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SOME FACTORS AFFECTING THE FORMATION OF COLONIES IN *GONIUM PECTORALE*

LYNN B. GRAVES, JR.* AND WENCEL J. KOSTIR

Department of Zoology and Entomology, The Ohio State University, Columbus 10

INTRODUCTION

Gonium pectorale O. F. Müller, 1773, a chlorophyll-bearing colonial phytomonad, is frequently encountered in many bodies of fresh water. When found in abundance in nature, almost all the colonies are normal and 16-celled. Occasionally, 8- and 4-celled colonies of *G. pectorale* have been observed in nature (as by Harper, 1912; and Pascher, 1927); but any person who has attempted to maintain *Gonium* in the laboratory will be familiar with these and the many other non-16-celled forms which occur under such conditions. A culture which is more than a week old may contain forms with every number of cells from 16-celled to 1-celled. As the culture grows older, the relative number of these abnormal forms increases, until, by the end of a month, little else but 1-celled forms remains. However, a subculture made at any stage, even one consisting chiefly of 1-celled forms, goes through the same stages as did the original culture.

The formation of colonies with fewer than the normal number of cells has been reported for other colonial Phytomonadida when cultured under laboratory conditions. It has been described for *Eudorina elegans* (Hartmann, 1924), *Astrephomene gubernaculifera* (Stein, 1958a), and for a number of species of *Gonium* (Pocock, 1955). We have also observed it in *Pandorina morum* and in *Pleodorina californica*.

In *G. pectorale* this phenomenon was first reported by Harper (1912). He observed some of the 16-celled colonies split in half to form two 8-celled colonies each, and saw other colonies break apart "with a succession of sudden jerks." Hartmann (1924, 1928) found that the degeneration he observed in *Gonium* occurred sooner in more concentrated culture medium than in less concentrated. He suggested that accumulation of toxic substances was probably responsible for these abnormalities. Crow (1927), who studied the production of abnormal colonies in an evaporating culture of *Gonium* in Knop solution, believed that this progressive degeneration was due to the increasing concentration of the medium and the accumulation of organic material from the decay of colonies.

It was the purpose of the present study to investigate as fully as possible at least some of the factors which are necessary for normal colony formation in *G. pectorale*, and at the same time to explain the abnormalities so frequently encountered.

MATERIALS AND METHODS

The organisms used in this study were from a clone which was isolated from a culture supplied by Carolina Biological Supply Company, Elon College, North

*Present address: Department of Zoology, Montana State University, Missoula, Montana.

Carolina. The original 16-celled colony from which the clone was established was freed from all other protozoa by repeated isolation and transfer to sterile culture medium. Other clones of *G. pectorale* have been established from collections made in and around Franklin County, Ohio, but these show no observable differences from each other or from the clone used.

The basic culture medium used was 0.05 percent (w/v) Knop solution as used and described by Hartmann (1928). This solution was modified slightly by using a ferric citrate-citric acid solution instead of ferric chloride, and by using, except where otherwise stated, a 1/88 M carbonate buffer (1/176 M Na_2CO_3, 1/176 M K_2CO_3) (Österlind, 1949). Enough 0.54 N HCl was added to give the solution an initial pH of about 6.5. One hundred ml of culture medium were placed in each flask, except when compressed gases were used; then the volume of culture medium was 150 ml. Reagent-grade salts were used for all solutions.

All solutions were made up with water that was freshly redistilled in an all-pyrex glass still. The solutions were then filtered through a millipore filter into 250-ml Erlenmeyer flasks (Corning #5000) which had previously been sterilized by autoclaving. The flasks were then stoppered with standard-taper ground-joint stoppers (Corning #7570), or with gas inlet tubes (Corning #98100) which had Corning #39533 fritted cylinders attached on the bottom of the inlet tubes. Standard taper "Teflon" sleeves were used on all ground glass joints.

The organisms to be used as an inoculum were washed by three successive centrifugations in sterile fluid. After the flasks were inoculated they were placed in a constant temperature water bath which was kept at $20 \pm 0.1°$ C. The flasks were illuminated by eight 40-watt standard warm white fluorescent lamps which were placed under the glass bottom of the water bath. A light intensity of 600 ft-c and a photoperiod of 12 hr light and 12 hr darkness were used.

In one experiment, the cultures were aerated with compressed gases containing various concentrations of CO_2. Three mixtures of gas were used; the compressed air was obtained from the compressed air line in the laboratory; the 4 percent CO_2 (mixture with 19.9 percent O_2, 76.1 percent N_2) and the 1.2 percent CO_2 (mixture with 19.9 percent O_2, 78.9 percent N_2) were obtained from Ohio Chemical and Surgical Equipment Company, Cleveland, Ohio. The gases were bubbled through sterile distilled water and passed through sterile cotton filters before they entered the culture flasks. A flow rate of 3 ft^3/hr was maintained through each flask. All pH values were measured with a Beckman Model "G" pH meter.

On each day of an experiment, an estimation was made of the relative abundance of normal 16-celled colonies and the number of cells in each culture. A random sample was removed from each flask, fixed by heating, and placed in a Sedgwick-Rafter counting cell (A. H. Thomas No. 9945). Fifty random fields were counted on each slide with the aid of a Whipple Disk. The number of colonies observed and the total number of cells in each colony were recorded. All cultures were made in duplicate, and the values reported are the averages of the values for a pair of similar cultures.

The samples were taken at approximately the same time each day, usually the middle of the morning, but never after the middle of the afternoon. This variation in time of sampling the culture did not appreciably affect the relation of one day's growth to the next day's growth, because this clone divided only at night, usually starting about 10:30 PM. Pocock (1955) noted a similar phenomenon with the *Gonium* with which she worked; but she discovered that different populations of *Gonium* might divide at different times of day, although the time of the day at which a given strain divided remained relatively constant.

RESULTS

The influence of added carbonate.—During some preliminary experiments, it was found that the growth of *Gonium* in the ordinary 0.05 percent (w/v) Knop

solution was somewhat erratic and unpredictable, considerable differences appearing among cultures supposedly identical. Measurements of the pH of these cultures showed that most of the values were below the pH 5.5 level that Österlind (1949) had found to be critical in the growth of *Scenedesmus* in media without added carbonate. Also, preliminary experiments showed that passing CO_2, as expired air, through growing cultures of *Gonium* greatly increased the relative abundance of 16-celled colonies and produced a much richer culture. Therefore, in order to obtain a richer and more predictable growth, carbonate was added to the culture medium. Equimolar amounts of Na_2CO_3 and K_2CO_3 were added to give a final concentration of 1/88 M carbonate. This acted both as a buffer and as a source of CO_2.

The growth of cultures in carbonate-containing culture medium was compared with that in carbonate-free medium; the results are shown in table 1. As can be seen from the table, the normal 16-celled colonies are relatively much more abundant, and the rate of growth appreciably greater in the carbonate-containing medium than in the carbonate-free medium.

TABLE 1

Influence of carbonate.—A comparison of the relative abundance of normal 16-celled colonies, and of rate of growth, between cultures in 0.05% (w/v) Knop solution and cultures in 0.05% (w/v) Knop solution to which 1/88 M carbonate has been added.

Treatment	Day	Percent of total cells as 16-celled forms	Total no of cells per mm³
No carbonate added to 0.05% Knop solution	1	42.96	1.49
	2	43.25	2.22
	3	50.41	2.89
	4	38.31	5.02
	5	39.45	9.72
	6	20.38	17.36
1/88 M carbonate added to 0.05% Knop solution	1	51.77	1.53
	2	62.09	3.11
	3	70.87	7.47
	4	63.87	12.28
	5	57.86	28.22
	6	49.43	97.20

The influence of the carbon dioxide concentration.—Cultures were grown in media which were aerated continuously with different concentrations of CO_2. The results, shown in table 2, indicate that rates of growth in 1.2 percent CO_2 and in 4 percent CO_2 were approximately the same, while that in compressed air was considerably slower. It can also be seen that the relative abundance of normal 16-celled colonies increased with the increasing concentration of CO_2.

The influence of filtrates from aged cultures.—Hartmann (1924) believed that toxic substances were produced in *Gonium* cultures, and that these substances were responsible for the abnormalities which were encountered in aging cultures. Pratt (1940) and Swanson (1943) found a growth inhibiting substance in cultures of *Chlorella vulgaris*, and Jørgensen (1956) found that *Nitzschia palea*, *Scenedesmus quadricauda*, and *Chlorella pyrenoidosa* all produced autotoxic substances.

It was decided, therefore, to investigate the influence of filtrates of aged cultures on young cultures which were in their logarithmic phase of growth. The filtrate was prepared by centrifuging the cells from an aged culture, and then filtering

the supernatant fluid through a millipore filter. Filtrate from a culture of the same age as the experimental culture was used as a control for the procedure. In addition, filtrates were prepared from cultures 1.5 weeks old, 2.5 weeks old, 3.5 weeks old, and 4.5 weeks old. On the fourth day of growth, these 5 filtrates were added to 5 different pairs of cultures, 5 ml to a flask, each pair of flasks receiving the filtrate from a differently aged culture. A sixth pair of cultures to which no filtrate was added was used as a control.

The results of this experiment are shown in table 3. It can be seen that the rate of growth drops off more rapidly in those cultures to which filtrates have been added from older cultures than in the control cultures. Moreover, a comparison of the relative abundance of 16-celled colonies shows that those cultures which had been treated with filtrates from older cultures seemed to be harmed by the filtrates. This effect was in approximate proportion to the age of the culture from which the added filtrate had been taken.

The influence of the pH of the medium.—There was some reason to suspect that the toxicity of the aged culture medium might be related to the pH of the medium.

TABLE 2

Influence of carbon dioxide.—A comparison of cultures in carbonate-buffered 0.05% (w/v) Knop solution which were aerated with gases containing different concentrations of CO_2.

Treatment	Day	Percent of total cells as 16-celled forms	Total no. of cells per mm^3
Compressed air (0.03% CO_2)	4	26.42	7.96
	5	37.55	14.48
	6	33.57	42.88
	7	25.62	78.05
1.2% CO_2 (in 19.9% O_2+78.9% N_2)	4	40.64	12.94
	5	43.60	33.76
	6	48.55	95.10
	7	26.70	223.80
4.0% CO_2 (in 19.9% O_2+76.1% N_2)	4	59.81	12.61
	5	76.34	29.00
	6	65.99	73.48
	7	49.28	239.40

Therefore, the pH of the culture medium of the control to which no filtrate had been added, in the experiment described above, was taken each day. In addition, the pH of all cultures was taken on the eighth day. These are also listed in table 3, and from these it can be seen that the colonies seem to degenerate more rapidly, and the rate of growth is retarded, when the pH reaches a value much above 9.0.

It was desirable, therefore, to know what effect the pH of the culture medium may have on the form and the rate of growth of the colonies. Using predetermined volumes of 0.54 N HCl to regulate the pH, cultures having initial pH values of 8.4, 6.8, and 5.0 were prepared.

The results of this experiment, listed in table 4, show that the relative abundance of normal 16-celled colonies and the rates of growth are similar in the two cultures having initial pH values of 6.8 and 5.0, respectively, until the eighth day. Then both the percentage of 16-celled colonies and the rate of growth drop off more sharply in the cultures having the higher pH than in the other cultures. In the cultures having an initial pH value of 8.4, both the percentage of normal colonies and the rate of growth are considerably lower than they are in the other cultures.

The loss of ions through precipitation.—One visible difference between culture medium which has a pH value above 8 and that having a pH value somewhat below 8 is presence of precipitate in the former. Nutrient salts are precipitated from Knop solution above pH 8, thereby decreasing the concentration of these salts in the medium.

In order to determine the influence of the lessened concentration of nutrient salts caused by their precipitation in alkaline medium, the following experiment was undertaken. The culture medium was made up at pH 9.2 and allowed to stand over night. The precipitate which then formed was filtered off, and the

TABLE 3

Influence of filtrate of aged culture.—A comparison of the relative abundance of normal 16-celled colonies, and of rate of growth, between cultures to which filtrates of more aged cultures had been added and cultures which had no filtrate added. Five ml of a particular filtrate was added on the fourth day of growth of a given culture. The medium used was carbonate-buffered 0.05% (w/v) Knop solution.

Treatment	Day	pH	Percent of total cells as 16-celled forms	Total no. of cells per mm³
No filtrate added	1	7.44	18.48	6.04
	2	7.23	8.88	9.01
	3	7.33	7.37	21.73
	4	7.43	11.29	42.54
	5	7.58	27.84	81.10
	6	7.80	57.59	242.24
	7	8.30	51.04	386.46
	8	9.09	41.11	397.40
Filtrate added from culture of same age	6		60.15	182.12
	7		52.85	391.30
	8	9.35	42.77	376.90
Filtrate added from a 1.5-week-old culture	6		56.16	225.38
	7		47.56	316.45
	8	9.55	23.14	322.00
Filtrate added from a 2.5-week-old culture	6		44.73	251.44
	7		31.80	370.20
	8	9.36	9.40	308.70
Filtrate added from a 3.5-week-old culture	6		45.92	169.70
	7		32.66	279.00
	8	9.54	7.88	271.70
Filtrate added from a 4.5-week-old culture	6		46.96	171.08
	7		22.92	245.10
	8	9.55	10.37	287.85

pH of the medium was lowered to pH 6.8, well within the normal range for *Gonium*. Nutrient salts, in half their original concentrations, were added to one portion of this medium before it was filtered into the culture flasks; no salts were added to the other portion. Both sets of flasks were then inoculated, and kept under the usual conditions.

The results of this experiment, listed in table 5, show very strikingly the detrimental effect which the loss of precipitated salts had on the form of the colony and the rate of growth. For, in the cultures with the added nutrient salts, over 75 percent of the cells were in normal 16-celled colonies on the sixth day,

while in the other culture less than 20 percent of the cells were in 16-celled colonies on the same day. Further, the cultures containing the added salts had about 21 cells/mm^3, while the other cultures had only about 1/10 that much growth on the sixth day.

The influence of the iron salt.—The requirement of iron for the growth of algae is well substantiated. Pringsheim (1946) attributed some of the success of his soil-water medium to the ability of humic acids to maintain iron in an available state. Rodhe (1948) discussed the problem of the utilization of iron by certain

TABLE 4

Influence of initial hydrogen ion concentration.—A comparison of the relative abundance of normal 16-celled colonies, and of the rate of growth, among cultures having different initial hydrogen ion concentrations. The medium used was carbonate-buffered 0.05% (w/v) Knop solution.

Initial pH	Day	Percent of total cells as 16-celled forms	Total no. of cells per mm^3	pH
8.4	5	39.61	17.37	8.5
	6	47.42	41.56	8.7
	7	49.89	52.56	8.8
	8	32.90	65.30	9.0
6.8	5	54.13	63.86	7.0
	6	71.25	152.62	7.2
	7	61.60	349.50	7.7
	8	37.36	361.20	9.2
5.0	5	56.21	67.44	5.6
	6	62.72	186.54	5.9
	7	61.57	378.30	6.3
	8	54.03	509.95	7.2

TABLE 5

Influence of loss of nutrient salts by precipitation.—An investigation of the influence which the loss of nutrient salts might have on the form of the colony and on rate of growth if these salts were made unavailable to the organisms because of precipitation in the alkaline culture medium. Carbonate-buffered 0.5% (w/v) Knop solution was made up at pH 9.2, and the precipitate which formed was filtered off. The pH of the medium was then lowered to the normal range. One-half of the medium was inoculated without further change, while nutrient salts in half their original concentration were added to the other half before it was inoculated.

Treatment	Day	Percent of total cells as 16-celled forms	Total no. of cells per mm^3
Precipitate removed, no salts added	4	26.67	0.72
	5	13.80	0.85
	6	18.46	1.90
	7	9.15	2.69
Precipitate removed, one-half original concentration of salts added	4	67.69	6.22
	5	73.13	11.52
	6	76.93	21.42
	7	57.12	44.34

algae, and found that the organisms he tested could not utilize colloidal iron. He recommended the use of ferric citrate, which stayed in a form available to the algae much longer than other iron salts he used. Others (Österlind, 1949; Hewitt, 1951; and Ketchum, 1954) have discussed the role of iron in plant mineral nutrition.

In order to determine the influence of an iron deficiency on the form of the colony and on the rate of growth, cultures in a medium containing no added iron were compared with cultures containing the usual amount of ferric citrate, 0.27 mg/100 ml Knop solution.

The results of this experiment, listed in table 6, show that though the reagent-grade salts used probably had a slight "impurity" of iron in them, nevertheless those cultures containing the added iron not only had relatively more of the normal 16-celled colonies, but also grew at an appreciably faster rate. Moreover, a subculture made on the fifth day from the iron-containing culture into fresh medium with added iron had 79 percent of its cells in normal 16-celled colonies and 19.4 cells/mm^3; while a subculture from the iron-free culture into fresh iron-free medium had only 44 percent normal colonies and 11.3 cells/mm^3 when both subcultures had been growing 4 days.

TABLE 6

Influence of iron.—An investigation of the influence of iron (as ferric citrate) in carbonate-buffered 0.05% (w/v) Knop Solution on the form of the colony and on rate of growth.

Treatment	Day	Percent of total cells as 16-celled forms	Total no. of cells per mm^3
0.27 mg ferric citrate added per 100 ml of solution	1	51.77	1.53
	2	62.09	3.11
	3	70.87	7.47
	4	63.87	12.28
	5	57.86	28.22
	6	49.43	97.20
No added ferric citrate	1	33.81	1.42
	2	62.39	2.31
	3	46.21	4.16
	4	48.18	9.62
	5	42.02	22.84
	6	29.18	66.45

The influence of the calcium ion concentration.—Another of the ions precipitated in alkaline Knop solution is the calcium ion. Though Rodhe (1948) found that 0.001 mg of calcium per liter allowed *Ankistrodesmus* to grow well, and Österlind found that this concentration of calcium also gave good growth in *Scenedesmus*, both authors found that optimum growth occurred at calcium ion concentrations of 1.0 to 5.0 mg/l. Ketchum (1954), however, states that calcium is not essential for the growth of *Chlorella*, and Arnon (1958) states that it is needed only in microquantities by green algae.

In order to determine the influence of the calcium ion concentration on the form of the colony and on the rate of growth, an experiment was planned with a series of cultures having six different calcium ion concentrations. The concentrations used were: (1) 70.0 mg/l, the amount usually in Knop solution, (2) 35.0 mg/l, (3) 7.0 mg/l, (4) 0.7 mg/l, (5) 0.07 mg/l, and (6) no added calcium. The nitrate level in the culture was kept constant by adding an equivalent amount of KNO_3 to replace the $Ca(NO_3)_2$ which would have normally been in the medium.

The results of this experiment are listed in table 7. During the period of the experiment, 5 days, the culture that contained no added calcium showed no growth. Further, by the second day, the only multicellular forms remaining were a few two-celled forms. However, all cultures containing added calcium showed appreciable growth, and all these except the culture with only 0.07 mg Ca/l, contained normal 16-celled colonies. The highest number of cells observed in a colony in the latter culture was 14 cells. In the four highest calcium concentrations, the percentage of normal 16-celled colonies increased approximately in a straight line relationship to the logarithm of the calcium ion concentration. A repetition of this experiment, using a modified Beijerinck's solution (Stein, 1958b), gave essentially the same results except that there was some slight growth in the solution that contained no added calcium.

Growth of new cultures in filtrates of aged cultures.—As stated above, Hartmann (1924) and Crow (1927) believed that toxic substances were produced in *Gonium* cultures, and that these substances were responsible for the abnormalities which they encountered in aging cultures. One of the experiments described

TABLE 7

Influence of calcium.—The relationship of the form of the colony and of the number of cells per mm^3 to the calcium ion concentration in carbonate-buffered 0.05% (w/v) Knop solution.

Treatment	Day	Percent of total cells as 16-celled forms	Total no. of cells per mm^3
No calcium	4	0.00	2.43
	5	0.00	2.29
0.07 mg Ca/liter	4	0.00	18.14
	5	0.00	24.92
0.7 mg Ca/liter	4	7.13	21.68
	5	3.59	26.86
7.0 mg Ca/liter	4	47.41	30.16
	5	53.13	56.52
35 mg Ca/liter	4	62.93	37.85
	5	65.71	66.71
70 mg Ca/liter	4	76.11	41.24
	5	79.87	61.30

above tested the influence of filtrates from aged cultures on young cultures. Though these filtrates produced a detrimental effect, other experiments indicated that the factors responsible for this effect were probably the depletion of the CO_2, the alkalinity of the medium, and the precipitation of nutrient salts rather than the presence of a toxic substance. Therefore, correcting in large measure for these factors, another experiment was performed, in order to determine whether anything did, in fact, accumulate in an aging culture which would be detrimental to normal colony formation and normal growth.

A culture of *Gonium* was maintained for 3 weeks in 1500 ml of culture medium in a 2-l flask. At the end of this time, the pH of the solution was 9.7, and the culture consisted almost exclusively of one-celled forms, with only an occasional 2- or 3-celled form being seen. The pH of the solution was lowered to 6.5 with HCl in order to redissolve the precipitated salts. The cells were then removed by centrifugation, and the fluid was filtered through a millipore filter. Then some of the solution was again filtered, this time into sterile flasks with no further change; to another portion, half the original concentration of carbonate was first added before filtration into flasks; and to a third portion, both carbonate and

mineral salts, in half their original concentration, were added before filtration. A control in fresh carbonate-buffered Knop solution was also prepared.

The results of this experiment, listed in table 8, show that a considerable percentage of normal 16-celled colonies was produced in every culture. Further, the two sets of cultures in aged medium, to which either carbonate alone or carbonate and other salts had been added, contained almost the same percentage of normal 16-celled colonies, and grew almost as rapidly in the first 3 days as did the control in fresh medium. On the fourth day, the control had an appreciably higher percentage of normal colonies and almost one-third more cells per cubic mm than these cultures. The culture in unaltered-aged filtrate showed both normal colony formation and growth, although it did so to a lesser degree than did the other cultures.

TABLE 8

Influence of old filtrate with pH adjusted to 6.5.—The form of the colony and rate of growth of *G. pectorale* in a new culture made in the sterile filtrate of a three-week-old culture (originally carbonate-buffered 0.05% (w/v) Knop solution). The filtrate was adjusted to pH 6.5 before inoculation.

Treatment	Day	Percent of total cells as 16-celled forms	Total no. of cells per mm^3
Filtrate, no salts added	1	8.89	3.60
	2	19.19	5.14
	3	37.66	19.13
	4	29.37	29.52
Filtrate, 1/176 M carbonate added	1	34.51	3.72
	2	35.54	6.69
	3	50.70	26.93
	4	23.11	44.30
Filtrate, 1/176 M carbonate, and ½ original conc. of nutrient salts added	1	29.58	3.77
	2	35.95	7.56
	3	52.66	24.97
	4	34.12	51.04
Fresh culture medium	1	32.20	3.46
	2	42.51	7.51
	3	58.74	27.33
	4	55.06	72.18

DISCUSSION

The main concern of this study has been to obtain a better understanding of those factors which promote the growth of normal colonies in *G. pectorale.* It was believed that a knowledge of factors which interfere with normal colony formation would provide information about conditions which are necessary for the growth of normal colonies.

Two main sources of non-16-celled colonies were observed. It was found, as noted also by Harper (1912) and Crow (1927), that many non-16-celled forms were produced by the break-up of pre-existing colonies. Other colonies, including most of the 8-, and many of the 4-, and 2-celled ones, seem to have been the result of what Harper (1912) called "dwarfed development in reproduction." Since a 16-celled colony is formed from a single cell in a series of four divisions, an 8-, 4-, or 2-celled colony would be produced if one or more of these divisions failed to occur. Under the normal conditions of culture used in the present study, as

represented by the controls in most experiments, the majority of the cells not included in 16-celled colonies were in colonies probably produced by this "dwarfed development." To state this unequivocally would be almost impossible, but observations made before, during, and after division strongly support this view. It would seem reasonable, therefore, to assume that those factors which influence cell reproduction also directly influence colony formation.

One of the most important of these factors is the availability of CO_2 (or carbonate) to the growing cell. Results of experiments (tables 1 and 2) seem to indicate that whenever the CO_2 concentration is sufficient for maximum growth, the majority of the dividing cells form 16-celled colonies. But, as the CO_2 supply becomes relatively less available for each cell, an increasing number of the cells stop dividing before the end of the fourth division.

Another consequence of the decreasing concentration of CO_2 throughout the life of a culture is an increase in the pH of that culture. It was found that both colony formation and reproduction are normal at pH values between about pH 5 and pH 8 (table 4). But as the pH value increases above pH 8, non-16-celled forms become more abundant and the rate of growth decreases. At pH values much above pH 9 little growth occurs, and many of the existing colonies break up.

At pH values above pH 8 some of the mineral salts of the culture medium are precipitated; the loss of these salts is detrimental both to the form of the colony and to the rate of growth (table 5). It is known that iron salts are generally insoluble in alkaline media (Granick, 1958). We have found that a shortage of iron not only results in a decreased rate of reproduction, as would be expected, but it also results in a decrease in the relative abundance of normal 16-celled colonies (table 6). Another ion which is precipitated in alkaline Knop solution is the calcium ion. Although it was found that the calcium ion is needed only in minute amounts for multiplication, it was also found that it is required in macroamounts for the maximum production of normal 16-celled colonies. Conversely, decreasing amounts of calcium result in the production of increasing numbers of non-16-celled forms, mainly through the failure of the cells of a colony to adhere to each other. Robertson (1941) and Spiegel (1954) stressed the importance of the calcium salt bridge in promoting the stability of intercellular matrices. It seems probable that the intercellular cement of *Gonium* is of the calcium salt bridge type.

Hartmann (1924) believed that the increasing inadequacy of the culture medium and the abnormalities which this produced were caused by an accumulation of toxic substances. In the present study, it appears that the detrimental effect of filtrates of aged cultures is due chiefly to the alkalinity of the filtrates (table 3). Further, if the pH of the filtrate of an aged medium is lowered to the optimum range for *Gonium*, the medium seems to be capable of supporting normal colony formation and growth. This is especially true if a replacement is made of the carbonate and the nutrient salts which have been lost to the medium either by precipitation or by utilization by the organisms (table 8). No evidence has been found for the production of an autotoxin.

SUMMARY

In ordinary laboratory cultures of *Gonium pectorale*, many or most of the colonies are abnormal, in contrast to their usual state in nature. Moreover, the proportion of these abnormal colonies increases with the age of the culture, until most are reduced to single cells.

Several factors have been found to contribute to this effect. (1) During reproduction a deficiency of CO_2 tends to inhibit cell division, resulting in 8-, 4-, or even 2-celled colonies. (2) At pH values above 8, reproduction is retarded, and forms with fewer than 16 cells become relatively more abundant. In such alkaline media some of the nutrient salts precipitate, causing a deficiency of several ions, principally iron and calcium. (3) A shortage of iron results not only in a lower

rate of cell reproduction, but also in relatively fewer normal 16-celled colonies being produced. (4) A deficiency of calcium, on the other hand, affects colony formation much more drastically than it does cell reproduction. While only a trace of calcium, 0.07 mg/1, is required for reproduction, a thousand times that amount, 70 mg/1, is required for normal colony formation. In calcium-free media, colonies will break apart.

No evidence was found to support the hypothesis that a specific autotoxin is formed in *Gonium* cultures.

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