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The constant culture of *Stentor coeruleus.*

By

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(With a chart in the text.)

Introduction.

Stentor coeruleus has proven a difficult object to control in cultures maintained for experimental investigation. The difficulties, particularly in attempts at isolation culture, are brought out in the papers by HARTMANN (1921) and by STOLTE (1922).

The technique described here involves not only a constant environment, but also one which is favourable to such an extent that the organisms look and act like those in the normal environment.

Material.

The *Stentors* used in these studies were collected from vicinities near Woods Hole, Mass., Stanford University, California, and the University of Chicago. The organisms used in this work were genetically heterogeneous, although no consistent differences were observable.

Method.

The series of media whose description follows were tested for *Stentor* with a uniform technique. The criteria of suitability were: (1) division-rate in isolation over a period of seven days, and (2) behaviour of the *Stentors* in Mass-culture. Mass-cultures were made up of a few organisms in about 100 cc. of medium to which was added a slight excess of food. All isolations were made in

1.5 cc. of medium to which was added .02 cc. concentrated (centrifuge) *Colpidium campylum*. Transfers were made every 24 hours. The isolation dishes were kept in moist chambers in which the temperature varied between 18° and 20°. No attempts were made to keep the isolations sterile but the dishes, pipettes, etc., were kept uniformly clean. The intervals required for division in these isolations are marked off against time in the chart (p. 124/125). Each vertical line represents an organism in isolation, the figures intervals required for division.

The Medium.

Water. Ordinary distilled water is invariably toxic. Double distilled water (Pyrex or block tin delivery) is satisfactory if the first and last fractions are discarded. Results were checked with charcoal-treated conductivity water described by Daniel (1908).

I. Knop's Solution.

$Ca(NO_3)_2$	6.8 %	10. cc.	
KNO_3	5. „	3.3 „	To be diluted to a
$MgSO_4$ $7H_2O$	5. „	5. „	total salt concentra-
KH_2PO_4	5. „	5. „	tion of .05 %.
KOH (.05 M.) to p_H of 6.8			

This is suggested by Bělař (Péterfi, 1928) as a medium for fresh-water ciliates. One third of the KNO_3 of the Knop's solution was omitted and .05 M. KOH was added to regulate the p_H to 6.8. The chart (p. 124/125) shows the intervals required for division of five isolations over a period of seven days. These were chosen as representative out of the thirty-six which were run. The *Stentors* fail to survive consistently in this medium.

II. Balanced Physiological Medium (BM). .0937 M. solutions are combined in the following proportions:

1000 NaCl	
78 $MgCl_2$	
38 $MgSO_4$	To be diluted ten times, to a
22 KH_2PO_4	total salt concentration of .06 %.
20 $CaCl_2$	
.05 M. NaOH to p_H of 6.8	

Osterhout (1906, 1907), has shown that a variety of fresh-water organisms will tolerate this medium even when the total salt concentration is as high as .6 %. *Stentor* will not survive in a

concentration above .1 %. (Fresh waters are commonly about .01 % total salt.)

The chart indicates irregular division intervals. The first four isolations were transferred every eight hours, but all others are twenty-four hour transfers. The organisms appear to feed for a period, then remain quiescent for hours. The behaviour in mass-culture is one of increase for twenty to forty hours followed by death of a portion and the appearance of dwarf and pale individuals. Even if transferred daily some of these aberrant forms persist: *Stentor* cannot be maintained for more than a few months in this medium.

Hydrogen-ion concentration. In an attempt to discover the essential requirements of *Stentor* a series of infusions (hay, wheat, lettuce) were made up and inoculated from a variety of natural waters. The observation of PETERS (1904) that *Stentor* invariably appears on the alkaline side of the infusion-cycle was confirmed. Further, a rather sharp increase in the numbers as well as an improvement in appearance was observed at a p_H of approximately 7.7. Those with a p_H of 7.7 to 8 supported great numbers of *Stentors* of excellent appearance. It had previously been determined that the common forms appearing in the acid (earlier) phase of the cycle (*Colpidium*, *Glaucoma*, *Paramecium*, etc.) are readily consumed by *Stentor*. Accordingly the NaCl of the BM. was replaced by (.0468 M.) Na_2HPO_4 giving an effectively buffered medium of approximately p_H 8. This proved toxic to *Stentor*, killing it in a few hours.

PETERS' Medium.

$CaCl_2$	.00055 M.
$NaNO_3$	.00015 „
$MgSO_4$	.00015 „
K_2HPO_4	.00015 „

PETERS (1904) modified PFEFFER's medium (a nutritive medium for plants) for *Stentor*. The p_H of this medium is exceedingly unstable, varying according to contact with air from p_H 7.5 to 6.8 (roughly). This is doubtless a reason why PETERS failed to achieve a dependable culture medium.

III. PETERS' Medium (Modified). In order to overcome this difficulty the medium was modified, the anions being introduced as bicarbonates as follows:

$Ca(HCO_3)_2$	.00055 M.	
$NaHCO_3$	.00015 „	$p_H = 8.$
$Mg(HCO_3)_2$	.00015 „	
$KHCO_3$	.0003 „	

The chart shows the intervals repuired for division of five isolations in this medium. The division-rate is regular. The behaviour in mass-culture is uniform. No aberrant specimens appear, and they will remain normal in appearance for over a month when starved. This medium proves effectively buffered for the twenty-four hour isolation interval, a change in p_H of about .1 (Phenol Red) being sometimes appreciable if the dishes are kept over for forty-eight hours.

IV. Comparison with Balanced Medium. Simultaneously a series of five isolations were run on BM. buffered to a p_H of 8 by partial replacement of the NaCl with $NaHCO_3$ as follows: NaCl 750; $NaHCO_3$ 250.

Division rate is slow as well as conspicuously irregular. The organisms in the original BM. were undoubtedly stimulated to rapid division which could not be maintained. The fact that they tended to die off in the mass-cultures indicates the very unstable equilibrium with the environment involved. In this alkaline BM. the *Stentors* behave uniformly in mass-culture, but the average size is somewhat smaller than in PETERS' medium.

V. PETERS' Ca-K Medium. Particular interest attaches to the following, which PETERS considers favourable, but not as satisfactory as the four-salt system:

$Ca(HCO_3)_2$	.00055 M.	(The anions are again modi-
$KHCO_3$	.00045 „	fications).

This medium compares favourably with the four-salt medium as shown by the chart. Since the K was present in the original as K_2HPO_4 PETERS had a more alkaline system here, and one in which the K was more concentrated than the Ca. The K was deliberately halved for the above experiment. (See discussion.)

Preparation of PETERS' Medium. An aqueous medium is desired in which:

$$[Ca^{++}] = .00055 \text{ M.}$$
$$[K^{+}] = .0003 \text{ „}$$
$$[Mg^{++}] = .00015 \text{ „}$$
$$[Na^{+}] = .00015 \text{ „}$$
$$[H^{+}] = .10^{-8}$$

and containing as large a concentration as possible of HCO_3^- and $CO_3^=$ to act as buffers to maintain the $[H^+]$. The equivalents of positive ions are therefore to be:

$$\begin{aligned} 2[Ca^{++}] &= .0011 \text{ N.} \\ [K^+] &= .0003 \text{ „} \\ 2[Mg^{++}] &= .0003 \text{ „} \\ [Na^+] &= .00015 \text{ „.} \end{aligned}$$

The total number of equivalents of cations in one liter $= 1.85 \times 10^{-3}$ must equal the total number of equivalents of anions.

$$\therefore 2[CO_3^=] + [HCO_3^-] = 1.85 \times 10^{-3} \qquad \text{I}$$

According to the second ionization constant of H_2CO_3 (at 25°):

$$\frac{[10^{-8}][CO_3^=]}{[HCO_3^-]} = 3.7 \times 10^{-11} \text{ [1]}$$

$$\text{Thus } [CO_3^=] = 3.7 \times 10^{-3} [HCO_3^-]. \qquad \text{II}$$

Substituting equation II in I:

$$[.0074][HCO_3^-] + [HCO_3^-] = 1.85 \times 10^{-3}$$
$$[HCO_3^-] = 1.84 \times 10^{-3}$$
$$[CO_3^=] = 6.8 \times 10^{-6}.$$

This $[CO_3^=]$ is not large enough to exceed the solubility product of $CaCO_3$ at 18° i. e.:

$$[.00055][6.8 \times 10^{-6}] < 10^{-8}.$$

At 25° the first ionization constant of H_2CO_3 is:

$$\frac{[10^{-8}][HCO_3^-]}{[H_2CO_3]} = 3.5 \times 10^{-7} \text{ [1]}$$

$$\therefore [H_2CO_3] = \frac{[HCO_3^-] \times 10^{-6}}{3.5 \times 10^{-7}} = \frac{[HCO_3^-]}{35} = 5.2 \times 10^{-5}.$$

This concentration of H_2CO_3 i. e. of dissolved CO_2, is only .0013 times that in equilibrium at 18° with CO_2 gas at a pressure of 74.5 cm. of Hg., namely .0406 M. According to HENRY's law this solution would be in equilibrium with CO_2 gas at about a pressure of $.0013 \times \frac{74.5}{76.0} = .0013$ atmosphere.

In other words the solution described is in equilibrium with air containing about .13 volume % of CO_2 (DALTON's law of partical pressures). This partial pressure could be maintained by making up the water at the bottom of the moist chamber as a similar but more concentrated bicarbonate system. Without this, however, in

[1]) International Critical Tables VII p. 244.

the presence of *Stentor* and food organisms, the isolation volumes suffer no appreciable change in p_H (Phenol Red) in the 24 hour interval. Probably the tendency to become more alkaline (assuming the moist chamber air is less than .13 % CO_2) is balanced by the CO_2 produced by the organisms.

To maintain a $[H^+]$ of 10^{-8} the $[CO_3^=]$, $[HCO_3^-]$, and $[H_2CO_3]$ must be in the ratios calculated. The absolute amounts of each may be increased or descreased if desired. An increase in the total cation concentration must of course accompany in the case of an increase, while a decrease may be accomplished either by descreasing the cation concentrations or substitution of another anion such as Cl^-.

The medium is made up as follows: (1) 2.65 L. of CO_2-free water is prepared by boiling and allowing to cool without disturbing. (2) 100 cc. of water is saturated with CO_2 at 18°. (3) The magnesium carbonate and 100 cc. of water saturated with CO_2 are added. After the few minutes required for solution of the magnesium the hydroxides and carbonates of the other metals are added as summarized below:

	Mols/L	g/2.75 L	Mols/L H_2CO_3 req.
$Ca(OH)_2$	.00055	.112	.0011
K_2CO_3	.00015	.057	.00015
$MgCO_3$	.00015	.035	.00015
Na_2CO_3	.000075	.021	.000075
	.000925	= .0082 %	.001475

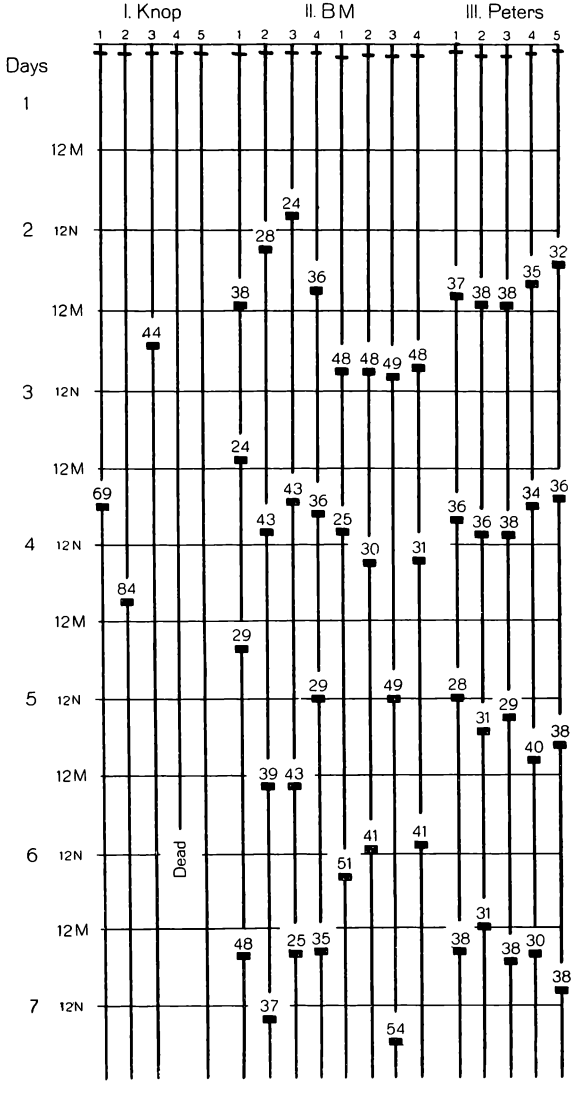
The calcium may be added as 69.5 cc. of the saturated solution (20°) if desired. The two-salt medium (Ca-K) is made similarly with the omission of the magnesium and sodium.

VI. Control. A control medium was attempted by filtering several lettuce cultures at a time when they were eminently favourable to *Stentor* through a Seitz Bacteriological filter [1]), thus deriving a clear sterile filtrate which could be preserved in the cold-room. The *Stentors* did not divide very uniformly in this medium (see chart) probably because the organic matter present supported some bacterial metabolism following introduction into the isolation dishes. The mass-cultures were similar to those of Peter's media.

[1]) Great care must be exercised in the use of this filter if sterile filtrates are desired. They should always be checked for sterility after allowing to stand a day or two.

The Food.

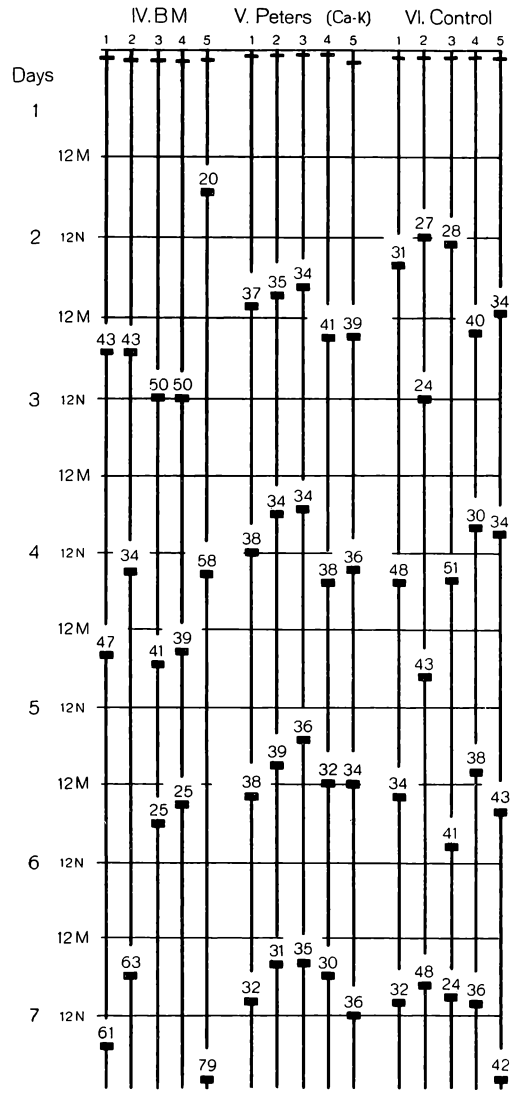
In order to achieve a constant environment the technique suggested by BĚLAŘ (PÉTERFI, 1928) of feeding the *Stentors* with organisms grown in separate cultures was followed.



Autotrophs. BĚLAŘ specifies *Gonium pectorale* grown on KNOP-agar for *Stentor*. This was used in an extensive series of trials. *Gonium pectorale* is eaten sparingly by *Stentor* when hungry but as satiation is approached it is refused altogether. This behaviour was duplicated toward several species of *Euglena*, *Trachelomonas*, and some small diatoms cultivated in the same manner. Two species of *Chlamydomonas* were never ingested by *Stentor*.

Ciliates. In contrast to this reaction to autotrophs *Stentor* ingests avidly many of the ciliates (no Hypotrichida were tried). An exception exists in the form of a species of *Prorodon* whose contact occasions violent contraction and withdrawal on the part of *Stentor*.

Schaeffer (1910) describes a selection by *Stentor* of different autotrophic forms, but does not bring out the general preference for ciliates, nor does he indicate selection within this group.



The following organisms were grown in sterile culture[1]) on Yeast Extract[2]) and tried as food for *Stentor*.

[1]) Sterilizing technique. As outlined by Parpart (1928), with following modifications: 1. No hood used. If air is quiet brief removal of Petri dish cover for transfer is safe. 2. About 1.5 cc. sterile wash medium instead of 6 drops kept in Protozoa Isolation Dishes. 3. Pipettes may be ordinary medicine droppers drawn out and sterilized by flaming. 4. Organisms were introduced at one edge of above volume and removed following migration through sterile fluid at other side. 5. The dishes were kept below 22° during 5 hour delay. 6. Several washed organisms were introduced into final dish and allowed to stand 12 hours before introduction, with surrounding medium, into 10 cc. of sterile Yeast Extract in a test tube.

[2]) Preparation. One pound of pressed yeast (Baker's yeast) is well ground in small amounts at a time in 500 cc. of tap-weter. Place in one liter flask, plug with cotton, and incubate at 50° for 24 hours. Boil and add NaOH to pH 7. (Dilute sample 1 : 10 for this purpose.) Filter and place in small flasks; sterilize at 15 pounds for 15 minutes. In filtering, the first portions must be filtered over; the process requires about 12 hours. This is called Yeast Autolysate. Yeast Extract is a 10% solution.

Colpidium campylum	(OEHLER, 1924)
Chilomonas (*paramecium* ?)	(PRINGSHEIM, 1921)
Glaucoma (*scintillans*?)	(LWOFF, 1924)
Loxocephalus sp.	

Colpidium campylum, washed free of nutritive substance (centrifuge), was the food source for the isolations referred to in the Table. This organism and *Glaucoma* are easily obtained by adding wheat to water and decaying matter collected from ponds in which protozoa abound. Although normally adjusted to an acid environment with great numbers of bacteria *Colpidium* is very resistant and will survive a few days in PETERS' medium.

Chilomonas, a saprophytic Cryptomonad, survives well in PETERS' medium. It seems adequate as a nutritive source for *Stentor*, but is always rejected in favor of ciliates if both are present.

Glaucoma does not grow in the enormous numbers which characterize the other three forms. Its normal environment resembles that of *Colpidium*.

Loxocephalus is well adjusted and is a good food. As in the case of the other forms, it will grow on Peptone glucose also.

Colpidium colpoda [1]) was grown on *Bac. subtilis* in pure culture (wheat grains are satisfactory for supplying food for the bacteria) and used for food. It appears to be more tolerant of the *Stentor*-medium than the smaller *C. campylum*. However, unless very thoroughly washed their addition causes a radical disturbance to the isolations.

Urocentrum turbo grows under the same infusion conditions as, and is readily eaten by, *Stentor*. Unfortunately attempts to cultivate *Urocentrum* failed.

In the case of each organism to be tried as food .02 cc. of the washed and concentrated mass was added to a series of isolations. The actual numbers varied according to size: eg. 500 *Chilomonas* = 150 *Colpidium colpoda*.

Thorough washing of food-organisms, regardless of source, is essential. Extensive bacterial growth promptly kills *Stentor*.

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[1]) Repeated efforts to grow this organism in sterile culture failed. PETERS' media failed (1921). *C. campylum*, which corresponds more closely to PETERS' description of his organism, failed to increase on his media. BRESSLAU (1923) describes the genus *Colpidium*.

Discussion.

That an independent investigation of an optimum salt medium for the fresh water flatworm *Planaria dorotocephala* should arrive at results closely similar to those of PETERS is an interesting and possibly fundamental fact.

Miss MURRAY (1928) found that this planarian would not tolerate the usual balanced media used for Vertebrate tissues; that the proportion of calcium and potassium, not calcium and sodium, is the crucial factor; and that the calcium should exist in quantities about equal to or greater than the potassium. The molecular proportions defining the limits for a favourable medium for *Planaria* are compared with those of PETERS' media.

MURRAY: Max. K 1.6 to Ca 1
Min. K 1 to Ca 11 (in presence of sodium)
PETERS: Max. K 1.6 to Ca 1
Min. K 1 to Ca 1.8

PETERS did not attempt to determine the limits so his figures are merely suggestive. Greater identity as to conclusions could hardly have been expected in view of the contrast in approach of the two investigators.

With regard to the H^+ concentration, Miss MURRAY used $NaHCO_3$ to regulate to a p_H of 7.5.

It must be kept in mind in considering the possible signifigance of these results that of the extensive work on balanced physiological media almost none has concerned the avascular freshwater animals.

The slight growth of bacteria which follows isolation in the small volumes is the important factor limiting the degree of uniformity to be obtained in the technique outlined. The bicarbonate medium cannot be autoclaved, nor is this desirable if it is made up in clean vessels.

The term "constant" is relative: isolation in an inorganic medium is constant relative to isolation in a nutritive medium in which bacteria grow. Even if bacterial growth is negligible, however, the technique outlined is not exactly eonstant, since products of metabolism accumulate during the 24 hour transfer period. Almost any desired degree of constancy could be obtained by the use of a string siphon for the continuous addition and removal of medium as described by PETERS (1901).

Just as the Balanced Physiological Medium is roughly similar to sea-water, so is the four-salt medium of PETERS when modified to a bicarbonate system typical of many fresh waters. Sometimes the potassium of fresh waters is much lower in proportion to the calcium, but this is always in the presence of a complex of other salts including those of sodium and magnesium which may tend to balance the system.

I wish to express my appreciation of the facilities enjoyed at the Whitman Experimental Laboratory of the University of Chicago through the courtesy of Prof. W. C. ALLEE.

I am indebted to Dr. T. F. YOUNG of the department of chemistry for help with the preparation of PETERS' medium, and to Dr. S. A. KOSER of the department of bacteriology for testing my sterile cultures.

Summary.

1. Artificial media for *Stentor* must be made up in conductivity water.

2. *Stentor* will not survive in KNOP's solution.

3. *Stentor* is not adjusted to dilute artificial sea-water.

4. It is adjusted to a cation ratio of: Ca 2 to K 1.

It will tolerate at least a 100 % alteration in this proportion.

5. A possible general signifigance of this system is indicated.

6. *Stentor* is adjusted to a p_H of 7.7—8.

7. It will not tolerate .0045 M. $HPO_4^=$.

8. In general, it prefers ciliates to autotrophic forms for food.

9. Many of the common ciliates make satisfactory food for *Stentor* in laboratory culture.

10. Bacterial growth is generally harmful to *Stentor*.

11. *Stentor* will divide regularly every 28—40 hours in isolation when supplied with an excess of a suitable food in PETERS' medium (Vol. 1.5 cc., temp. 19°).

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