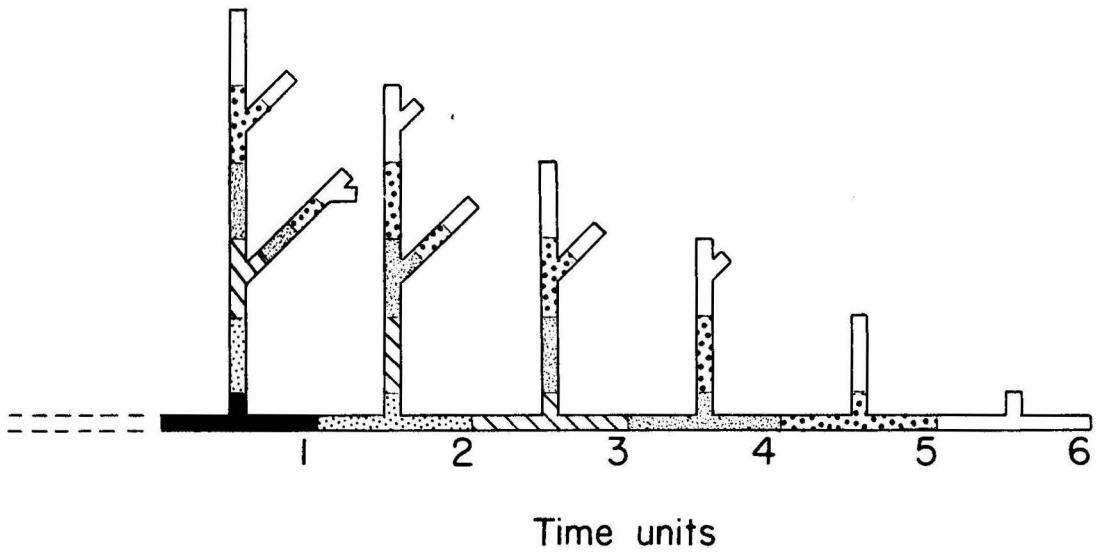


Figure 30. Model B. For description see text.

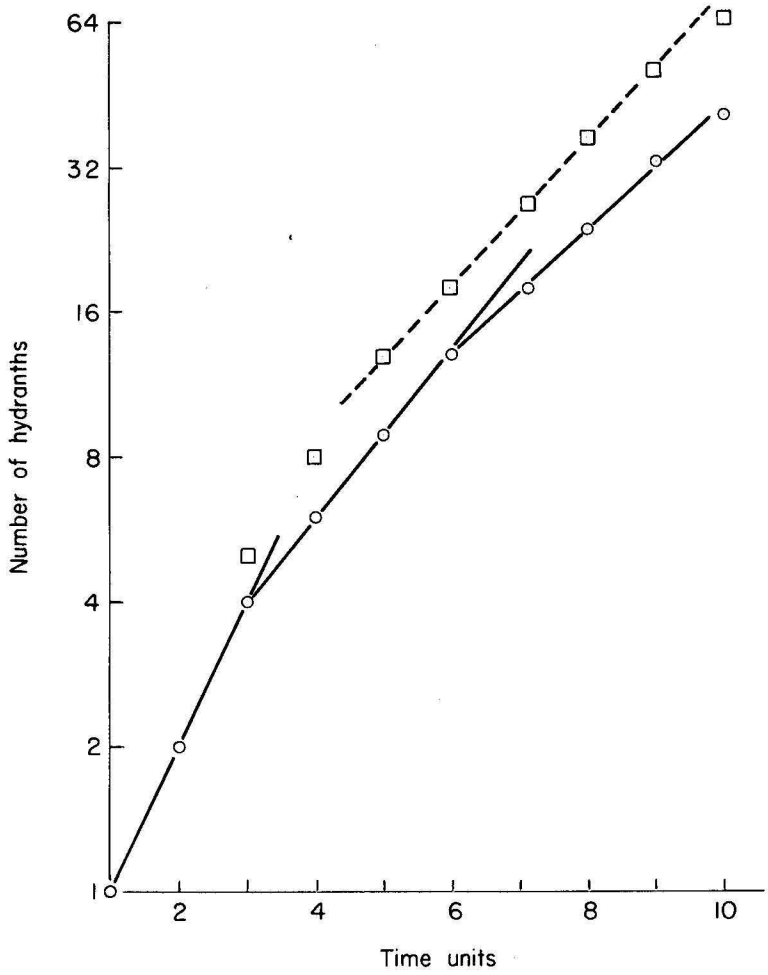


Figure 31. Curve A (solid line) depicts the growth curve predicted by Model B, while Curve B (dotted line) indicated the results of introducing a secondary stolon, as described in the text.

But on the other hand Model B provides a colony pattern which fairly closely parallels that seen in the laboratory, and the data on which the model is based (linear growth of parts, constant spacing of side tubes, essentially exponential growth of the whole) must be considered established.

Several considerations make possible the resolution of this difficulty While the model as stated does not give rise to perfectly exponential growth, during the usual range of a growth experiment (about 10 to 60 hydranths), the deviation of the growth curve predicted by the model (Figure 31) from exponential would not be striking.[1] Further, it is possible to introduce factors into Model B which yield growth more closely approximating exponential. For example, assume that the colony of Model B (Figure 30) gives off a secondary stolon during the second unit of time. This would not, presumably, influence the further development of the colony as shown in Figure 30, but would simply introduce a second growing stolon. The results of this factor would be:

Unit time	1	2	3	4	5	6	7	8	9	10
No. of hydranths (1° stolon)	1	2	4	6	9	13	18	24	33	41
No. of hydranths (2° stolon)			1	2	4	6	9	13	18	24
No. of hydranths (total)	1	2	5	8	13	18	27	37	51	65.

As shown in Figure 31 (Curve B), the addition of this one factor obliterates the tendency of the growth curve to fall away from exponential in the range where growth is normally followed. The critical point is that the developing colonies regularly introduce just such factors (e.g., consider the number of stolons per colony in Table 33).

IV. Examination of colony development

The growth rates and spacing of the tubes comprising a colony have been described, and a model developed to resolve the linear growth of parts into exponential growth of the whole. These considerations permit the intelligent examination of the development of shape in individual colonies.

---

[1] In Chapter III, data were presented which indicated that the average growth curve deviates somewhat from ideality, resulting in uncertainty in repeated interpolation of doubling time from the same growth curve of about a tenth of a day. No evidence has been found, however, that the deviations from ideality follow the growth curve predicted by Model B. In fact, many growth curves have been followed from a hydranth number of one, and such growth curve can be extrapolated back to one hydranth -- a result not predicted by Model B (Figure 31).

Colony no. 267 has been selected for detailed presentation because its pattern is relatively simple. The colony was a cut back older colony, which was marked with trypan blue and then grown for an additional 4.3 days under standard conditions. Part of the colony is illustrated in Figure 32. After the period of growth, all parts of the colony were measured.

During the 4.3 days after the colony was marked, the number of hydranth increased from 19 to 58, giving a doubling time of 2.6 days and a growth rate, k, of 0.27. The number of uprights increased from 19 to 40, and the final hydranth to upright ratio is 1.5. All side branches developed on uprights which were present at the time of marking. There were 7 stolon tips present at the beginning of the experiment, and by chance no new stolons developed. These results all correspond well to those obtained with other cut back older colonies (cf. Table 33).

A portion of the measurements of Colony no. 267 are given in Table 35. Each stolon tip and each upright were given a number, and from the measurements and drawings of the colony two-dimensional maps of the colony were prepared. The map of the stolons, showing the position of the uprights, is given as Figure 33. The new perisarc which develops can readily be distinguished from that present at the beginning of the experiment because of the trypan blue marking. From the data given in Table 35, the mean interupright distance were calculated. For the old parts of the colony, the interupright distance had a mean of 3.32 mm., while for the new parts the mean was 2.95 mm. The mean for the colony is 3.14 mm. on the basis of 33 measurements.

The seven stolons grew an average of 10.5 mm., with variation from 4.9 to 14.2 mm. (Table 36). The mean growth rate is 0.10 mm. per hour.

A map of the uprights and branches coming from five of the seven stolon of the colony is shown in Figure 34. This figure and Figure 33, provide a scale diagram of the entire colony with the exception of seven uprights. The data from which the map of the uprights was constructed (cf. Table 35) permit the computation of several aspects of upright development, all of which are shown graphically in Figure 34.

In the first place, the distances between branches as well as the distance between the first branch and the stolon (cf. Figure 25) are relatively erratic compared to interupright distances. The distance from the first branc to the stolon (Figure 25D) varies from as little as 1.8 to as much as 11.9 mm.

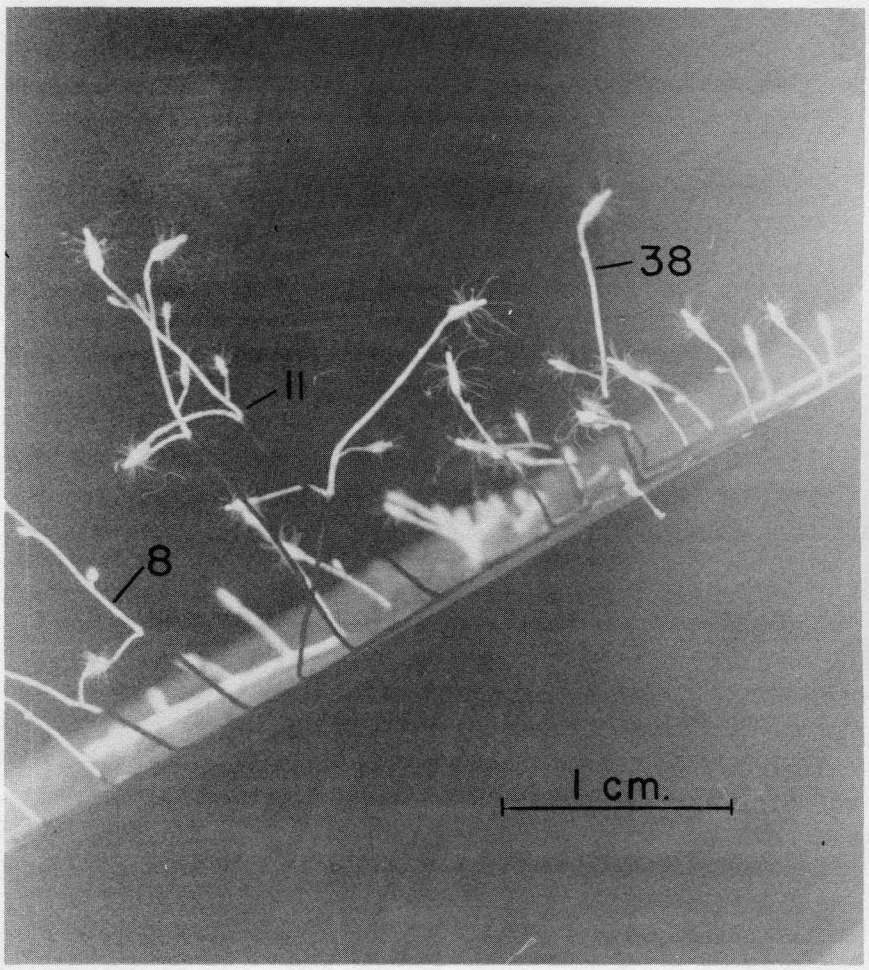


Figure 32. Colony no. 267, viewed from the left side as mapped in Figure 33. Three uprights have been numbered to correspond with Figures 33 and 34.

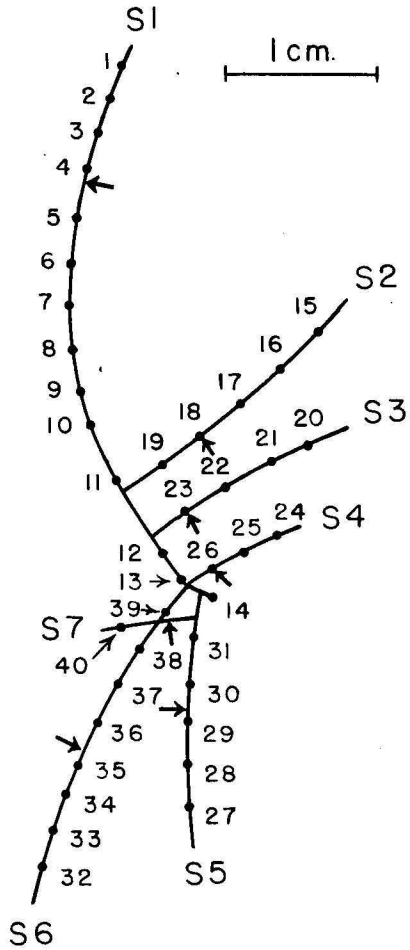


Figure 33. Scale map of the stolons of Colony no. 267. The positions of the uprights are marked and numbered. The heavy arrows indicate the points to which the perisarc was marked with trypan blue.

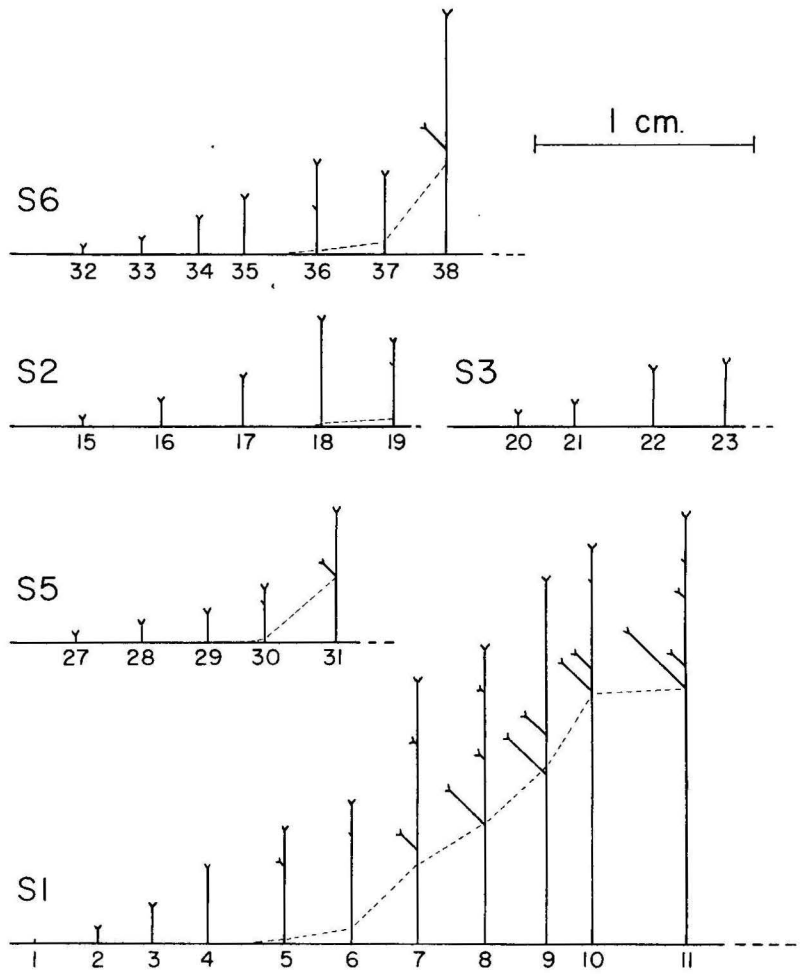


Figure 34. Scale map of the uprights of Colony no. 267. The uprights occurring on five of the seven stolons are shown; the numbers correspond to those in Figure 33. The dotted lines indicate the limits of the trypan blue marking.

TABLE 35

Measurements of spacing in Colony no. 267

The experimental procedure is described in the text. The numbers of the uprights are the same as used in Figures 33 and 34. All measurements are in mm.

Upright no.	Interupright distance[1]	Upright length[2]		Distance to first branch[3]
		pre TB	post TB	
1	(s 1.1)	---	bud	---
2	2.9	---	0.6	---
3	2.5	---	1.7	---
4	2.5	---	3.5	---
5	3.5	0.2	5.0	3.7
6	3.1	0.7	5.7	5.0
7	3.0	3.7	8.4	4.4
8	3.1	5.6	8.0	5.6
9	2.8	8.2	8.6	7.8
10	2.3	11.6	6.7	11.7
11	4.3	11.9	7.8	11.9
12	6.0	4.0	2.6	---
13	2.1	3.0	2.3	3.7
14	2.3	aberrant		---
15	(s 3.1)	---	0.3	---
16	3.6	---	1.1	---
17	3.7	---	2.2	---
18	3.6	0.1	4.8	---
19	3.3	0.3	3.6	2.8
20	(s 3.2)	---	0.5	---
21	2.6	---	1.0	---
22	3.6	---	2.6	---
23	3.3	---	2.9	---
24	(s 1.8)	---	0.3	---
25	2.6	---	0.7	---
26	2.4	---	0.8	---
27	(s 3.0)	---	0.3	---
28	3.0	---	0.8	---
29	3.0	---	1.3	---
30	2.6	0.1	2.3	1.8
31	3.3	3.0	3.0	3.1
32	(s 3.3)	---	0.3	---
33	2.7	---	0.6	---
34	2.6	---	1.6	---
35	2.1	---	2.5	---
36	3.3	0.1	4.0	2.0
37	3.1	0.6	3.0	---
38	2.8	4.1	7.8	4.8
39	3.1	2.0	1.8	---
40	(s 1.4)	---	0.3	---

[1] Figure 25 (B). Distance to preceeding upright; numbers in parentheses indicate distance from upright to tip of stolon.

[2] Distance present at time of trypan blue marking (pre TB), Figure 25 (D); increase after labelling (post TB), Figure 25 (C).

TABLE 36

Stolon growth in Colony no. 267

The growth represents the length added to the stolon during the 105 hours of the experiment, measured as in Figure 25 (A).

Stolon no.	Increase in length (mm.)	Growth rate (mm./hr.)
S1	10.9	0.104
2	14.2	0.135
3	12.9	0.122
4	6.9	0.066
5	11.0	0.105
6	12.7	0.121
7	4.9	0.046
mean	10.5	0.10

TABLE 37

Relative growth of first branches in Colony no. 267

The lengths given were determined as indicated in Figure 25 (F and G). The ratio of the two values allows an approximation of the relative rates of growth.

Upright no.	Length in mm. of: First branch	Upright distal to branch	Ratio: branch / upright
5	0.1	1.5	0.067
6	bud	1.4	-----
7	1.0	7.7	0.130
8	2.1	8.0	0.262
9	2.4	9.0	0.266
10	1.8	6.6	0.273
11	3.4	7.8	0.436
13	0.3	1.6	0.188
19	bud	1.1	-----
30	bud	0.6	-----
31	0.7	2.9	0.241
36	bud	2.1	-----
38	1.3	7.1	0.183
mean			0.227

with the mean of 13 measurements being 5.3 mm. There is one disturbing feature a tendency of the first branches in longer uprights to appear immediately after trypan blue treatment (Figures 32, 34). This result has been observed in other colonies marked with trypan blue, as has the tendency, also shown in the figures, of the second branch of an upright to appear within a short distance (as little as 0.9 mm.) of the first.

The growth of uprights is more regular than their branching. During the experiment, the increase in length of 15 uprights which were present at the start of the experiment averaged 5.25 mm., giving a rate of 0.05 mm. per hour. Thus the growth rate of uprights is half the rate of stolons.

If the uprights and stolons grow at the same rate, as in Model A, they form a 45 degree angle at the stolon tip. With Colony no. 267, where the uprights grew at half the rate of stolons, the tangent of the angle formed would be 0.50, or an angle of about 26 degrees. Thus one should be able to determine the relative rates of growth in colonies simply by measuring this angle. This may not always be feasible, however, as illustrated by the drawings of Figure 34. In none of the stolons shown can a straight line be drawn through the lengths of the uprights; instead the curve tends to bend upward, suggesting that newly formed uprights do not grow as rapidly as older uprights. This is not unreasonable, and the simplest explanation is that after an upright bud makes its appearance, it must first develop a hydranth, and then that hydranth must grow to adult size before the upright begins to increase in length at a constant rate.

It is clear from the measurements taken and mapped in Figure 34 that the relative growth of branches is still less than that of uprights. The ratio of the length of a branch to the length of the upright above that branch (Figure 25, F/G) gives and index of the relative growth rates of the two tubes. For the 13 first branches listed in Table 37 this index has a mean value of 0.23, indicating that branches grow only about one-fourth as rapidly as their parent uprights.

The quantitative description of the shape of Colony no. 267 is compared with that of another colony from the same experiment (Table 38). It is apparen that the values obtained for the two colonies agree fairly well.

TABLE 38

Parameters of size and shape in two Cordylophora colonies

Two cut back older colonies were grown 105 hours after marking with trypan blue. Numbers in parentheses indicate the number of measurements from which the mean was calculated.

Colony number	267	281
Increase in hydranth no.	19 to 58	28 to 62
Growth rate (k)	0.27	0.19
Final no. of stolon tips	7	4
Final no. of uprights	40	24
Hydranth-upright ratio	1.5	2.6
Interupright distance (mm.)	3.14 (33)	2.50 (21)
Mean growth of stolons (mm.)	10.5 (7)	9.45 (5)
Mean stolon growth rate (mm./hr.)	0.10	0.090
Mean growth of uprights present at start of experiment (mm.)	5.25 (15)	6.29 (12)
Mean upright growth rate (mm./hr.)	0.050	0.059
Upright-stolon growth ratio	0.50	0.66
Branch-upright growth ratio	0.23 (9)	0.20 (11)

V. Conclusions

We can now formulate a preliminary descriptive picture of the development of shape in a Cordylophora colony. A colony depicted as a series of tubes of uniform diameter, and the shape of such a colony to result from the relative rates of growth of the tubes with respect to one another, the intervals at which the tubes branch, and the angles at which the tubes leave one another. On the basis of the observations, there are three classes of tubes, stolon tubes and hydranth-bearing stem tubes of two types. All of these tubes increase in length at a constant rate; their special properties may be conveniently tabulated:

Tube	Source	Relative growth rate	Distance between	Angle
Stolon	Stolon	1	erratic	90°, along substratum
Upright	Stolon	½	3 mm.	90°, away from substratum
Branch	Upright	1/8	ca. 3 mm.	45°, away from substratum.

This colony pattern tends to closely approximate that predicted by Model B (Figure 30), though it is clear that there is too much variability in the colonies to permit useful mathematical formulation of the development of shape in a colony. Model B may thus be considered a formalization of the actual development of Cordylophora colonies.

The relationship between the developmental pattern and exponential increase in hydranth number with time has also been clarified. It is clear that the phrase essentially exponential growth as used in Chapter III is a necessary one, since if the formal presentation of development as Model B is a correct interpretation of the data, the increase in hydranth number in a Cordylophora colony cannot be considered to be perfectly exponential as in the case of microbial and hydra cultures (Monod, 1942; Loomis, 1954). Instead, Model B predicts a growth curve which continually falls away from exponential, though the organism can introduce factors to recover the exponential curve (Figure 31). It is clear, however, that within the countable range of hydranth number, the essentially exponential growth predicted by Model B provides a suitable analytical tool as employed in Chapters III and IV.

DISCUSSION

The development of shape in a Cordylophora colony has been clarified. The study has revealed that the development of a colony which at first glance

appears to be a forest of little trees (Figures 4, 32) occurs in an orderly and regular fashion: the growth and ramification of a series of tubes.

Few systems offer such simplicity. The complex changes in the geometry of man and other metazoa during development (Medawar, 1945; Thompson, 1943) offer little promise for analysis with present tools. The fungi, many of which have a pattern of development superficially similar to that of Cordylophora, have as yet received very little attention from that point of view. Perhaps the closest parallel in recent years to the present study are the studies of Erickson (cf. 1959) on the integrated development of higher plants.

Colonial hydroids have been the object of many careful observations by Berrill (e.g., 1949, 1952, 1953). Unfortunately, his observations on the asexual development of these organisms have all been made with material brought in from nature, and while his inferences about the dynamics of development are suggestive they require laboratory analysis.

All of the present studies have been made under one set of culture conditions. Kinne (1956, 1958a,b) has greatly extended Roch's observation that the shape of Cordylophora colonies can be profoundly influenced by salinity.[2] Colonies grown in water of moderate salinity (ca. 50 per cent sea water) were found to be taller than colonies grown in fresh water. At the end of the experiments, the total lengths of stems and stolons were measured. In fresh water, the stolon length was found to account for 76 per cent of the total length of the tubes, whereas in 50 per cent sea water the stolons only accounted for 16 per cent.

Using a very different hydroid, the calyptoblast Campanularia, Crowell (1957) found profound alterations in colony pattern were produced in response to a reduction in the amount of food provided. Colonies fed few Artemia showed only moderate reduction of the amount of stolon growth but greatly reduced stem growth compared to control colonies.

From the point of view developed in the present study, the observations of Kinne and Crowell suggest that the relative growth rates of stolon vs.

---

[2] Kinne devoted primary attention to the profound influences of salinity (and temperature) on the size and shape of the hydranth and its component cells. This aspect of Cordylophora has not been touched in the present inquiry

stem tubes can be influenced by environmental conditions. The author has also found that the pattern of Cordylophora colonies can be altered by varying environmental factors, including composition of the aqueous environment and nutrition. These observations offer a challenge for further investigation using the techniques and concepts of the present study.

Chapter VIII

General Discussion: The Dynamic Biology of *Cordylophora*

In Chapter II, the static biology of Cordylophora was reviewed. We are now in a position to examine more dynamic aspects of the life of the organism, in particular as a result of the thesis research. Emphasis is placed on developmental aspects of the biology of Cordylophora, with considerable speculation and discussion of prospects for future study.

Cordylophora may be viewed as a black or at best gray box living and growing in a defined aqueous environment. Living prey is captured and taken into the coelenteron, where it is converted into nutrients. These nutrients are circulated through the colony by peristalsis and the movement of flagella, and are taken into the cells of the organism, where they become metabolites.

Metabolites yield energy for maintenance, including morphostasis (the maintenance of form, Weisz, 1951). It would appear that Cordylophora has considerable morphostatic ability, for there is little or no sign of regression of parts when colonies are maintained under adequate conditions. In contrast to the calyptoblastic hydroid Campanularia, where the hydranths develop, age, and regress quite rapidly (Crowell, 1953), the hydranths of Cordylophora remain healthy indefinitely. Kinne (1956) followed single hydranths for more than 140 days without sign of regression.

This is reminiscent of the situation in hydra, where the studies of Brien (1953) led him to conclude that a single hydra is eternal. Hydra was found to develop new cells (by cell division) in the distal region, and to continually shed old cells at its base. Cordylophora cannot shed old cells in this manner because of its structure, and one wonders what a colony does with aged cells.

In addition to energy for morphostasis, metabolites yield energy for morphogenesis -- the synthesis of new form. Morphogenesis can take two forms: (a) the production of new like tissue (growth) via cell division, and (b) the divergence of common tissue (differentiation) into new structures. Let us consider what factors might regulate these two forms of morphogenesis.

As discussed in Chapter VII, growth in length of stem and stolon tubes at a constant rate can best be explained by assuming that the tubes have regions of cell division of constant size (growth zones or meristems). The rate at which these meristems produce new tissue would be regulated by metabolites, and that in turn controlled by peristalsis. Since peristalsis is proximally-oriented one might envision that stolon meristems receive more nutrients than stem

meristems, and thus stolons grow more rapidly than stems. This is probably a great over-simplification, however, and does not readily explain why uprights increase in length more rapidly than their side branches.

As a stolon, upright or branch grows, it branches at intervals. The factors determining these intervals are of considerable interest. In Chapter VII, it was noted that secondary stolons develop at a distance from the tips of stolons, and that they tend to develop at the bases of uprights. It has been found possible to induce stolon development from stem tissue by tying a ligature around an upright. A successful ligature severs the continuity of coenosarc and coelenteron between the upright and the rest of the colony. When this is done, in all cases, a stolon develops after several days from the upright just above (distal to) the ligature. This stolon grows out normally, whether free or attached to the substratum, and produces uprights at intervals. If the ligature breaks loose, allowing a redevelopment of continuity, no stolon develops, indicating that it is not the injury itself which produces the stolon. These experiments have been interpreted to indicate that a "stolon-inducing substance" is produced by hydranths (or uprights), and that wherever a critical level of this substance accumulates a new stolon develops. A successful ligature would force accumulation of this substance just distal to the ligature. At the present stage, other interpretations are possible, though this interpretation perhaps helps to explain why secondary stolons develop at erratic positions in a colony, and usually at the bases of uprights.

In contrast to secondary stolons, uprights arise from stolons (and branches from uprights) at fairly regular intervals and only make their appearance just behind growing tips. It seems fairly clear that the primary factors determining the spacing of hydranth-bearing tubes are internal rather than external. The factors could either be viewed as promoters or inhibitors -- a combination which is often difficult to distinguish experimentally (Thimann, 1956). From the view of promotion, one might postulate that old uprights use up a vital factor promoting upright development, and only when a sufficient distance is reached can a new upright develop, while inhibition would suggest that new uprights can only develop below a threshold level of restraining substance produced by old uprights. The author tends to favor the view that the regular spacing of uprights is due to influences restraining the tendency of coenosarc tissue to become hydranth tissue. This is supported by the observation that coenosarc tissue isolated from any part of a colony,

whether as lengths of stem or stolon or as tissue fragments, develops hydranth Only in the colony does it remain coenosarc tissue.

Another aspect of asexual colony development is the angle at which the various tubes leave one another. Tubes arising from stolons (uprights, second ary stolons) leave at a right angle, while tubes arising from uprights (branch leave at a 45 degree angle. The reason for this is obscure, though physical forces suggest themselves and could readily be studied.

This view of asexual development of a Cordylophora colony at the tissue level unquestionably greatly oversimplifies the phenomena, but provides testable hypotheses for study. At this point Cordylophora colonies would appear very well suited for just such studies -- at the tissue level. The colonies do not appear as promising for analyses of development at the cellular or chemical level, though one might hope eventually to isolate and identify such substances as the "stolon-inducing principle" if they exist. On the cellular level, the difficulty is that with our present knowledge, the cells of Cordylophora or any other coelenterate, when isolated from their tissues, simply round up into spheres and become necrotic. On the chemical level, the difficulty is due to asexual development itself. While a growing asexual colony shows divergence as well as increase in mass, as measured chemically there are no changes -- i.e., like yields more of like. One system which might be of biochemical utility is the differentiation of hydranths from isolated coenosarc tissue, for here like yields unlike.

Cordylophora colonies show, on the one hand, some independence of parts -- such that, for example, one can consider hydranths as units or treat them as individuals with respect to prey capture and feeding -- but at the same time the colonies show considerable integration of parts into the whole. The two most striking examples of integration which come from the present study are the coordinated and orderly peristalsis and the regular developmental pattern of colonies which results in a regular growth rate.

Sexual differentiation is the major known phenomenon of the life of Cordylophora which the author has not yet observed in the laboratory. Sexuality is seen in nature in response to seasonal influences (in Woods Hole Cordylophora become sexual in the spring and begin to become asexual at the end of July), but all attempts to induce it in the laboratory have been unsuccessful. Kinne (1956) reports that his colonies became sexual in the

laboratory at 10 to 20°C, in from fresh water to 27 per cent salinity. In correspondence, he could only suggest that experiments be started in the spring. It seems fairly clear that the inducing factor, unlike that in Hydra (Loomis, 1957) is not an increased partial pressure of carbon dioxide. They become sexual in nature in rapidly-flowing, clean, shallow water, in which the pCO_2 probably does not often rise above atmospheric. Further, unlike Hydra, cultures may be grown to considerable densities without becoming sexual (Chapter III).

The inducing stimulus or stimuli is still being sought. Sexual uprights from nature grew in the laboratory, but gradually became asexual. At first the new portions of the colonies which developed in the laboratory developed gonads (gonophores), but as growth continued fewer and fewer and then none developed. This suggests a sex-hormone which is gradually diluted out in the absence of inducing stimulus.

The fact that all hydroids appear to have a seasonal sexual phase even though the bulk of their growth is asexual suggests that sexuality plays an important role in the long-term existence of the organisms. One wonders if this role is simply gene-reshuffling over the course of hundreds of years, or if hydroids are like Paramecium and certain other organisms in that they age unless allowed to enter a sexual phase (cf. Sonneborn, 1954). Asexual Cordylophora clone A has been grown continuously in the laboratory since August 1957. If one assume extremes of doubling times between 3 and 14 days, then clone A has grown a minimum of 68 and a maximum of 316 generations without sexuality. There has been no indication of any decline of vigor during this period.

The development of a procedure by which the sexual differentiation of Cordylophora could be obtained reproducibly in the laboratory would allow a number of studies with this conceptually simple organism. One could seek the identity of the factor or factors inducing sexuality, or even analyze the events of sexual differentiation, for as with Hydra (Loomis, 1957) one would expect to be able to control the simultaneous sexual differentiation of a population of Cordylophora. One could attempt an analytical study of the embryonic development of this organism. Finally, one might be able to examine the genetics of the organism and even to obtain developmental mutants to further the study of asexual colony development.

References

References

Abe, N. 1938 Feeding behavior and the nematocyst of Fungia and 15 other species of corals. Palao Trop. Biol. Station Studies (Japan) 3: 469-521.

Allman, G. J. 1843 Synopsis of the genera and species of zoophytes inhabitin the fresh waters of Ireland. Report Brit. Assoc. Adv. Sci. 13: 77.

_____ 1853 On the anatomy and physiology of Cordylophora, a contribution to our knowledge of tubularian zoophytes. Phil. Trans. Roy. Soc. (London 143: 367-384.

_____ 1871 A monograph of the gymnoblastic or tubularian hydroids. I. The Hydroida in general. London, The Ray Society. pp. 1-154.

_____ 1872 Ibid. II. The genera and species of the Gymnoblastea. London, The Ray Society. pp. 155-450.

Beadle, L. C., and F. A. Booth 1938 The reorganization of tissue masses of Cordylophora lacustris and the effect of oral cone grafts, with supplementary observations on Obelia gelatinosa. J. Exp. Biol. 15: 303-326.

Benassi, C. A., G. Colombo, and G. Peretti 1959 Free amino acids of the hemolymph of Anacridium aegyptium L. (Orthoptera). Experientia 15: 457-458.

Berrill, N. J. 1949 Growth and form in gymnoblastic hydroids. I. Polymorphic development in Bougainvillia and Aselomaris. J. Morph. 84: 1-30.

_____ 1952 Ibid. III. Hydranth and gonophore development in Pennaria and Acaulis. J. Morph. 90: 5-20.

_____ 1953 Ibid. VII. Growth and reproduction in Syncoryne and Coryne. J. Morph. 92: 273-302.

Beutler, R. 1924 Experimentelle Untersuchungen über die Verdauung bei Hydra. Z. Wiss. Biol., Abt. C, 1: 1-56.

_____ 1926 Beobachtungen an Gefütterten Hydroidpolypen. Z. Wiss. Biol., Abt. C, 3: 737-775.

Bonner, J. T. 1952 Morphogenesis: an essay on development. Princeton, Princeton University Press. pp. 121-128.

Brachet, J. 1959 New observations on biochemical interactions between nucleu and cytoplasm in Amoeba and Acetabularia. Exp. Cell Res., suppl. 6: 78-9

Brien, P. 1953 La pérennité somatique. Biol. Rev. 28: 308-349.

Buchsbaum, R. 1948 Animals without backbones, second edit. Chicago, Univers of Chicago Press.

Burnett, A. L. 1959 Histophysiology of growth in hydra. J. Exp. Zool. 140: 281-341.

Burnett, A. L. and M. Garofalo 1960 Growth pattern in the green hydra, Chlorohydra viridissima. Science 131: 160-161.

Calvin, M. 1954 Mercaptans and disulfides: some physics, chemistry, and speculation. in Glutathione (S. P. Colowick, et al., eds.). New York, Academic Press. pp. 3-30.

Cavanaugh, M. W. 1955 Pulsation, migration and division in dissociated chick embryo heart cells in vitro. J. Exp. Zool. 128: 573-589.

Chaberek, S., and A. E. Martell 1959 Organic sequestering agents. New York, John Wiley.

Chalkley, H. W., and H. D. Park 1947 Methods for increasing the value of hydra as material in teaching and research. Science 105: 553.

Chapman, G. B., and L. G. Tilney 1959 Cytological studies on the nematocysts of hydra. J. Biophys. Biochem. Cytol. 5: 69-84.

Chinard, F. P. 1952 Photometric estimation of proline and ornithine. J. Biol. Chem. 199: 91-95.

Clarke, S. F. 1878 A new locality for Cordylophora. Amer. Nat. 12: 232-234.

Cliffe, E. E., and S. G. Waley 1958 Effects of analogues of glutathione on the feeding reaction of hydra. Nature 182: 804-805.

Cochrane, V. W. 1958 Physiology of fungi. New York, John Wiley.

Cohen, A. I. 1952 Studies on the pigmentation changes during reconstitution in Tubularia. Biol. Bull. 102: 91-99.

Cronkite, E. P., V. P. Bond, T. M. Fliedner, and J. R. Rubine 1959 The use of tritiated thymidine in the study of DNA synthesis and cell turnover in hemopoietic tissues. Lab. Invest. 8: 263-275.

Crowell, S. 1953 The regression-replacement cycle of hydranths of Obelia and Campanularia. Physiol. Zoöl. 26: 319-327.

_____ 1957 Differential responses of growth zones to nutritive level, age, and temperature in the colonial hydroid Campanularia. J. Exp. Zool. 134: 63-90.

Dempster, R. P. 1953 The use of larval and adult brine shrimp in aquarium fish culture. California Fish and Game 39: 355-364.

Dougherty, E. C. 1959 Introduction to axenic culture of invertebrate metazoa: a goal. Ann. New York Acad. Sci. 77: 27-54.

Duchâteau, G., and M. Florkin 1953 Teneur en acides aminés non protéinique du plasma de l'hémolymphe des chemilles de Cossus cossus et de deux saturnidés africains (Imbrasia macrothyris et Pseudobunaea seydeli). Arch. Intern. Physiol. 61: 232-233.

Eagle, H. 1956 The salt requirements of mammalian cells in tissue culture. Arch. Biochem. Biophys. 61: 356-366.

_____ 1959 Amino acid metabolism in mammalian cell cultures. Science 130: 432-437.

Edsall, J. T., and J. Wyman 1958 Biophysical chemistry. I. Thermodynamics, electrostatics, and the biological significance of the properties of matter. New York, Academic Press.

Emerson, S. 1950 The growth phase in Neurospora corresponding to the logarithmic phase in unicellular organisms. J. Bact. 60: 221-223.

Erickson, R. O. 1959 Integration of plant growth processes. Amer. Nat. 93: 225-235.

Ewer, R. F. 1947 On the functions and mode of action of the nematocysts of hydra. Proc. Zool. Soc. (London) 117: 365-376.

Fitzgerald, P. J., E. Simmel, J. Weinstein, and C. Martin 1953 Radio-autography: theory, technic, and applications. Lab. Invest. 2: 181-222.

Fowden, L. 1956 Azetidine-2-carboxylic acid: a new cyclic imino acid occurring in plants. Biochem. J. 64: 323-332.

Fruton, J. S., and S. Simmonds 1958 General biochemistry, second edit. New York, John Wiley.

Geyer, R. P., and R. S. Chang 1958 Bicarbonate as an essential for human cells in vitro. Arch. Biochem. Biophys. 73: 500-506.

Hadzi, J. 1909 Über das Nervensystem von Hydra. Arb. Zool. Inst. (Univ. Wien.) 17: 225-268.

Ham, R. G., and R. E. Eakin 1958 Time sequence of certain physiological events during regeneration in hydra. J. Exp. Zool. 139: 33-53.

Ham, R. G., D. C. Fitzgerald, Jr., and R.E. Eakin 1956 Effects of lithium ion on regeneration of hydra in a chemically defined environment. J. Exp. Zool. 133: 559-572.

Hammen, C. S., and P. J. Osborne 1959 Carbon dioxide fixation in marine invertebrates: a survey of major phyla. Science 130: 1409-1410.

Hammett, F. S. 1943 The role of amino acids and nucleic acid components in developmental growth. Part one: the growth of an Obelia hydranth. Growth 7: 331-399.

Hand, C., and G. F. Gwilliam 1951 New distributional records for two athecate hydroids, Cordylophora lacustris and Candelbrum sp. from the west coast of North America, with revisions of their nomenclature. J. Washington Acad. Sci. 41: 206-209.

Hargitt, C. W. 1897 Notes upon Cordylophora lacustris. Zoöl. Bull. 1: 205-208.

Harvey, E. B. 1956 The American Arbacia and other sea urchins. Princeton, Princeton University Press.

Harvey, H. W. 1957 The chemistry and fertility of sea waters, second edit. Cambridge, University Press.

Hauenschild, C. 1954 Genetische und Entwicklungsphysiologische Untersuchungen über Intersexualität und Gewebeverträglichkeit bei Hydractinia echinata Flemm. (Hydroz. Bougainvill.). Roux' Arch. Entwcklngsmechn. Organ. 147: 1-41.

_____, and A. Kanellis 1952 Kulturversuche mit Hydractinia echinata Flemm. Prakt. Akad. Athenon. 27: 11-18.

_____, and _____ 1953 Experimentelle Untersuchungen an Kulturen von Hydractinia echinata Flemm. zur Frage der Sexualitat und Stockdifferenzierung. Zool. Jahrb., Abt. Physiol. 64: 1-13.

Hauschka, T. 1944 The rôle of hydroplasmic pressure in hydroid growth. Growth 8: 321-336.

Henschel, J. 1935 Untersuchungen über den chemischen Sinn der Scyphomedusen Aurelia aurita und Cyanea capillata und der Hydromeduse Sarsia tubulosa. Wiss. Meeresuntersuch., Abt. Kiel, N.F. 22: 21-42.

Hirs, C. H. W., S. Moore, and W. H. Stein. 1954 The chromatography of amino acids on ion exchange resins. Use of volatile acids for elution. J. Amer. Chem. Soc. 76: 6063-6065.

Hoffman-Berling, H. 1959 The role of cell structures in cell movements. in Cell, organism and milieu (D. Rudnick, ed.). New York, Ronald Press. pp. 45-62.

Hutchinson, G. E. 1957 A treatise on limnology. Vol. I. Geography, physics, and chemistry. New York, John Wiley.

Huxley, J. S., and G. R. de Beer 1923 Studies in dedifferentiation. IV. Resorption and differential inhibition in Obelia and Campanularia. Quart. J. Micr. Sci. 67: 473-495.

Hyman, L. H. 1937 Hydras. in Culture methods for invertebrate animals (J. G. Needham, ed.). New York, Comstock Publishing Co. pp. 140-142.

_____ 1940 The invertebrates: Protozoa through Ctenophora. New York, McGraw Hill.

Kinne, O. 1956 Über den Einfluss des Salzgehaltes und der Temperatur auf Wachstum, Form und Vermehrung bei dem Hydroidpolypen Cordylophora caspia (Pallas), Thecata, Clavidae. Zool. Jahrb., Abt. Physiol. 66: 565-638.

_____ 1958a Über die Reaktion erbgleichen Coelenteratengewebes auf verschiedene Salzegehalts- und Temperaturbedingungen. Zool. Jahrb., Abt. Physiol. 67: 407-486.

_____ 1958b Adaptation to salinity variations - some facts and problems. in Physiological adaptation (C. L. Prosser, ed.). Washington, American Physiological Society. pp. 92-106.

Kirchner, H.-A. 1934 Die Bedeutung der interstitiellen Zellen für den Aufbau von Cordylophora caspia Pall. Z. Zellforsch.u. Mikr. Anat. 22: 1-19.

Kühn, A. 1909 Sprosswachstum und Polypenknospung bei den Thecaphoren. Studien zur Ontogenese und Phylogenese der Hydroiden. Zool. Jahrb., Abt. Anat. 28: 387-476.

Lenhoff, H. M. 1958 Hydra and Planaria. Carnegie Inst. Wash. Year Book 57: 157-162.

_____ 1959 Migration of ^{14}C-labelled cnidoblasts. Exp. Cell Res. 17: 570-573.

_____, and J. Bovaird 1959a Requirement of extracellular sodium ion by Hydra littoralis for growth and cnidoblast migration. Anat. Rec. 134: 598-599.

_____, and _____ 1959b Requirement of bound calcium for the action of surface chemoreceptors. Science 130: 1474-1476.

_____, and E. S. Kline 1958 The high imino acid content of the capsule from Hydra nematocysts. Anat. Rec. 130: 425.

_____, E.S.Kline, and R. Hurley 1957 A hydroxyproline-rich, intracellular, collagen-like protein of Hydra nematocysts. Biochim. Biophys. Acta 26: 204-205.

_____, and W. F. Loomis 1957 Environmental factors controlling respiration in hydra. J. Exp. Zool. 134: 171-181.

_____, and H. A. Schneiderman 1959 The chemical control of feeding in the Portugese Man-of-war, Physalia physalia L. and its bearing on the evolution of the Cnidaria. Biol. Bull. 116: 452-460.

Li, H. P., and T. Yao 1945 Studies on the organizer problem in Pelmatohydra oligactis. III. Bud induction by developing hypostome. J. Exp. Biol. 21: 155-160.

Loomis, W. F. 1953 The cultivation of hydra under controlled conditions. Science 117: 565-566.

_____ 1954 Environmental factors controlling growth in hydra. J. Exp. Zool. 126: 223-234.

_____ 1955 Glutathione control of the specific feeding reactions of hydra. Ann. New York Acad. Sci. 62: 211-227.

_____ 1957 Sexual differentiation in Hydra. Control by carbon dioxide tension. Science 126: 735-739.

_____ 1959a Feedback control of growth and differentiation by carbon dioxide tension and related metabolic variables. in Cell, organism and milieu (D. Rudnick, ed.). New York, Ronald Press. pp. 253-294.

_____ 1959b pCO_2 inhibition of normal and malignant growth. J. Natl. Cancer Inst. 22: 207-217.

Loomis, W. F. 1959c Further studies on cellular differentiation in hydra. Fed. Proc. 18: 276.

_____, and H. M. Lenhoff 1956 Growth and sexual differentiation of hydra in mass culture. J. Exp. Zool. 132: 555-573.

Lwoff, A., and J. Monod 1947 Essai d'analyse du role de l'anhydride carbonique dans la croissance microbienne. Ann Inst. Pasteur 73: 323-347.

MacLeod, R. A., and E. E. Snell 1948 The effect of related ions on the potassium requirement of lactic acid bacteria. J. Biol. Chem. 176: 39-52.

McConnell, C. H. 1932 The development of the ectodermal nerve net in the buds of Hydra. Quart. J. Micr. Sci. 75: 495-509.

_____ 1933 Mitosis in Hydra. Mitosis in the ectodermal epitheliomuscular cells of Hydra. Biol. Bull. 64: 86-95.

Medawar, P. B. 1945 Size, shape, and age. in Essays on growth and form (W. E. L. Clark and P. B. Medawar, eds.). London, Oxford University Press. pp. 157-187.

Meister, A. H. 1957 Biochemistry of the amino acids. New York, Academic Press.

Monod, J. 1942 Recherches sur la croissance des cultures bactériennes. Paris, Hermann and C. 210 p.

_____ 1949 The growth of bacterial cultures. Ann. Rev. Microbiol. 3: 371-394.

Moore, J. 1952a The induction of regeneration in the hydroid Cordylophora lacustris. J. Exp. Biol. 29: 72-93.

_____ 1952b Interstitial cells in the regeneration of Cordylophora lacustris. Quart. J. Micr. Sci. 93: 269-288.

Moore, R. C., ed. 1956 Treatise on invertebrate paleontology. Part F: Coelenterata. Lawrence, University of Kansas Press.

Morgenstern, P. 1901 Untersuchungen über die Entwicklung von Cordylophora lacustris Allman. Z. Wiss. Zool. 70: 567-591.

Mueller, J. F. 1950 Some observations on the structure of hydra, with particular reference to the muscular system. Trans. Amer. Micr. Soc. 69: 133-147.

Osgood, E. E., and M. L. Krippaehne 1955 The gradient tissue culture method. Exp. Cell Res. 9: 116-127.

Pantin, C. F. A., and A. M. P. Pantin 1943 The stimulus to feeding in Anemonia sulcata. J. Exp. Biol. 20: 6-13.

Pauly, R. 1900 Untersuchungen über den Bau und die Lebenweise von Cordylophora lacustris Allman (Vorl. Mitteilung). Zool. Anz. 23: 546-551.

_____ 1902 Untersuchungen über den Bau und die Lebenweise von Cordylophora lacustris Allman. Jenaische Z. Naturwissensch. 36: 737-780.

Plaut, W., and L. A. Sagan 1958 Incorporation of thymidine in the cytoplasm of Amoeba proteus. J. Biophys. Biochem. Cytol. 4: 843-844.

Prosser, C. L., ed. 1950 Comparative animal physiology. Philadelphia, W. B. Saunders Co. pp. 447-470.

Provasoli, L., J. J. A. McLaughlin, and M. R. Droop 1957 The development of artificial media for marine algae. Arch. Mikrobiol. 25: 392-428.

_____, and K. Shiraishi 1959 Axenic cultivation of the brine shrimp Artemia salina. Biol. Bull. 117: 347-355.

Rae, K. M. 1958 Parameters of the marine environment. in Perspectives in marine biology (A. A. Buzzati-Traverso, ed.) Berkeley, University of California Press. pp. 3-16.

Ramsay, J. A. 1952 A physiological approach to the lower animals. Cambridge, University Press.

Rees, W. J. 1957 Evolutionary trends in the classification of capitate hydroids and medusae. Bull. British Museum (Natural History) 4: 453-534.

Richards, A. G., and L. K. Cutkomp 1946 Correlation between the possession of a chitinous cuticle and sensitivity to DDT. Biol. Bull. 90: 97-108.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten 1953 Studies of biosynthesis in Escherichia coli. Carnegie Inst. Wash. Publ. 607. 521 p.

Roch, F. 1924 Experimentelle Untersuchungen an Cordylophora caspia (Pallas) (=lacustris Allman) über die Abhängigkeit ihrer geographischen Verbreitung und ihrer Wuchsformen von den physikalisch-chemischen Bedingungen des umgebenden Mediums. Z. Morph. u. Ökologie der Tiere 2: 350-436, 667-670.

Rulon, O., and C. M. Child 1937 Observations and experiments on developmental pattern in Pelmatohydra oligactis. Physiol. Zoöl. 10: 1-13.

Ryan, F. J., G. W. Beadle, and E. L. Tatum 1943 The tube method of measuring the growth rate of Neurospora. Amer. J. Bot. 30: 784-799.

Saint-Hilaire, K. 1930 Morphogenetische Untersuchungen der nichtzellulären Gebilde bei Tieren. Periderm der Hydroiden. Zool. Jahrb., Abt. Physiol. 47: 511-622.

Schmalz, P. 1913 Cordylophora lacustris Allm. im Aquarium. Blätter f. Aquarien- u. Terrarienkunde 24: 452-454.

Schneiderman, H. A., and L. I. Gilbert 1958 Substances with juvenile hormone activity in Crustacea and other invertebrates. Biol. Bull. 115: 530-535.

Schulze, F. E. 1871 Über den Bau and die Entwicklung von Cordylophora lacustris (Allman). Leipzig, Wilhelm Engelman. 52 p.

Schulze, P. 1921 Die Hydroiden der Umgebung Berlins mit besonder Berücksichtigung der Binnenlandformen von Cordylophora. Biol. Zentr. 41: 220-237.

Simpson, J. W., K. Allen, and J. Awapara 1959 Free amino acids in some aquatic invertebrates. Biol. Bull. 117: 371-381.

Slautterback, D. B., and D. W. Fawcett 1959 The development of the cnidoblasts of Hydra. An electron microscope study of cell differentiation. J. Biophys. Biochem. Cytol. 5: 441-452.

Smith, I., ed. 1958 Chromatographic techniques. Clinical and biochemical applications. New York, Interscience Publishers.

Smith, J. H. 1923 On the apical growth of fungal hyphae. Ann. Bot. 37: 341-343.

Snedecor, G. W. 1956 Statistical methods, fifth edit. Ames, Iowa State College Press.

Sonneborn, T. M. 1954 The relation of autogamy to senescence and rejuvanescence in Paramecium aurelia. J. Protozool. 1: 38-53.

Sorokin, C., and R. W. Krauss 1959 Maximum growth rates of Chlorella in steady-state and in synchronized cultures. Proc. Nat. Acad. Sci. 45: 1740-1744.

Steinberg, M. S. 1958 On the chemical bonds between animal cells. A mechanism for type-specific association. Amer. Nat. 92: 65-81.

Stein, W. H., and S. Moore 1954 The free amino acids of the human blood plasma. J. Biol. Chem. 211: 915-926.

Stocking, C. R., and E. M. Gifford, Jr. 1959 Incorporation of thymidine into chloroplasts of Spirogyra. Biochem. and Biophys. Research Comm. 1: 159-164.

Tallan, H. H., S. Moore, and W. H. Stein 1954 Studies on the free amino acids and related compounds in the tissues of the cat. J. Biol. Chem. 211: 927-939.

Tessier, G. 1931 Etude expérimentale du développement de quelques hydraires. Ann. Sci. Nat. Zool. 14: 4-60.

Thimann, K. V. 1955 The life of bacteria. New York, Macmillan.

_____ 1956 Promotion and inhibition: twin themes in physiology. Amer. Nat. 90: 145-162.

Thompson, D'Arcy W. 1943 On growth and form. Cambridge, University Press.

Tracey, M. V. 1955 Chitin. in Modern methods of plant analysis. Vol. II. (K. Paech and M. V. Tracey, eds.). Berlin, Springer-Verlag pp. 264-274.

Trembley, A. 1744 Mémoires pour servir à l'histoire d'un genre de polypes d'eau douce, à bras en forme de cornes. Leide, Verbeek. 324 p.

Umbreit, W. W., R. H. Burris, and J. F. Stauffer 1957 Manometric techniques, third edit. Minnesota, Burgess Publishing Co.

Weill, R. 1934a Contribution a l'étude des Cnidaires et de leurs nématocystes. I. Recherches sur les nématocystes. Travaux Station Zool. Wimereux 10: 1-347.

_____ 1934b Ibid. II. Valeur taxonomique du Cnidome. Ibid. 11: 349-701.

Weiss, P. 1955 What is growth? in The hypophyseal growth hormone, nature and actions (R. W. Smith, Jr., et al., eds.). New York, McGraw Hill. pp. 3-16.

Weisz, P. B. 1951 A general mechanism of differentiation based on morphogenetic studies in ciliates. Amer. Nat. 85: 293-311.

Wieland, T. 1954 Chemistry and properties of glutathione. in Glutathione (S. P. Colowick, et al., eds.). New York, Academic Press. pp. 45-57.

Woolley, D. W. 1952 A study of antimetabolites. New York, John Wiley.

Zick, K. 1932 Die Entladung der Nesselkapseln durch Protozoen. Zool. Anz. 98: 191-197.