Environmental Factors Influencing the Growth of *Cordylophora*[1]

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A simple method has been developed for cultivation of the colonial hydroid *Cordylophora* (Fulton, '60). The method was perfected by studying the influence of environmental factors on growth rate, an approach similar to that used by Loomis ('54) for the solitary hydroid *Hydra*. The major results of the study are reported here.

ORGANISM AND CULTURE METHOD

Cordylophora. The work has been done with the descendants of a single hydranth isolated in August 1957 from a colony growing in Nye Pond, North Falmouth, Massachusetts (Clone A).[3] This is a male clone, but it has remained asexual in the laboratory. A number of other clones have also been isolated; all grew under the conditions described below. Clone A agrees in every particular with the descriptions of *C. lacustris* (Allman, 1853; Schulze, 1871; Hand and Gwilliam, '51), and *Cordylophora* refers to that species.

Cordylophora is unusually vigorous under laboratory conditions, which is not surprising since it lives in fresh or brackish water and must tolerate greater fluctuations in its habitat than its marine relatives. Allman (1872), Hargitt (1897), and others reported that colonies survived well in the laboratory. Two previous studies of the laboratory growth of *Cordylophora* (Roch, '24; Kinne, '56, '58a, b) are discussed below.

Culture method. Cordylophora is sessile, aquatic, and carnivorous; these three properties delimit the minimal conditions for successful cultivation. Colonies were grown for these experiments as previously described (Fulton, '60) and illustrated in figure 1. Secondary colonies of clone A were cultured on 1 × 3 inch microscope slides slanted in 100 ml beakers filled with CCS5. The cultures were fed to saturation once each day with freshly hatched *Artemia* larvae (cf. Loomis and Lenhoff, '56), and the culture solution changed one hour thereafter and again six to eight hours later. Between feedings the cultures were maintained in the dark at a constant temperature of 22°C.

CCS5 was normally prepared in demineralized water, but could be prepared in distilled water if 1.5×10^{-4} M disodium ethylenediamine tetraacetate (versenate) was added to sequester heavy metal ions. *Cordylophora* - versenated - distilled water (CVD) was used whenever precise definition of the aqueous environment was unnecessary.[4]

Evaluating growth. Growth is measured in terms of a growth rate (k), as

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[3] I am indebted to the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, and especially to Mr. Milton Grey, for repeatedly taking me to the sites where *Cordylophora* grows.

[4] The critical variable in this method appears to be the culture solution. Consistent results have been obtained in my laboratory if demineralized water for the preparaion of CCS5 is made with a Barnstead Bantam demineralizer, and on the basis of its conductivity has less than 0.1 ppm salts (as NaCl). The first few liters of effluent which leave the column are frequently toxic to *Cordylophora*, and therefore are discarded each time the demineralizer is used. In some localities, Cordylophora may be cultivated in versenated tap water (CVT), prepared in the same manner as CVD (Fulton, '60). Tap water must be used with caution, however, since in Waltham it remains toxic to *Cordylophora* even after the addition of versenate. In Waltham demineralized or distilled water has replaced tap water in all operations, including the hatching of *Artemia* larvae.

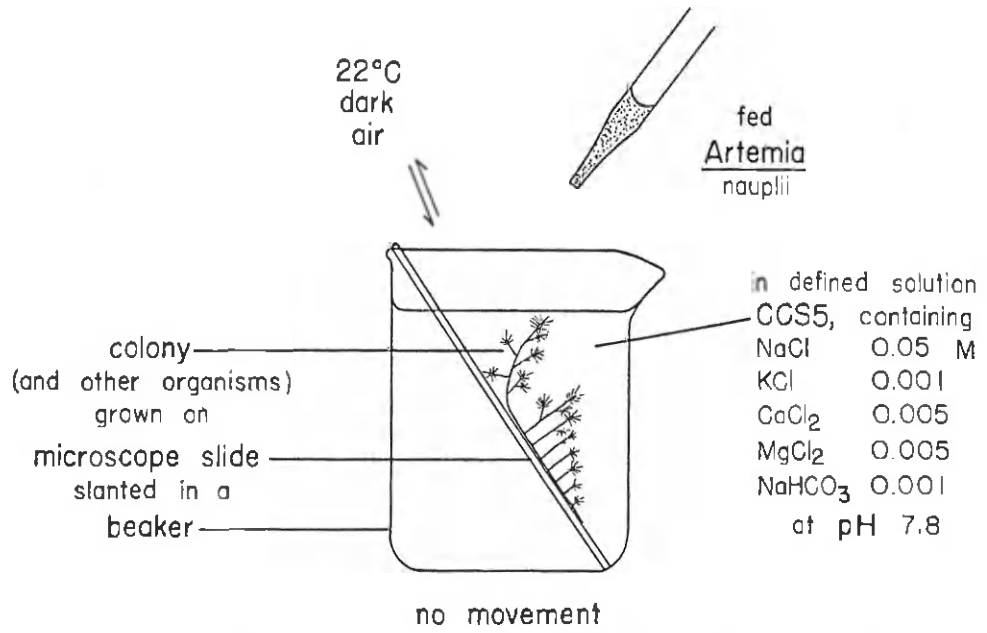

Fig. 1 A schematic illustration of the essential features of the culture method. In preparing CCS5, $KHCO_3$ at 0.001 M was frequently substituted for the KCl and $NaHCO_3$.

described below. Under decidedly unfavorable conditions, all of the hydranths of a colony are either resorbed or fall off, yielding a hydranth number of zero. For convenience, such negative growth (decrease in the 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 cultures are transferred to experimental conditions is termed day 0.

PATTERN OF COLONY GROWTH

Cordylophora colonies grow by budding, a process which increases the number of countable units rather than the size of a single unit. The units are hydranths, arranged on stems and stolons in a simple and regular pattern (fig. 2). The hydranths are of a single type, and appear to be perennial, unlike the hydranths of *Campanularia*, which regress about a week after they form (Crowell, '53). Kinne ('56) found that individual *Cordylophora* hydranths live for at least 140 days, and I have never observed regression of hydranths in healthy colonies.

Secondary colonies are started by tying a single upright (stem with attached hydranths; Crowell, '57) to a microscope slide. The explanted upright develops a stolon at its proximal end; this stolon attaches to the slide and begins to grow along the substratum. The stolon produces new stolons at irregular intervals, and uprights at regular intervals (fig. 2). The uprights develop hydranths at their apices, 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; this distal growth shows essentially the same pattern as an upright in an older colony. Figure 3 illustrates colonies of various ages. A detailed, quantitative description of colony development constitutes a separate study (Fulton, '62).

Although the present experiments on growth concern young colonies, usually with no more than 70 hydranths, it is possible to grow colonies to considerably greater densities (fig. 3C). Old colonies can reach wet weights of as much as a gram, which has been found equivalent to about 2,000 hydranths. With fastidious attention, such colonies remain healthy, though indirect evidence indicates that they grow very slowly.

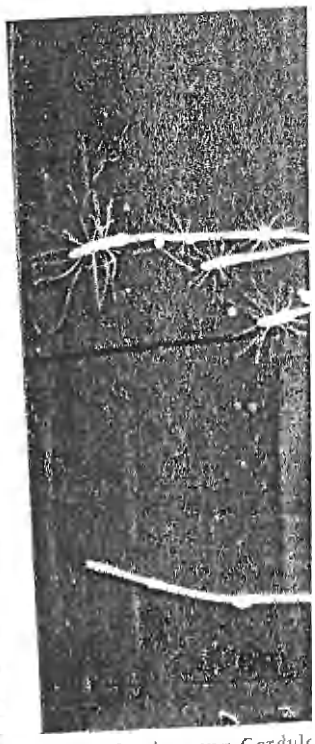

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Fig. 3 Three *Cordyl*
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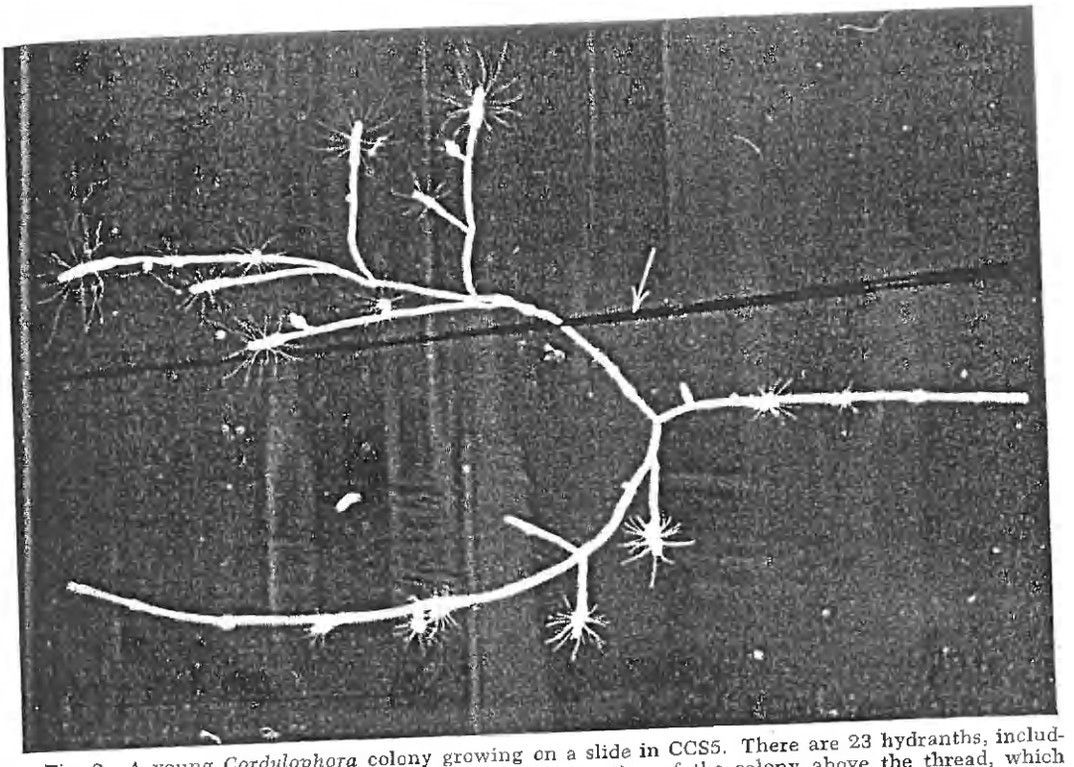

Fig. 2 A young *Cordylophora* colony growing on a slide in CCS5. There are 23 hydranths, including buds. The thread is indicated by an arrow. The portion of the colony above the thread, which developed from the explanted upright, is unattached, while the portion below is attached and has four stolons. There are two main stolons, with one having seven and the other four uprights, and two young stolons.

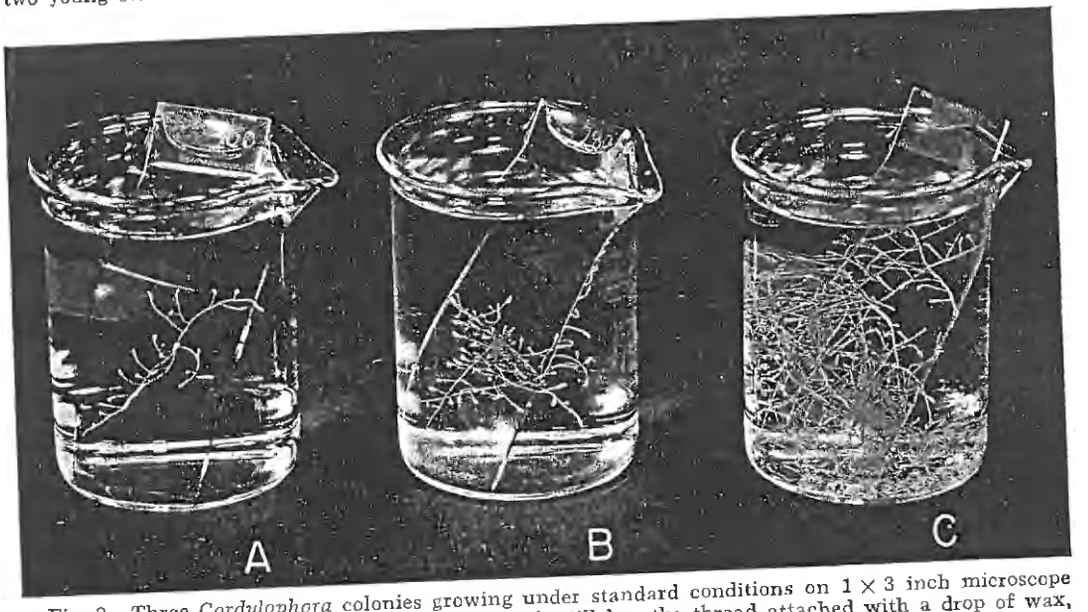

Fig. 3 Three *Cordylophora* colonies growing under standard conditions on 1 × 3 inch microscope slides slanted in 100 ml beakers. Colony A, which still has the thread attached with a drop of wax, was started from a single hydranth 13 days before the photograph was taken, and has 21 hydranths. Colony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

EXPONENTIAL GROWTH AND GROWTH RATE

Increase in hydranth number with time in *Cordylophora* colonies provides a convenient, quantative measure of growth (Fulton, '60). With colonies growing under standard conditions, hydranth number increases exponentially (fig. 4). *Hydra* also grows exponentially (Loomis, '53), but *Cordylophora* achieves exponential growth by a more circuitous route than does its solitary relative (Fulton, '62).

The growth rate of a *Cordylophora* colony is determined using 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: $dn/dt = kn$. This may be integrated to yield: $\ln (n/n_0) = kt$, where n_0 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, '54): $k = \ln 2/T = 0.693/T$.

In practice, the number of hydranths in a colony is counted on a series of successive days. These data are plotted on semilogarithmic paper, and the points interpolated to give a straight line, from which the doubling time T is determined to the nearest tenth of a day, and the growth rate calculated.

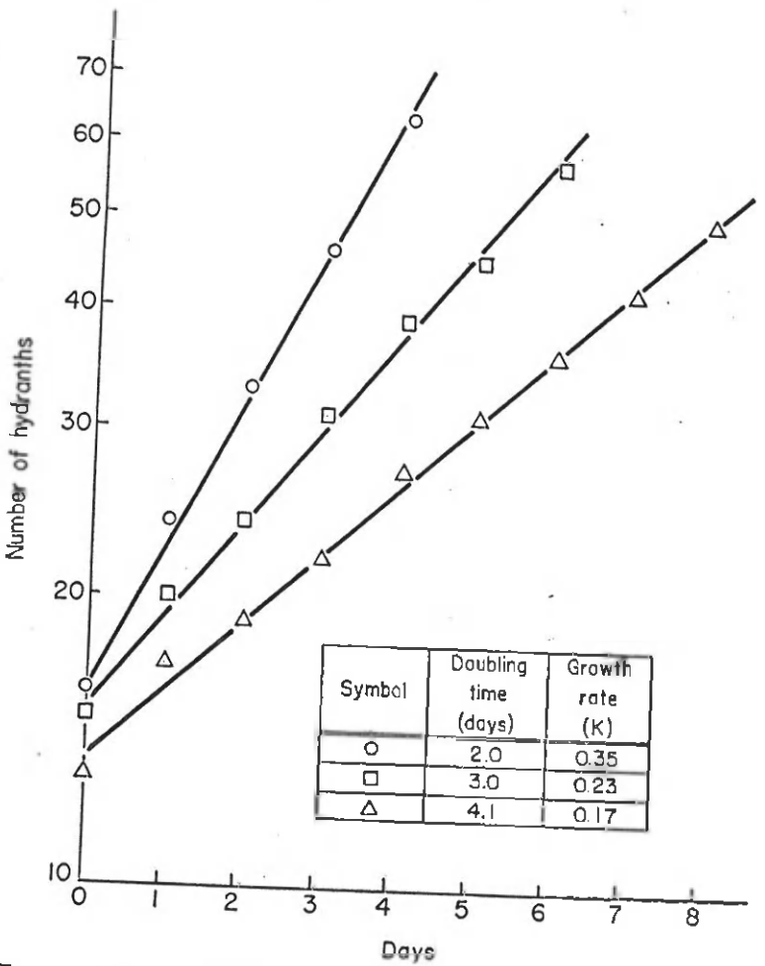

Fig. 4 Exponential increase in hydranth number. Three growth curves selected to illustrate extremes of variation in growth rate under standard conditions.

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Group no.	No. of culture
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2	3
3	2
4	3
5	3
6	8
7	4
8	8
9	3
10	2
11	5
12	5
13	5
14	2
15	2
16	3
17	2
18	3
19	4
20	3
21	2
22	3
Mean values	
"Within group"	3
All cultures as a group	78

Even under standard conditions, growth rates can vary as much as twofold (fig. 4). The regularly observed growth rates ($k \sim 0.2$) represent a doubling time of about three days. Growth rates of 0.30 or higher have been observed in less than 1% of the several hundred colonies whose growth rates have been determined, and such growth rates have been eliminated in current work (see below). With the exception of these high growth rates, the major deviations in growth rate came between rather than within experiments, unless significant variations in conditions were introduced within an experiment. To illustrate this, data from 22 experiments in which the growth rates of two or more replicate colonies were followed have been evaluated (table 1). The standard deviation from the mean growth rate ($k = 0.21$) of the 79 cultures is 0.038. If each of the 22 groups is calculated separately, the mean growth rate varies from 0.11 to 0.29, and the range from 0.00 to 0.10. The "within group" standard deviation, calculated from the ranges, has a mean of about 0.02, or about half the variability of the growth rates taken as a whole.

The range of growth rates within an experiment (table 1) 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% of the time a difference in growth rate of 0.08 or more between two cultures is significant.[5]

[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, '56).

TABLE 1

Growth rates of replicate cultures

The table is a collection of data from all experiments prior to January 1960 in which growth rates were determined for two or more cultures growing under standard conditions.

Group no.	No. of cultures	Observed growth rates of replicate cultures	Mean growth rate ($\bar{k}$)	Range
1	3	0.11, 0.11, 0.11	0.11	0.00
2	3	0.15, 0.16, 0.17	0.16	0.02
3	2	0.18, 0.19	0.19	0.01
4	3	0.18, 0.19, 0.20	0.19	0.02
5	3	0.19, 0.19, 0.20	0.19	0.01
6	8	0.17, 0.17, 0.17, 0.18, 0.18, 0.19, 0.22, 0.23	0.19	0.06
7	4	0.16, 0.18, 0.19, 0.26	0.20	0.10
8	8	0.15, 0.19, 0.20, 0.21, 0.21, 0.22, 0.22, 0.22	0.20	0.07
9	3	0.20, 0.22, 0.22	0.21	0.02
10	2	0.20, 0.22	0.21	0.02
11	5	0.20, 0.20, 0.20, 0.21, 0.22	0.21	0.02
12	5	0.17, 0.18, 0.19, 0.24, 0.25	0.21	0.08
13	5	0.18, 0.21, 0.22, 0.23, 0.23	0.21	0.05
14	2	0.21, 0.24	0.23	0.03
15	2	0.22, 0.24	0.23	0.02
16	3	0.22, 0.23, 0.24	0.23	0.02
17	2	0.22, 0.25	0.24	0.03
18	3	0.22, 0.22, 0.29	0.24	0.07
19	4	0.23, 0.24, 0.26, 0.27	0.25	0.04
20	3	0.25, 0.25, 0.27	0.26	0.02
21	2	0.25, 0.27	0.26	0.02
22	3	0.27, 0.29, 0.30	0.29	0.03
Mean values				
"Within group"	3.5		0.21	0.035
All cultures as a group	78		0.21	0.19

ENVIRONMENTAL FACTORS INFLUENCING GROWTH

I. *Intrinsic factors*

Of many variables which might be expected to influence the growth rate of *Cordylophora* colonies, the extrinsic or environmental variables related to the culturing procedure have been examined most thoroughly. These variables prove especially susceptible to variation and control for experimental purposes. However, the colonies themselves, as well as associated organisms, may also be expected to influence growth rate. Although the effects of such intrinsic factors have not been explicitly studied, at least four ways are known in which such factors could affect growth rate.

1. The growth rates of several clones have been compared with that of clone A under standard conditions. Of six other clones compared in repeated experiments, three grew at about the same rate as clone A colonies, but three definitely grew more slowly.

2. A variety of microorganisms have been found associated with clone A, and these may influence growth rate. By treatment of *Cordylophora* with antibiotics under special conditions, it has been possible to obtain colonies unable to grow under standard conditions, suggesting the normal presence of symbiotic organisms providing growth factors or removing toxic by-products.

3. The immediate previous history of a strain can influence the growth rate observed in short-term experiments. The rare cases of growth at exceptional rates ($k > 0.30$) observed in early experiments have recently been shown to result from a preceding period without growth; such exceptional cases have been completely eliminated by strict standardization of conditions.

4. The particular pattern of individual colonies (e.g., the number of stolons) can influence growth rate, as described in Fulton ('62).

All of these intrinsic factors — strain of *Cordylophora*, associated organisms, previous history of strain, and colony pattern — may thus be expected to influence growth rate. Each warrants further investigation, completion of which may permit further control over the reproducibility of growth rate. At present such factors, especially random variations in colony pattern, probably account for some of the variability of growth rates within and between experiments. Within the framework of the results on extrinsic factors reported here, however, there has been no indication that the variables of the organism would invalidate the results of the experiments in any manner.

II. *Methodological variables*

For convenience, extrinsic factors fixed by the basic method of culture, such as substratum, are treated separately from the more general environmental factors, such as ionic composition of the aqueous environment.

Culture container and substratum. The culture chamber must accommodate a sessile organism, permanently attached to its 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 though the slides in old beaker cultures become covered with a thin film of bacteria, the film never becomes as pronounced as in cultures grown horizontally.

Horizontal cultures are often useful, however, as in the control of gaseous environment described below. For a few days, colonies under such conditions are able to grow almost as rapidly as in beaker cultures, but the growth rate falls off rapidly (table 2). If continued, horizontal cultures soon become necrotic, whereas beaker-slide cultures can be grown almost indefinitely.

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

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TABLE 2

Comparison of growth in beaker vs. petri dish

Slide cultures grown in beakers were transferred to either 100 ml beakers or 20 × 100 mm petri dishes containing 80 ml CCS5. The cultures were fed daily and maintained at 22°C, with a second culture solution change. Growth rate was determined from the growth curve to day 9; on day 13 the colonies were dissected to obtain accurate counts of hydranth number.

Culture container	No. of hydranths on day:										Growth rate (k)
	1	2	3	4	5	6	7	8	9	13	
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

on slides which have been pre-washed, etched in hydrofluoric acid or strong alkali, or on pieces of Lucite plastic.

Culture solution. CCS5 and CVD have been used interchangeably in the maintenance of stock cultures and in growing up young colonies; the growth rate is not significantly influenced by the water used or the addition of versenate. If CCS5 is prepared in distilled water without the addition of versenate, the colonies fail to grow and become necrotic. Though the cause of this has not been traced, it is probably copper, as is the case with hydra (Chalkley and Park, '47; Loomis and Lenhoff, '56). If cupric chloride is added to CCS5 to a concentration of 10^{-6} M, prey capture is much reduced and breaks develop in the coenosarc tissue. 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. *Cordylophora* is a carnivore, and must be fed living food. It would be difficult to find a more suitable source than washed larvae or nauplii of the brine shrimp *Artemia*, introduced as food for coelenterates by Crowell ('53), Hauenschild and Kanellis ('52), and Loomis ('53). Large quantities of dried *Artemia* eggs may be purchased, and these eggs may be readily hatched to produce virtually unlimited quantities of uniform food. Various other organisms have been tested; all were less suitable for handling as a food source and none gave growth at a better rate than washed *Artemia* larvae. For example, the white worm *Enchytraeus*, recommended as a nutrient for *Cordylophora* by Kinne ('56, '58a, personal communication) has to be cut up into packets of the right size and hand fed individually to each hydranth. In one experiment, *Cordylophora* fed *Enchytraeus* daily grew with a k of 0.15 while cultures fed *Artemia* grew with a k of 0.22.

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. Three different lots of *Artemia* eggs (probably representing different species, Dempster, '53) were compared in repeated experiments and found to give similar growth rates.[6] *Artemia* eggs were sterilized by the method of Provasoli and Shiraishi ('59), and hatched in autoclaved A solution; the *Cordylophora* cultures fed sterile nauplii grew at rates similar to those of control cultures (e.g., growth rates of 0.22 vs. 0.19).

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

[6] One of the many batches of *Artemia* eggs that have been used led to very poor growth of *Cordylophora*, but permitted growth of *Hydra littoralis* at a normal rate. The cause of difficulty was not traced.

falls off sharply. If the second medium change is omitted in dense cultures, they become necrotic and regress.

Since the beaker-slide cultures grow without agitation, it seemed possible that localized gradients might accumulate in the microenvironment around the colonies, and that such gradients might stimulate or inhibit growth (cf. Osgood and Krippaehne, '55). However, colonies agitated 15 times a minute on an improvised shaker grew at the same rate as standing cultures.

III. *Ionic requirements*

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. The results of a single set of experiments are shown in figure 5.

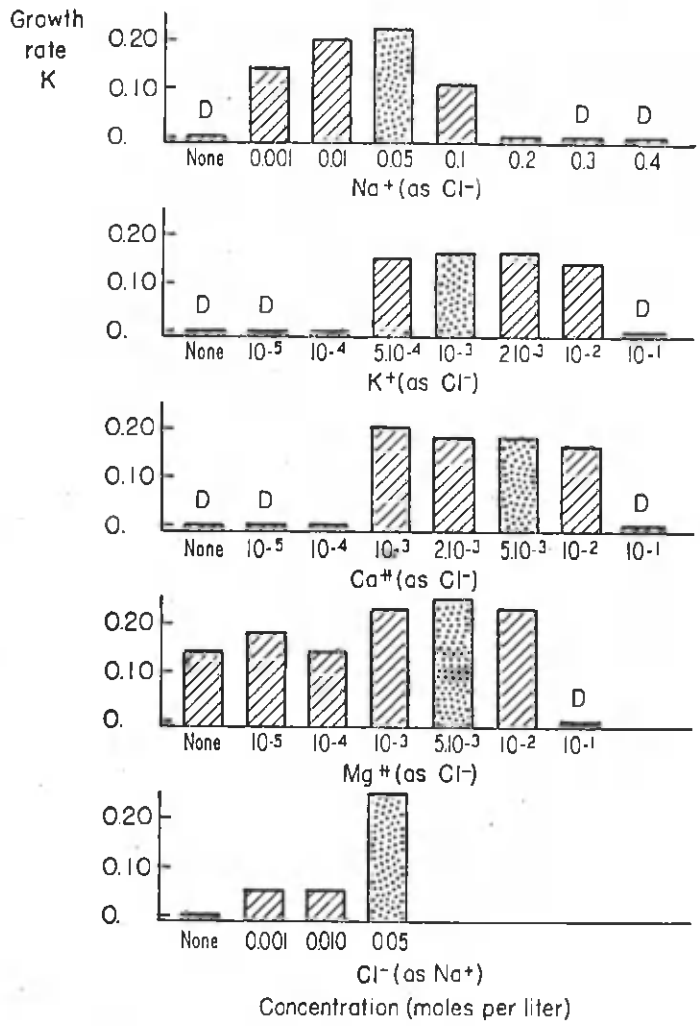

Fig. 5 Influence of the ionic constituents of CCS5 on the growth rate of *Cordylophora*. The histograms indicate the growth rates observed with varying concentrations of each required ion. The basic solution contained NaCl, 0.05 M; $KHCO_3$, 0.001 M (or KCl and $NaHCO_3$, each 0.001 M); $CaCl_2$, 0.005 M; and $MgCl_2$ 0.005 M. To evaluate the cation requirements, NaCl, KCl, $CaCl_2$, or $MgCl_2$ were individually varied. The chloride requirement was determined as described in table 3, Experiment II. In the graph, a "D" indicates that the hydranths regressed; the stippled blocks indicate the approximate ionic composition of CCS5.

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Na^+ requirement. Sodium ions are an absolute requirement for the growth of *Cordylophora*. In the absence of sodium ions, the ability of 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. 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} M KCl is insufficient to allow continued maintenance of hydranths. 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 original upright of the colony.

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

Ca^{++} requirement. In the absence of sufficient calcium ions, the tissue gradually dissociates. Low calcium (10^{-4} M) 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 is to bind the cells together, as has been suggested for other systems (cf. Steinberg, '58). In most unicellular organisms the Ca^{++} requirement is low (cf. Eagle, '56), whereas the cells of metazoan tissues tend to dissociate in the absence of calcium. Calcium is the only required ion the absence of which results in dissociation rather than resorption of hydranths.

Mg^{++} requirement. The results from one experiment in which the concentration of magnesium ion was varied are presented in figure 5. In the absence of added magnesium growth continued, but at about half the rate of cultures to which magnesium was added. In another experiment, in which colonies were grown from one to about 70 hydranths, the control culture grew with a $k = 0.20$ while the culture in magnesium-free CCS5 grew for 24 days with a $k = 0.12$. Similar results have been obtained in six 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, '56) or from *Artemia* nauplii.

Excess magnesium causes dissociation of the hydranth tissue.

Cl^- requirement. Determination of a chloride 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, '56). It was possible, however, to compound suitable media for *Cordylophora*, and to demonstrate an absolute requirement for chloride (table 3). In the first experiment illustrated, the culture solution was more dilute than CCS5, but adequate for growth. All relevant combinations of the ingredient anions were 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, gave 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 *Artemia* larvae.

Conclusion. 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 (fig. 5).

TABLE 3

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. (M)	Na+ as		Ca++ as		No. hydranths on day:							Growth rate (k)
	Cl^-	$SO_4^=$	Cl^-	NO_3^-	1	2	3	4	5	6	7	
0.012	+		+		12	13	15	17	19	24	25	0.13
0.002		+	+		13	16	17	19	21	24	27	0.13
0.010	+			+	10	11	11	12	15	18	20	0.12
0.000		+		+	8	8	9	9	9	9	9	0.00
0.001	+	+		+	9	10	12	13	14	16	18	0.12
0.010	+	+		+	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. (M)	Na+ as		No. hydranths on day:							Growth rate (k)
	Cl^-	$SO_4^=$	1	2	3	4	5	6	7	
0.000		+	18	19	16	16	16	16	16	0.00
0.001	+	+	13	14	17	16	17	17	17	$<$ 0.10
0.010	+	+	12	14	16	16	18	18	22	$<$ 0.10
0.050	+		13	18	21	26	34	37	45	0.25

Roch's medium. The work of Roch ('24) includes the only previous study of the ionic requirements of *Cordylophora*. Roch found that if Na^+, K^+, or Cl^- were omitted from a dilute artificial sea water, the colonies perished. Mg^{++} ions were found dispensable, and "calcium salts present a certain impediment for the development of *Cordylophora*" (p. 371). Since Roch's methods of observation were qualitative (see Discussion), it is not surprising that no influence of magnesium ions was detected, but his observation that calcium ions, if anything, were harmful disagrees with the observations reported here, as well as with the general observation that calcium is required for the maintenance of metazoan tissue integrity.

The effect of calcium on the growth of colonies in Roch's medium has been reexamined. In the complete absence of calcium, or in 10^{-5} M calcium, the hydranths rapidly dissociated, as they do in calcium-deficient CCS5 (table 4). The composition of Roch's medium does not mitigate the calcium requirement; in Roch's experiments enough calcium probably was present as an impurity in his calcium-free medium to permit survival.

Slow growth usually occurred in Roch's medium whenever the concentration of calcium ions was adequate; the addition

TABLE 4

Growth of 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 the same as Roch's Ca-free medium ('24, 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	$NaHCO_3$ M	No. hydranths on day:						Growth rate (k)
		1	2	3	4	5	6	
10^{-5}	—	1	0					0.00
5×10^{-3}	—	23	25	25	30	35	38	$<$0.1
5×10^{-3}	10^{-3}	10	12	20	29	32	43	0.26

of a buffer permitted growth in Roch's medium at rates comparable to those observed in CCS5 (table 4). Probably the absence of a buffer in Roch's medium accounts for the apparent toxicity of Ca^{++} he observed. His medium, containing Na^+, K^+, Mg^{++}, and Cl^- in amounts that should be adequate on the basis of studies with CCS5 (cf. table 4 and fig. 5), permits growth at a rate similar to that observed with CCS5 if Ca^{++} and a buffer are added.

Trace requirements. A 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 replaced without effect (cf. below, table 6). Other cations and anions, added to CCS5 at subtoxic concentrations, have not influenced growth rate. Natural sea water, a rich source of trace ions, gives the same growth rate as artificial sea water (Harvey, '56) at the same concentration. 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 (Edsall and Wyman, '58). The situation is similar to that observed by Eagle ('56), where no trace requirements could be demonstrated for mammalian cells in culture in media containing 1 to 5% dialyzed serum. *Artemia* nauplii must supply *Cordylophora* with an adequate source of trace ions, as well as other required, although completely unknown, nutrients.

IV. *Ionic interactions*

Since the experiments reported have indicated requirements for five ions, there could be as many as ten interactions of pairs of ionic species. Two of the more likely of these have been examined.

Na^+ vs. K^+. With CCS5 as base, growth ceases completely when the concentration of NaCl reaches 0.2 M (fig. 5). Yet it has been known for many years, both from observations in nature and in the laboratory (cf. Roch, '24; Kinne, '56), that *Cordylophora* will tolerate a wide range of salinities. This has been confirmed for clone A by evaluating growth of colonies in serial dilutions of artificial sea water (Harvey, '56) in demineralized water; all dilutions were adjusted to 0.001 M $NaHCO_3$. Colonies died in 0.0% sea water, and grew very slowly in 2.5% sea water, but in 5, 10, 20, 40 or 80% sea water they grew about equally well, with a mean growth rate of 0.20 and a range from 0.17 to 0.23 in one experiment. The NaCl concentration of 80% sea water is 0.34 M.

To determine which constituents of CCS5 become limiting as NaCl is increased, a medium was prepared having the composition of CCS5 except for a 5-fold increase in NaCl from 0.05 to 0.25 M. Each other constituent of the medium was then varied; only K^+ could reverse the effect of high Na^+ (table 5). If the concentration of Na^+ was increased 5-fold, the concentration of K^+ also had to be increased 5- to 10-fold to permit growth, indicating a definite interaction between Na^+ and K^+ (cf. MacLeod and Snell, '48).

Ca^{++} vs. Mg^{++}. Interactions between calcium and magnesium ions are encountered frequently, and since growth

TABLE 5

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[1]	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

[1] Concentration of KCl in CCS5.

will continue in a medium without added magnesium (fig. 5), it was of interest to see what effect a magnesium-free medium would have on the calcium requirement. The results of such experiments showed that Ca^{++} is required in the same amounts in the absence as in the presence of Mg^{++}, thus failing to indicate any interaction between the two divalent cations.

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. The study has already yielded three such media: CCS5, which contains 0.05 M NaCl; a medium containing 0.01 M NaCl (table 3, Expt. I); and one containing 0.25 M NaCl (table 5). All three solutions permit growth at a similar rate.

V. *Hydrogen ion concentration*

For study of the influence of pH on growth, phosphate was used to buffer CCS5 below pH 7 and Tris for pH 7 and above. Neither buffer exerted any significant influence on growth rate (table 6). The growth of *Cordylophora* colonies is quite indifferent to the pH of CCS5, the growth rate being very similar between pH 6.3 and 8.6 (table 6). At pH 5.1 the colonies survived but failed to grow.

VI. *Physical factors influencing growth*

Temperature. Kinne ('56) found that *Cordylophora* colonies remain healthy from about 8 to 24°C, and that the range tolerated is influenced by salinity (% sea water). In CCS5, the hydranths are resorbed at temperatures below 8°C, but the coenosarc remains viable for extended periods and can regenerate hydranths on return to a favorable temperature. From 10 to 14°C, the colonies remain healthy but grow very slowly (table 7).

The most useful range for growth is 18 to 26°C; a number of experiments have revealed no striking difference in the growth rates obtained within this range of temperatures. In one experiment, for example, in which successive 2°C intervals were compared from 18 to 26°C, growth rates ranged, without order, from 0.27 to 0.30. It is possible that slight differences in growth rate between 18 and 26°C have been masked by the variability of k.

Clone A can grow slowly at 30°C in beaker-slide cultures if the medium is changed twice daily, but at higher temperatures the hydranths regress (table 7).

Light. Allman (1872) considered *Cordylophora* to be a "light-shunning animal," and others, including Roch ('24) 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. For example, a pair of colonies were grown at room temperature (22–25°C) about 4 inches from a 30 watt daylight fluorescent bulb, left on continuously. One culture,

TABLE 6

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 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 (k)
		1	2	3	4	5	
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

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TABLE 7

Influence of temperature on growth of Cordylophora

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

Temperature	No. hydranths on day:							Growth rate (k)
	1	2	3	4	5	6	7	
°C								
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

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 lighted culture grew with a k of 0.22, the other with a k of 0.19.

Oxygen tension. Study of the influence of oxygen tension on growth has been accomplished by adapting a method of Loomis ('59). *Cordylophora* colonies, growing on microscope slides, were placed in petri dishes and covered with CCS5 to a depth of about 1 cm. These dishes were placed individually in vacuum desiccators having a volume of about 2,000 ml (Corning no. 3118); 50 ml of water or appropriate solution was placed in the bottom of the desiccators to maintain humidity or remove traces of gases. The desiccators were evacuated with a water aspirator attached to a mercury manometer, and refilled with appropriate gas mixtures to atmospheric pressure. After refilling, the desiccators were placed on a rocker table attached to a synchronous motor which rocked the table once every 30 seconds, assuring constant exchange between the culture and gaseous phase. Every 24 hours the dishes were removed for feeding, counting, and solution change, and then returned to the desiccators where the appropriate gas mixtures were re-established.

In the first experiment, oxygen tension was varied simply by growing colonies in mixtures of nitrogen and oxygen (table 8). Growth was clearly reduced in the absence of added oxygen, but was continuous throughout the experiment. One per cent oxygen permitted growth at a slightly reduced rate, whereas 4% oxygen (about 40% saturation) gave maximal growth.

This experiment indicated a low oxygen requirement; a second experiment was performed to determine if removal of all oxygen would reduce growth to zero. The oxygen absorbant selected, alkaline pyrogallol, removes not only O_2 but also CO_2 from the atmosphere. The experimental design included three desiccators, containing (a) alkaline pyrogallol (Umbreit et al., '57), (b) alkali (40% KOH), and (c) water. After each feeding, the desiccators were evacuated and refilled with nitrogen, and sealed with silicone grease and bunsen valves (Umbreit et al., '57). The results are presented in table 9. In

TABLE 8

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:						Growth rate (k)
		1	2	3	4	5	6	
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[1]	152	15	20	22	25	29	35	0.17

[1] Approximately the oxygen tension of air.

TABLE 9

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

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. Those hydranths which survived seven days of anerobiosis were highly contracted when first removed from the desiccator, but as oxygen re-entered the culture solution, they expanded and became able to capture and eat *Artemia* nauplii. There was no 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.[7]

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

VII. *Nutrition*

Because *Artemia* larvae have been selected as a suitable food source, the only nutritional variable is the amount that colonies are fed. This may be varied by (1) the length of time cultures are left with food, (2) the intervals between feeding, or (3) the number of larvae fed to each hydranth at each feeding (cf. Crowell, '57). Since the last is difficult to control with *Cordylophora*, cultures were fed to repletion with *Artemia* larvae during each feeding period.

The length of time cultures are left with food has no measurable influence on growth rate. Most hydranths capture a repletion level of *Artemia* (about 20–30 nauplii per hydranth) within the first few minutes after feeding. Colonies exposed to food for 15, 30, 60, or 120 minutes all grew at similar rates.

Variation of the length of time between feedings produces a dramatic effect on growth rate (table 10). In CCS5 at 22°C, one 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

[7] In three experiments *Cordylophora* has grown with a reduced k in CO_2-free air vs. normal air, but the effect has not been striking. Further increases of CO_2 above the level found in air (0.03%) have not enhanced growth, and Loomis ('61) reported that CO_2 above 1.5% inhibited the growth of *Cordylophora*. The difference between no CO_2 and traces of CO_2 under partial anaerobiosis (table 9) is similar to observations of Cohn and Horibata ('59), who found that *Escherichia coli* would not grow anaerobically in glucose unless CO_2 was added.

TABLE 10

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

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DISCUSSI

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[1] Lenhoff and Bovair

hydranth number to a steady state level which is maintained for an extended period.

DISCUSSION

The major advances of the culture method are (1) the growth of 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 ('54). The use of the exponential growth rate, k, has permitted quantitative evaluation of conditions influencing growth.

The method may be compared with those used by the two workers who previously studied laboratory cultures of *Cordylophora*. Roch ('24, p. 366) indicated 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 ('56, '58a), 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. The water was changed every few days. Kinne presents several growth curves, plotted linearly, which permit estimation of the growth rate he obtained. The most rapid growth was exhibited by one culture (Kinne, '58a, fig. 9) which grew with a doubling time of five days, giving a k of 0.14. Kinne's conditions, on the basis of my observations, were suboptimal.

The only similar organism (in a broad sense) to *Cordylophora* for which data on the growth parameters are available is *Hydra littoralis* (Loomis, '54). The two hydroids show essentially no differences in tolerances or optima to a variety of environmental variables — pH, temperature, oxygen tension, or rate of feeding (table 11). As would be expected (since *Hydra* is restricted to fresh water), *Cordylophora* can tolerate a tenfold or more greater salinity than *Hydra*. The major difference in growth requirements is in the aqueous environment. *H. littoralis* requires significant amounts only of Ca^{++}, and traces of Na^{+} (Lenhoff and Bovaird, '60) for growth.[8] The requirement for Ca^{++} is

[8] Ham, Fitzgerald and Eakin ('56) report that *Pelmatohydra oligactis* requires Na^{+} and K^{+} in addition to Ca^{++} for growth. These authors also found that *H. littoralis* requires Na^{+} and K^{+} for regeneration. Several workers have found recently that Mg^{++} substantially increases the growth rate of *Chlorohydra viridissima* (in Muscatine, '61).

TABLE 11

Comparison of growth conditions for Hydra littoralis and Cordylophora lacustris (Data for *Hydra* from Loomis, '54)

Factor compared	*Hydra*	*Cordylophora*
Organization	solitary	colonial
Growth curve	exponential	exponential
Growth rate (mean k)	0.37	0.23
Ionic requirements for growth		
a. absolute	Ca^{++}	Na^{+} K^{+} Ca^{++} Cl^{-}
b. less critical	Na^{+}[1]	Mg^{++}
c. Ca^{++} conc. required (M)	10^{-4}	10^{-3}
Range for growth		
a. hydrogen ion conc. (pH)	5.3 to 8.7	6.3 to 8.6
b. NaCl conc. (M)	traces[1] to 0.03	$<$ 0.001 to $>$ 0.25
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

[1] Lenhoff and Bovaird ('60).

about tenfold higher in *Cordylophora* than in *Hydra*. It would appear that *Hydra* is able to retain enough of the K^+, Cl^-, Mg^{++}, and most of the Na^+ obtained from *Artemia* to suffice for continued growth in their absence in the aqueous milieu, whereas *Cordylophora*, being a more open system with respect to these ions, must have them continually supplied in the aqueous environment.

The composition of the aqueous environment is probably the critical variable determining the ability of *Cordylophora* to live in a given body of water. *Cordylophora lacustris* has been found throughout the world, in habitats varying in their salinity from fresh to almost sea water (Roch, '24; Hand and Gwilliam, '51). The tendencies of fresh water to contain high calcium and carbonate but very little sodium and chloride (Hutchinson, '57, p. 555) may be a major factor in restricting *Cordylophora* to relatively few fresh water localities, while *Hydra* can be found in most fresh-water ponds.[8] 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 5). Although the requirements for colony growth include all the major ions of sea water except sulfate and bicarbonate, *Cordylophora* cannot grow in sea water because the total salt concentration is too great.

The typical habitat for *Cordylophora* appears to be that given by Allman (1853) in his original description of the species: "In fresh, calm water, living on various submerged objects, and preferring dark places." But *Cordylophora* has been found in strikingly different habitats, such as that described by Clarke (1878) in Baltimore, Maryland, where the hydroid grew "in the channel where the sunlight is strongest, . . . where the current is most rapid . . . , and changes in the surrounding conditions must be greatest." This 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 factors found in most bodies of water (Hutchinson, '57). The only other frequent limiting factor would be the amount of prey organisms available as food. The cosmopolitan nature of *C. lacustris* would support the idea that many bodies of water meet the necessary requirements.

SUMMARY

1. Under appropriate conditions, the hydranth number of cultures of the colonial hydroid *Cordylophora lacustris* increases exponentially, with a doubling time of about three days. This growth rate was used to examine the environmental variables influencing growth.

2. These sessile organisms grow well attached to microscope slides slanted in beakers if their standing aqueous environment is replaced twice daily, but grow poorly on a horizontal substratum or with a single daily solution change.

3. In a defined culture solution, *Cordylophora* shows an absolute requirement for Na^+, K^+, Ca^{++}, and Cl^- for growth, and in addition requires Mg^{++} for growth at a maximum rate. Sodium and potassium ions interact and must be in proper proportion for growth to occur.

4. These carnivores are fed *Artemia* larvae to saturation at each feeding; a maximum growth rate is obtained with one feeding per day.

5. The growth of colonies is relatively indifferent to several physical variables. Light is without measurable effect on growth, and the growth with wide variations in p 6 to 9) and temperatu *Cordylophora* has a real, l requirement for growth.

6. Growth conditions f are similar to those for with the exception of ion and help to explain the *Cordylophora* in nature.

[8] The water of Nye Pond, from which *Cordylophora* clone A was collected, has a very low salinity; analysis of the chloride ion of three different samples gave values of 2, 7, and 4 milliequivalents of Cl^- per liter. A more 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; the other ions were determined by titration. The results, in milliequivalents per liter, were: Na^+, 1.56; K^+, 0.123; Ca^{++}, 0.20; Mg^{++}, 0.83; Cl^-, 3.96; SO_4^{--}, 0.65; CO_3^{--}, 0.00; and HCO_3^-, 0.16. The anions are 21% 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. Further, the composition of the water cannot be expected to remain constant, since the pond receives some backflow of sea water at spring tides. In the laboratory, cultures grow slowly either in water from Nye Pond or in a solution based on the analysis.

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Hand, C., and G. F. Gw tributional records for

velopment of *Cordylo-*
ce the organism is re-
'e to temperature, pH,
ght, etc., and should
range of variation of
in most bodies of water
The only other fre-
r would be the amount
ıvailable as food. The
: of *C. lacustris* would
:hat many bodies of
:ssary requirements.

:MARY

riate conditions, the
f cultures of the co-
ylophora lacustris in-
ly, with a doubling
: days. This growth
xamine the environ-
fluencing growth.
ɔrganisms grow well
ɔpe slides slanted in
ling aqueous environ-
ɔice daily, but grow
l substratum or with
n change.
lture solution, *Cordy-*
olute requirement for
Cl^- for growth, and
Mg^{++} for growth at a
ium and potassium
t be in proper propor-
ur.
:s are fed *Artemia*
at each feeding; a
te is obtained with

:olonies is relatively
physical variables.
:asurable effect on

from which *Cordylophora*
very low salinity; analysis
e different samples gave
ıivalents of Cl^- per liter.
one sample of Nye Pond
um and potassium were
photometry through the
:reen of Rutgers Univer-
determined by titration.
ıts per liter, were: Na^+,
Mg^{++}, 0.83; Cl^-, 3.96;
and HCO_3^-, 0.16. The
icating some quantitative
ıults are atypical of fresh
ıajor cation and chloride
the composition of the
remain constant, since
:kflow of sea water at
:y, cultures grow slowly
d or in a solution based

growth, and the growth rate is similar with wide variations in pH (from about 6 to 9) and temperature (18–26°C). *Cordylophora* has a real, but low, oxygen requirement for growth.

6. Growth conditions for *Cordylophora* are similar to those for *Hydra littoralis*, with the exception of ionic requirements, and help to explain the distribution of *Cordylophora* in nature.

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