Growth and Differentiation of *Gonium* *

By
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Introduction

There has been considerable interest in plant developmental physiology and important problems on the internal and external mechanisms of development, differentiation, reproduction, morphogenesis, etc. have been investigated. Algae have been employed in many investigations which have significantly advanced our understanding of developmental physiology.

However, the developmental physiology of algae, i. e., the analysis of internal and external factors which determine of the development throughout their life cycle, has been barely touched upon. The reason for this is that algae are quite exacting in their cultural requirement so that the culturing of these forms poses a serious problem, particularly if one wants to use defined media and axenic conditions.

As a result of this situation, most physiological studies have been limited to a few species of algae: Chlorophyta; *Chlorella, Scenedesmus, Chlamydomonas* and *Volvox*, Cyanophyta; *Anabaena* and *Anacystis*, Euglenophyta; *Euglena*, and several other fresh water and marine phytoplankton. With the exceptions of *Chlamydomonas* and *Volvox*, morphogenetical studies have been restricted to asexual life cycles. These algae were used in the physiological studies, because it was possible to culture them in defined media and under axenic conditions, thus, making possible control of the life cycle and mass culture. Consequently, the quantitative and qualitative controls of mechanisms of growth, morphogenesis, reproduction, etc. have been defined.

The algal material used in physiological research should meet the following basic requirements:

(1) Have established the genetic stability, origin and maintenance methods for the algal strains.

(2) The algal strains must be easily cultured in axenic mass-culture under the controlled laboratory conditions.

(3) The life cycle of the algae must be controlled and completed in as short time as

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possible.

The above three requirements have been solved for the algae to be employed in the present study.

Considering the above items relating to growth, development and differentiation, the haploid colonial green alga *Gonium* provides a model system for the investigation of an array of physiological and morphogenetic problems. Namely, they possess a combination of characteristics which make them particularly favorable organisms for this research.

The taxonomy, morphology and cytology, including culture studies, of *Gonium* have been initially investigated by many workers: MÜLLER (1773), EHRENBERG (1838), ROSTAFINSKI (1875), WARMING (1876), MIGULA (1890), CHODAT (1894), LEMMERMANN (1898), SCHERFFEL (1901), SCHUSSING (1911), HARPER (1912), DANGEARD (1916), MAST (1916, 1928), KORSHIKOV (1923 a, b), HARTMANN (1924), SCHREIBER (1925), BOCK (1926), CROW (1927), SMITH (1931, 1950), FRITSCH (1935), PRESCOTT (1949), CAVE *et al.* (1951), STARR (1955), POCOCK (1955), PROCTOR (1957), STEIN (1958, 1959, 1965 a, b, 1966 a, b), PRINGSHEIM (1958, 1959), PRINGSHEIM *et al.* (1959), GRAVES *et al* (1961), LANG (1963) and HENRY-HISS *et al.* (1965). The physiology and biochemistry of *Gonium* has been investigated by HAIGH *et al.* (1964), BROWN (1967) and HENRY-HISS *et al.* (1969, 1971).

Nutritional studies on *Gonium* have been conducted only by PROCTOR (1957), PRINGSHEIM and *et al.* (1958, 1959) and STEIN (1966). However, the nutritional requirements and culture studies on this algal group are still unexplored in detail. This information is required if *Gonium* is to be used as a model of algal biology, particularly the unique and dramatic biology of volvocalean reproduction. Unfortunately, our knowledge of physiological and biochemical aspects of volvocalean reproductive pocesses is scantly with the exceptions of *Chlamydomonas* and *Volvox*. This is in part due to the limitation of techniques suited for culturing a large quantity of algae under controlled conditions.

Since 1967, the writer has been investigating the physiology and culture of several *Gonium* species under the guidance of Prof. Y. NAKAMURA at the Institute of Algological Research, Faculty of Science, Hokkaido University at Muroran.

The purpose of this investigation was to develop *Gonium* as a model for nutritional and morphogenetic studies. This investigation consisted of the following parts:

(1) Control vegetative growth, and define controlling factors and a morphogenetic substance of *G. multicoccum*.
(2) Define nutritional requirements of *G. multicoccum*.
(3) Control of the entire life cycle of *G. multicoccum* and define the controlling factors.
(4) Conduct comparative nutritional studies and palmelloid formation for several species of *Gonium*.

During the course of this study, the writer has published on the specific nutritional requirements, palmelloid formation and rapid growth culture media and techniques for *Gonium* (1972, 1974, 1975a, b, 1976, 1977). These data are contained herein.

Acknowledgements

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Materials and Methods

Experimental organisms

Gonium belongs to the family Volvocaceae, and is a motile, colonial, fresh water green algal group, characterized by typically 4, 8, 16 or 32 biflagellate cells arranged in a flat quadrangular plate.

The species of *Gonium* used during the course of this study are listed in Table 1. Twenty strains of six species were used in the present study. Among them, sixteen strains were obtained from the Indiana University Algal Culture Collection (=UTEX) (STARR, 1964) and are identified here by their collection number; *G. octonarium* (842, 843), *G. quadratum* (956), *G. multicoccum* (783), *G. pectorale* (13, 805, 806, 826, 827), *G. sacculiferrum* (822, 823) and *G. sociale* (936, 14, 15, 197).

Sexual strains of *G. multicoccum*, 7-1, 8-5 (+) and 7-2, 8-7 (−), were isolated by T. ICHIMURA and S. SAITO from mud samples which were collected from rain water pools located in the Southern Central Nepal in 1968 by T. ICHIMURA (unpublished data, cf. Plate I).

Clonal isolations of *G. multicoccum* from Nepal

A small quantity of mud sample was moistened with glass-distilled water in the presence of boiled pea cotyledon. Sixteen-celled or eight-celled colonies of pale colored *Gonium* were seen within 2 or 3 days. Then single colonies were isolated with a micropipette under a dissecting microscope. Washing five-times in sterile distilled water was sufficient to obtain an axenic culture. Testing for mating types was accomplished by mixing ten droplets (ca. 0.5 m*l*) of actively growing algal suspension in all possible combinations amongst the clones into test tubes containing 2 m*l* of distilled water. Sexually compatible pairs were recognized by the presence of stringy zygote assemblages around the rim of water surface in the test tubes. This occurred within 2 or 3 days. One heterothallic pair from one locality was

Table 1 Six species in the genus *Gonium*

Species	Strain number	Isolator	Remarks
Gonium multicoccum POCOCK	7-1	ICHIMURA & SAITO	(+) strain
G. multicoccum POCOCK	7-2	ICHIMURA & SAITO	(−) strain
G. multicoccum POCOCK	8-5	ICHIMURA & SAITO	(+)
G. multicoccum POCOCK	8-7	ICHIMURA & SAITO	(−)
G. multicoccum POCOCK	783	STEIN	(+)
G. octonarium POCOCK	842	STEIN	(−)
G. octonarium POCOCK	843	STEIN	(+)
G. pectorale MÜLLER	13	PRINGSHEIM	
G. pectorale MÜLLER	805	WILBOIS	(−)
G. pectorale MÜLLER	806	WILBOIS	(+)
G. pectorale MÜLLER	826	STEIN	(+)
G. pectorale MÜLLER	827	STEIN	(−)
G. quadratum PRINGSHEIM	956	PRINGSHEIM	
G. sacculiferrum SCHERFFEL	822	STEIN	(−)
G. sacculiferrum SCHERFFEL	823	STEIN	(+)
G. sacculiferrum SCHERFFEL	935	STEIN	homothallic
G. sociale var. *sociale*	14	STARR	homothallic
G. sociale var. *sociale*	15	PRINGSHEIM	
G. sociale var. *sociale*	197	HARTMANN	
G. sociale var. *sacculum* STEIN	936	STEIN	(+)

7-1, 7-2, 8-5 and 8-7 strains of *G. multicoccum* were originally isolated from dried mud samples collected in Southern Central Nepal. The other 16 strains of *Gonium* were obtained from Indiana University Algal Culture Collection and identified in that collection by the strain numbers.

Table 2 Intercrossing of heterothallic strains of *G. multicoccum* from Nepal.

		7-1	7-2	8-5	8-7	12-2	13-4
G. multicoccum	7-1 (+)		Z	−	Z	−	−
	7-2 (−)	Z		Z	−	Z	Z
	8-5 (+)	−	Z		Z	−	−
	8-7 (−)	Z	−	Z		Z	Z
	12-2 (+)	−	Z	−	Z		−
	13-4 (+)	−	Z	−	Z	−	

Z: intercross zygotes.
Strains 12-2 and 13-4 of *G. multicoccum* could not be maintained in culture.

Table 3 Sterility test media of *Gonium* (Bact-free tester media)

	ST-1	Pepton	Thioglycollate medium
Soil extract	10 m*l*	—	—
Trypticase	10 mg	—	—
Liver extract	1 mg	—	—
Yeast extract	1 mg	—	—
Trypton	—	1 g	1 g
Beef extract	—	300 mg	150 mg
Vitamin mix No. 8	0.1 m*l*	—	—
B_{12}	10 μg	—	—
L-cystin	—	—	10 mg
Glucose	—	—	500 mg
Na-thioglycollate	—	—	50 mg
Resazurin	—	—	0.16 m*l*
Carbon source mix II	2 m*l*	—	—
Ferric ammonium citrate	0.1 m*l*	—	—
NaCl	—	150 mg	250 mg
KNO_3	20 mg	—	—
$(NH_4)_2HPO_4$	2 mg	—	—
$MgSO_47H_2O$	1 mg	—	—
$CaCl_2$	0.05 mg	—	—
pH	7.0	7.0	7.0

Amounts are per 100 m*l* of medium.
Vitamin mix No. 8: see Table 5 (PROVASOLI, 1958).
Carbon source mix II (TATEWAKI & PROVASOLI, 1964) contains: glycine 100 mg; DL-alanine 100 mg; L-asparagine 100 mg; Na-acetate $3H_2O$ 200 mg; glucose 200 mg; L-glutamic acid 200 mg, H_2O 100 m*l*.

selected and maintained. Crossing amongst the four strains of these two pairs was accomplished by the same method. The results of testing for mating types and crossing amongst the strains are shown in Table 2. Table 2 shows that 7-1 and 7-2 strains or 8-5 and 8-7 strains of *G. multicoccum* are opposite mating types. Strains 12-2 and 13-4 of *G. multicoccum* could not be maintained in culture.

Axenic culture

Axenic culture was obtained by washing individual colonies in five changes of sterilized distilled water. The presence of bacteria and other biotic contaminations was tested for with the following sterility test media; ST-1 medium, Peptone medium, Thioglycollic acid medium, Medium 5 and Medium 6 (Tables 3 and 4). These media were microscopically examined after three weeks. If they contained only *Gonium* at that time, they were described as pure or axenic.

Table 4 Organic culture media for the growth of *Gonium*

	M-1	M-2	M-3	M-5	M-6
Na-acetate • $3H_2O$	100 mg	—	—	100 mg	100 mg
Trypticase	100 mg	10 mg	—	—	—
Tryptone	—	—	—	50 mg	50 mg
Yeast extract	10 mg	10 mg	—	10 mg	10 mg
Soil extract	10 m*l*	10 m*l*	10 m*l*	2 m*l*	—
Pea extract	—	—	10 m*l*	—	—
$MgSO_4$ • $7H_2O$	2 mg	2 mg	—	2 mg	5 mg
K_2HPO_4	2 mg	2 mg	—	2 mg	3 mg
NH_4NO_3	2 mg	2 mg	—	5 mg	10 mg
$CaCl_2$	—	—	—	2 mg	2 mg
Ferric ammonium citrate	0.1 mg	0.1 mg	—	0.1 mg	—
Metal mix G	—	—	—	—	0.5 m*l*
Vitamin mix No. 8	—	—	—	0.1 m*l*	0.1 m*l*
Glycylglycine	—	—	—	—	50 mg
pH	7.0	7.0	7.0	6.5	6.5

Amounts are per 100 m*l* of medium.
Medium 1 and Medium 2: (T. ICHIMURA, personal communication)
Metal mix G: see Table 5.
Vitamin mix No. 8: See Table 5.

Stock culture

Axenic stock cultures were maintained in Medium 5, Medium 6, or Medium 52 (Tables 4 and 5) for strains 7-1, 7-2, 8-5, 8-7, 842, 843, 956 and 783 of *Gonium*. The remaining strains were maintained in Medium T-3, Medium T-4, or Medium T-6 (see Table 17).

The algal stocks were grown at 14±1℃ at ca. 500 lux of light (cool-white fluorescent lamps) and an alternating 14-hr-light and 10-hr-dark cycle. The strains 7-1, 7-2, 8-5, 8-7, 842, 843, 956 and 783 were transferred every three weeks, and remaining strains were transferred every two or three months.

Preservation of zygote

Matured zygotes of Nepal strains of *G. multicoccum* have been capable of germination after ten-year preservation as a result of this study. In the future, it may be an important means of preservation.

Culture methods

All solutions were prepared with Pyrex glass-distilled water. Media were brought to the proper pH by adding 1 N KOH, NaOH or HCl, then dispensed in amounts of 10 m*l* per test tubes (18×130 mm) with plastic screw caps or 50 m*l* per 100 m*l*, 150 m*l* per 300 m*l* and 2000 m*l* per 5000 m*l* Erlenmeyer flasks with cotton plugs, and sterilized by autoclaving (120℃, 1 kg/cm^2) for 20 min. or sterilized using Millipore HA filters (maximum pore size 0.45 and

0.22 μm).

Unilateral light was provided by cool-white fluorescent lamps which maintained a light intensity of about 4000 lux at the surface of the culture vessels. The incubator was usually maintained at 25±1℃. However, light intensity and temperature were changed according to the purpose of the experiment. In this investigation batch cultures were employed except where the effects of air and CO_2 bubbling were studied.

Medium 51 (Table 5, modified T. ICHIMURA medium for *Chlamydomonas*) was used in

Table 5 Defined culture media for *G. multicoccum* (Amounts are per 100 m*l* of medium.)

	T. I medium	M 51	M 52	M-G
$(NH_4)_2HPO_4$	20 mg	20 mg	—	—
NH_4Cl	—	—	20 mg	20 mg
$MgSO_47H_2O$	10 mg	10 mg	5 mg	5 mg
KCl	10 mg	10 mg	—	—
$CaCl_2$	0.2 mg	0.5 mg	5 mg	5 mg
KH_2PO_4	—	—	15 mg	15 mg
Na-acetate • $3H_2O$	300 mg	100 mg	300 mg	300 mg
Tris-HCl buffer	1 m*l*	1 m*l*	1 m*l*	1 m*l*
P II metals	1 m*l*	1 m*l*	—	—
Metal mix G	—	—	1 m*l*	1 m*l*
Vitamin mix No. 8	0.1 m*l*	0.1 m*l*	0.1 m*l*	—
Thiamine	—	—	—	0.02 mg
B_{12}	—	—	—	0.00 1μg
pH	7.0	6.5	6.5	6.5

Tris-HCl buffer (pH 7.0): One m*l* of buffer contains Tris-aminomethane, 100 mg.
P II & Vitamin No. 8: PROVASOLI, 1958.
Metal mix G: SAITO, 1972.

P II metals (PROVASOLI, 1958). One m*l* of P II metal contains: Na_2-EDTA, 1 mg; Fe (as Cl), 0.01 mg; B (as H_3BO_3), 0.2 mg; Mn (as Cl), 0.04 mg; Zn (as Cl), 0.005 mg; Co (as Cl), 0.001 mg.

Metal mix G (SAITO, 1972). One m*l* of Metal mix G contains: Na_2-EDTA, 2 mg; Fe (as SO_4), 0.1 mg; Mn (as Cl), 0.01 mg; Zn (as Cl), 0.005 mg. Co (as Cl), 0.001 mg; Cu (as Cl), 0.002 mg; Mo (as $(NH_4)_6$), 0.005 mg. Adjust pH to 7.0.

Vitamin mix No. 8 (PROVASOLI, 1958). One m*l* of Vitamin mix No. 8 contains: thiamine HCl, 0.2 mg; nicotinic acid, 0.1 mg; putrescine 2HCl, 0.04 mg; Ca-pantothenate, 0.1 mg; riboflavin, 5.0 μg; pyridoxine 2HCl, 0.04 mg; pyridoxamine 2HCl, 0.02 mg; para-aminobenzoic acid, 0.01 mg; biotin, 0.5 μg; choline H_2 citrate, 0.5 mg; inositol, 1.0 mg; thymine, 0.8 mg; orotic acid, 0.26 mg; B_{12}, 0.05 μg; folic acid, 2.5 μg (delete folinic acid from original Vitamin mix No. 8).

preliminary experiment until producing Medium 52 (Table 5). Medium 51 was used successfully in our laboratory to grow other algae. After development, Medium 52 was used for preculture and all experimental basal media.

Gonium for nutritional studies was first precultured until it reached the exponential growth phase (ca. 35 hr). To minimize carry-over from the old media, the algal colonies were washed three times with glass-distilled water. When necessary, the levels of endogenous reserves were further reduced by incubating the colonies for 6 hrs to 15 days in the medium from which the compounds to be tested had been removed.

This algal suspension was then diluted with sufficient sterile water to reduce the concentration to an optical density of ca. 0.3-0.6. Two drops (ca. 0.05 m*l*) were inoculated with a glass micropipette into each culture tube containing 10 m*l* of sterilized experimental media. For other large scale culture, the inoculum was prepared in a ratio of 0.05 m*l* of algal suspension to 10 m*l* of experimental media. Agar media used were at a concentration of 0.5-1 g% of agar-agar.

For vitamin experiments all glassware coming in contact with solutions was baked at 250℃ for 3 hrs. For the trace element experiments, all glassware was washed with 1% Na_2-EDTA deionized water solution and then washed three times with Pyrex distilled water.

For the purpose of determination of vitamin requirements, vitamin tester supplement solution (Table 6) modified from STEIN (1966) was used. Each vitamin was used in the tester supplement solution at the same concentration as vitamin mix No. 8.

Growth was measured by dry weight (D. W.), packed cell volumes (P. C. V.) and changes in optical density (O. D.) at a wavelength of 560 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer. For the dry weight calculations, 100 m*l* of culture

Table 6 Compositions of Vitamin Mixtures (modified STEIN's vitamin tester solution, 1966)

	1	2	3	4	5
1	Riboflavin 5 μg	Thiamine 0.2 mg	Pyridoxine 0.04 mg	Pantothenate 0.1 mg	p-Aminobenzoic 0.01 mg
2		Nicotinic 0.1 mg	Choline 0.5 mg	Folic acid 2.5 μg	Biotin 0.5 μg
3			Inositol 1 mg	Pyridoxamine 0.02 mg	Orotic acid 0.26 mg
4				Cyanocobalamine 0.05 μg	Putrescine 0.04 mg
5					Thymine 0.8 mg

Concentration/1 m*l* H_2O. Used 0.1 m*l* of tester/100 m*l* Medium 52 vitamin-free.
Example: Vitamin mixture No. 2=Thiamine, Nicotinic, Choline, Folic acid, Biotin.
Compounds and concentrations of vitamins employed as in Vitamin mix No. 8 (delete Folinic acid) (PROVASOLI, 1958).

suspensions were centrifuged in a tared tube and the supernatant decanted. This treatment was repeated 3 times. The cellular precipitates were dried for 24 hrs at 100℃ and then weighed after perfect drying in a vacuum-drier. Packed cell volumes were determined by centrifuging 1-10 m*l* of culture suspension in a hematocrithead for 30 min. at 4000 rpm. (ca. 2600 g). There was a linear relationship between optical density and dry weight through the exponential growth phase. The results were given in terms of cell concentration as optical density within 60-hr culture unless stated otherwise.

Growth rate (K-doublings per day) was computed according to the formula of HOOGENHOUT and AMESZ (1965).

$$kg=\frac{\log_{10}\cdot N_2/N_1}{t_2-t_1} \qquad K=2.30/0.69.kg$$

If K is calculated on a 24-hr basis, the growth rate figure refers to the number of cell doublings per day.

N_1 and N_2: growth amount of O. D.

t_1 and t_2: culture time in days

All experimental results were the average of either two or four replicate culture vessels from at least two separate experiments.

Other experimental methods will be mentioned in detail in the materials and methods of each chapter.

Chemicals of special reagent grade were used for the preparation of all culture media.

I Nutritional Studies of *Gonium multicoccum*

PRINGSHEIM and PRINGSHEIM (1959) found that *Gonium multicoccum* was an obligate mixotroph and acetate served as a carbon source. However, there has been no research conducted on the nutrition and development of *G. multicoccum*.

The nutritional studies reported in this chapter were concerned with vitamins, and inorganic and organic nutritional requirements for vegetative growth of the organisms under conditions of continuous light and dark. The goal was a defined medium in which rapid and extensive growth could occur. Moreover, the nutrition and vegetative growth characteristics of *G. multicoccum* were studied in preparation for investigations of the factors regulating the life cycle and growth.

1. Materials and Methods

Culture experimental materials and methods are the same as described in previous chapter. The (+) strain of 7-1 and (−) strain of 7-2 of *G. multicoccum* were used in the experiments described in this chapter.

Each experiment employed two or four replicate cultures of each strain. Further, each experiment was conducted at least twice. The data reported here are the average of both strains for all repeated experiments.

2. Nutrition of vegetative growth

Inorganic nutrition

Preliminary experiments showed that Medium 5 containing unknown factors supported growth of 7-1 and 7-2 strains and Medium 51 also supported growth of these strains. Figure 1 shows the growth curves in Medium 51 under continuous light (ca. 3000 lux) and dark at 20±1℃. Omission of acetate from the basal medium resulted in no growth, demonstrating an acetate requirement as a carbon source.

Medium 51 was used as the initial basal medium for this nutrition study and the concentration of each component was systematically varied to determine the optimum Medium 52 and Medium G for each strain in terms of growth and final yield, under the continuous light and dark. Table 5 describes Medium 52 and Medium G which have been decided the final medium after systematic experiments to determine the optimum growth.

Figure 2 shows the growth response of *G. multicoccum* to different inorganic nitrogen sources. Ammonium and nitrate were required at a concentration of approximately 20 mg% for maximum growth under light and dark culture conditions. Ammonium was slightly

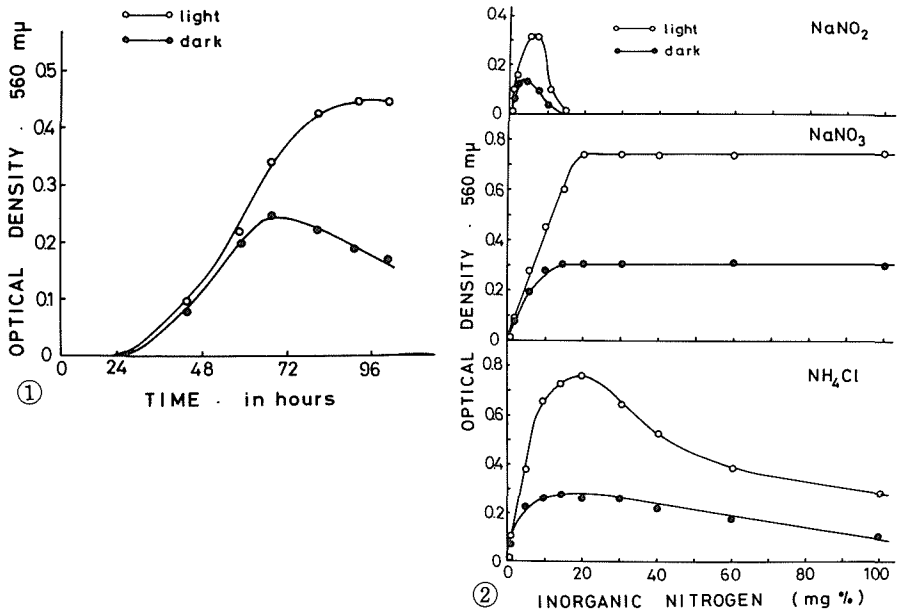
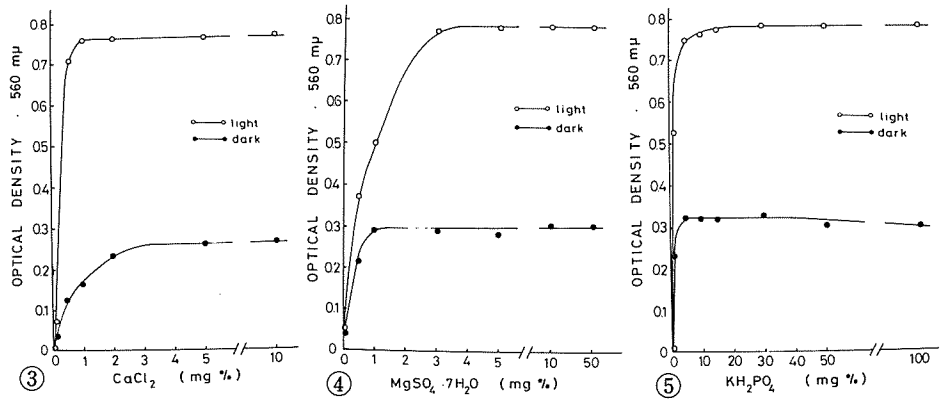


Fig. 1 Growth of *Gonium multicoccum* in Medium 51 at 20℃ in the light and dark. Initial pH was adjusted to 6.5.

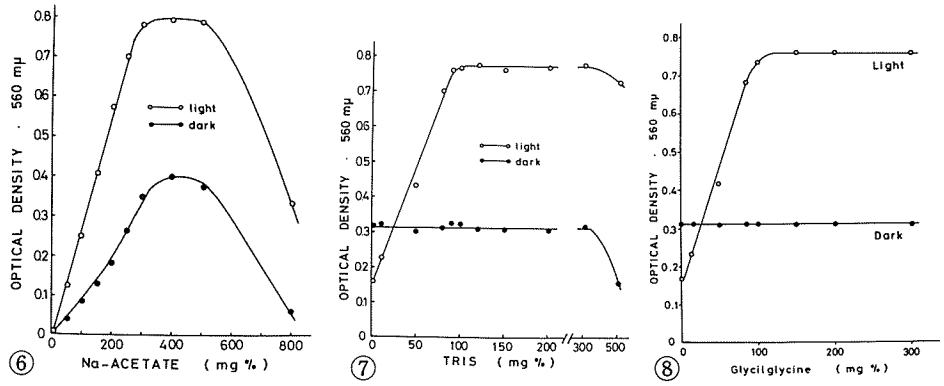
Fig. 2 Effects of $NaNO_3$, $NaNO_2$ and NH_4Cl on the growth of *G. multicoccum*. Basal medium : M-52 minus ammonia. Optical densities were measured after 60-hr incubation in the light and dark.

better at 20 mg% than nitrate, but at higher concentrations inhibited growth, whereas nitrate sustained maximum growth. Optimal growth occurred at approximately 8 mg% nitrite. However, the yield was poor and the range at which nitrite supported growth narrow.

Figures 3, 4 and 5 show the growth response of *G. multicoccum* to varying concentrations of the macro-nutrients $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4. Optimal growth occurred at approximately 1 mg% $CaCl_2$ in the light and between 2 and 5 mg% in the dark. Higher



Figs. 3–5 Growth of *Gonium multicoccum*: Fig. 3, Effects of Ca^{++} concentrations (Basal medium=M-52 minus $CaCl_2$); Fig. 4, Effects of Mg^{++} concentrations (M-52 minus $MgSO_4 \cdot 7H_2O$); Fig. 5, Effects of phosphate concentrations (M-52 minus KH_2PO_4). These optical densities were measured after 60-hr incubation in the light and dark.



Figs. 6–8 Growth of *Gonium multicoccum*: Fig. 6, Effects of acetate concentrations (Basal medium=M-52 minus acetate); Fig. 7, Effects of Tris (hydroxymethyl) aminomethane concentrations (M-52 minus Tris); Fig. 8, Effects glycylglycine concentrations (M-52 minus Tris). These optical densities were measured after 60-hr incubation in the light and dark.

concentrations (up to 10 mg%) did not inhibit growth. Conversely, $MgSO_4 \cdot 7H_2O$ supported maximum growth at 1 mg% in the dark, but at a concentration of 3 mg% in the light. This indicated that the $MgSO_4 \cdot 7H_2O$ requirement was greater in the light than the dark. Greater concentrations (up to 50 mg%) did not inhibit growth. The lowest concentration of KH_2PO_4 supporting maximum growth in light and dark was between 5 and 15 mg%. Growth was not inhibited at the highest concentration tested (100 mg%).

Acetate as a carbon source

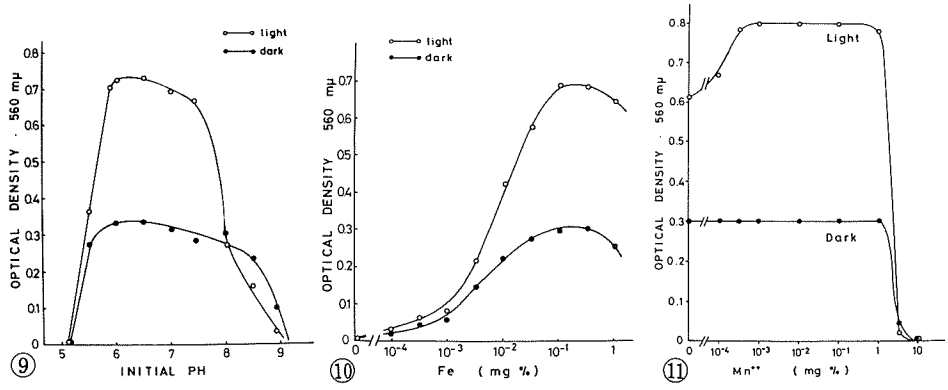
The growth response of *G. multicoccum* to varying concentrations of Na-acetate $\cdot 3H_2O$ is shown in Fig. 6. The two strains required 300 mg% to 500 mg% for maximum growth under light and dark conditions. Growth was almost linearly related to acetate concentration up to 300 mg%. Above 500 mg%, growth was inhibited. The two strains did not grow in acetate-free medium, even after 10 days.

Buffer

The growth response of *G. multicoccum* to varying concentrations of Tris (hydroxymethyl) amino methane and glycylglycine are presented in Figs. 7 and 8. Each solution of the two buffers was adjusted to pH6.6-7.2 after autoclaving. Each experimental medium contained concentrations of mg% as the volume of Tris-aminomethane or glycylglycine. Growth was almost linearly related to the Tris concentration up to 90 mg% in the light. Optimal growth in the light was maintained at Tris concentrations ranging from 90 to 300 mg%. Tris did not inhance growth in the dark. Tris tended to inhibit growth at concentrations exceeding 300 mg%. Growth response to glycylglycine was similar to Tris with the exception that there was no inhibition at the highest concentration tested (300 mg%).

pH-range

Figure 9 shows growth response to different initial pH values. The initial culture media



Figs. 9-11 Growth of *Gonium multicoccum*: Fig. 9, Effects of initial pH (Basal medium = M-52); Fig. 10, Effects of Fe^{++} concentrations (M-52 minus $FeSO_4 \cdot 7H_2O$); Fig. 11, Effects of Mn^{++} concentrations (M-52 minus $MnCl_2 \cdot 4H_2O$). Optical densities were measured after 60-hr incubation in the light and dark.

contained 100 mg% Tris-HCl buffer. The pH range of 6.0-6.5 supported optimum growth. At higher and lower pH values growth was inhibited, and no growth occurred below pH 5 or above pH 9. In the presence of 100 mg% of Tris, the pH values shifted from 6.5 to 10.0 after 60 hrs incubation in the light, and to 8.5 in the dark. It was supposed that these phenomena were affected by Na-acetate, and these high values of pH reversibly inhibited the growth.

Chelator

Na_2-EDTA and Na_3-EDTA chelators were tested on *G. multicoccum*. The two strains grew best in Na_2-EDTA. Further, the lag phase of growth was considerably shorter in Na_2-EDTA than in Na_3-EDTA.

Trace elements

Figure 10 shows growth response to varying concentrations of $FeSO_4 \cdot 7H_2O$ (Fe^{++}). Na_2-EDTA was used as the chelator in this study. The ferrous ion (as $FeSO_4 \cdot 7H_2O$) was required at higher concentrations for maximum growth than found in P II or P IV metal mixtures (PROVASOLI & PINTNER, 1959) in the light and dark conditions. Mn-ion (as $MnCl_2\ 4H_2O$) was required at the same concentration for maximum growth as found in P II and P IV metal mixtures (Fig. 11). The other trace elements compounded in P II and P IV metal mixtures were also required at the same levels given in the original prescriptions. However, Boron (contained in P II metal mixtures) was not required for growth. Figure 12 shows the effects of continuous transferring *G. multicoccum* from cultures with B (20 mg%) in Medium 52 to cultures without B. These results indicated no effective difference in growth with or without Boron in the light and the dark. From the above results, the metal mix G (Table 5) was developed from P II and P IV metal mixtures.

Vitamin nutrition

Table 7 shows that No. 1 and No. 2 tester supplement solutions (Table 6) supported the maximum growth, and No. 4 supported poor growth. Essentially no growth occurred in Nos. 3, 5 and the control culture (no vitamins). These results indicated that thiamine was required by two strains. The maximum and the minimum levels of thiamine were then determined (Fig. 13). Thiamine concentrations ranging 0.001-10 mg% provided for maximum growth. No growth was observed in thiamine-free cultures.

The stimulation effect of B_{12} on growth with only 0.02 mg% of thiamine in Medium 52 and excluding other vitamins is shown in Fig. 14. The results indicated that B_{12} was required at a concentration of about 0.0001 μg% or higher to reduce the lag time, but the final yield was not changed. This was particularly true for light conditions. Thus, a chemically defined medium, called Medium-G (Table 5) was finally established as a result of the above nutritional experiments for the two strains of *G. multicoccum*.

Organic nitrogen sources

The preceding experiments have shown that the two strains can utilize ammonium and nitrate as inorganic nitrogen sources for the growth. Utilization of organic nitrogen sources

Table 7 Effect of vitamins on the growth of *G. multicoccum*.

Vitamin mixture	Growth after 70 hrs (O. D. 560 mμ)			
	Light		Dark	
	7-1	7-2	7-1	7-2
No. 1	0.8	0.8	0.34	0.34
No. 2	0.8	0.8	0.32	0.32
No. 3	0.1	0.12	0.07	0.08
No. 4	0.32	0.19	0.12	0.06
No. 5	0.1	0.12	0.07	0.08
No-vitamin	0.1	0.1	0.08	0.08

Vitamin mixture: SAITO, 1972.

Vitamin mixture (concentration/1000 m*l* medium)

No. 1: riboflavin, 5 μg; thiamine HCl, 0.2 mg; pyridoxine 2HCl, 0.04 mg; pantothenate, 0.1 mg; para-aminobenzoic acid, 0.01 mg.

No. 2: thiamine HCl, 0.2 mg; nicotinic acid, 0.1 mg; choline H_2 citrate, 0.5 mg; folic acid, 2.5 μg; biotin, 0.5 μg.

No. 3: pyridoxine 2HCl, 0.04 mg; choline H_2 citrate, 0.5 mg; inositol, 1.0 mg; pyridoxamine 2HCl, 0.02 mg; orotic acid, 0.26 mg.

No. 4: pantothenate, 0.1 mg; folic acid, 2.5 μg; pyridoxamine 2HCl, 0.02 mg; cyanocobalamine, 0.05 μg; putrescine 2HCl, 0.04 mg.

No. 5: para-aminobenzoic acid, 0.01 mg; biotin, 0.5 μg; orotic acid, 0.26 mg; putrescine 2HCl, 0.04 mg; thymine, 0.8 mg.

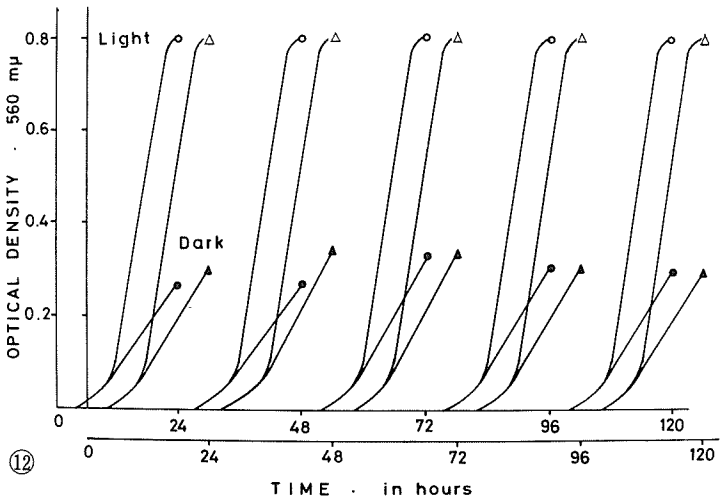
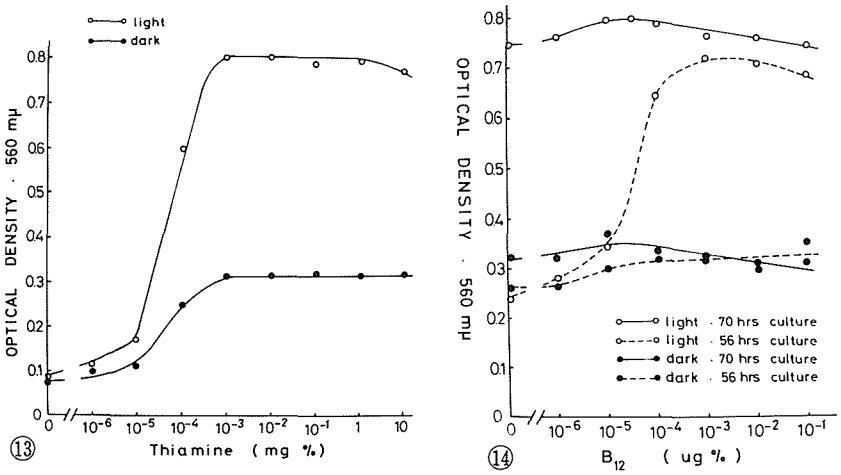


Fig. 12 Effects of continuous transferring cultures, with boron (0.1 mg%) or without, in Medium 52 on the growth of *Gonium multicoccum*. Medium 52 (Metal mix G, without boron), —○—, —●—; M-52 (Metal mix G-II, with boron), —△—, —▲—.



Figs. 13–14 Growth of *Gonium multicoccum* in the light and dark: Fig. 13, Effects of thiamine concentrations (Basal medium=M-52 minus Vitamin mix No. 8), Optical densities were measured after 70-hr incubation; Fig. 14, Effects of B_{12} concentrations (M-G minus B_{12}), Optical densities were measured after 56- and 72-hr incubations.

was tested under the light and dark culture conditions in Medium-52 with an inintial pH 6.8 -7.2.

Concentrations of each organic nitrogen source were at first tested at two levels: 5 mg% and 10 mg%. The following organic nitrogen sources were investigated: *Amino-acids*; L -arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-hydroxyproline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L -serine, L-threonine, L-tyrosine, L-valine and L-tryptophane. *Amids*; L-glutamine, L -asparagine and acetoamide. *Other compounds*; urea, uric acid, adenine, hypoxanthine, uracil, cytosine, thymine, xanthine and trypticase (casaamino acid).

Of the thirty organic nitrogen sources tested only six provided for growth of the two strains (Table 8). Urea supported growth at a level similar to ammonium and nitrate. Growth in arginine, L-glutamine, uric acid and trypticase was fairly good. Whereas the cell density for arginine was slightly lower than urea, it took 140 hrs culture to reach maximum density, suggesting a long lag phase.

Organic carbon sources

With the exception of acetate which was mentioned earlier (Fig. 6), the various compounds were tested at 100 mg% and 200 mg% in light and dark with an adjusted pH of 6.8–7.1.

The following organic carbon sources were investigated:

Sugars; glucose, fructose, galactose, maltose, sucrose, lactose, xylose, mannose, arabi-

Table 8 Effects of organic nitrogenous compounds on the growth of *G. multicoccum*.

	Concentrations for the optimum growth (mg/100 m*l* of medium)	Growth after 60 hrs (O. D. at 560 mμ)	
		Light	Dark
Urea	20 mg	0.75	0.30
L-glutamine	100 mg	0.22	0.15
Uric acid	20 mg	0.33	0.08
L-arginine	40 mg	0.51*	0.20*
L-cystine	20 mg	0.07	0.00
Trypticase	100 mg	0.22	0.15

* Growth was measured after 140 hrs culture.
Other 24 kinds of nitrogen compounds (see text) did not serve as a nitrogen source at concentration of both 5 and 10 mg%.

Table 9 Effects of organic carbon sources on the growth of *G. multicoccum*.

	Concentration for the optimum growth (mg/100 m*l* of medium)	Growth after 60 hrs (O. D. at 560 mμ)	
		Light	Dark
Na-pyruvate	500 mg	0.25	0.00
Na-lactate	600 mg	0.34*	0.00*
Na-acetate	300 mg	0.77	0.34

* O. D. after 10 days incubation.
Other 57 kinds of carbon compounds (see text) did not serve as a carbon source at concentrations of both 100 and 200 mg.

nose, ramnose and inulin.

Krebs-cycle acids; lactate, citric acid, succinic acid, fumaric acid, malic acid, oxalacetic acid, glycolic acid, isocitric acid, pyruvate, α-ketoglutaric acid, cis-aconitic anhydride, glyoxylic acid and maleic acid.

Fatty acids; formate, propionate, butyrate, valerate, oxalate, malonate and fumarate.

Alcohols; ethyl, glycerin, n-propyl, n-butyl and n-amyl alcohol.

Amino acids and other compounds; 18 kinds of amino acids, urea, L-asparagine, L-glutamate, D-aspartic acid and acetoamide.

Of the sixty carbon sources tested only Na-acetate, Na-lactate and Na-pyruvate supported growth in the light (Table 9). Of these Na-acetate was the superior carbon source. Na-lactate, which gave the second best results, required 10 days to obtain the maximum O. D. (0.34), indicating a long lag phase. Only Na-acetate supported growth in the dark.

Other carbon sources such as sugars, fatty acids, etc. tested, but did not support growth of two strains. These results suggest that the algal strains remarkably depend on acetate for the growth in the light and dark.

Sulphur sources for the growth

The following sulphate sources were tested : *Inorganic* ; $Na_2S \cdot 9H_2O$, $Na_2S_2O_5$, Na_2SO_4, $NaHSO_3$ and $MgSO_4 \cdot 7H_2O$.

Organic ; cystine, cysteine, DL-homocystine, L-methionine, and taurine. These sources were tested at a concentration of 10 mg% in Medium 52. Five mg% $MgCl_2 \cdot 6H_2O$ replaced the $MgSO_4 \cdot 7H_2O$ in the basal medium except in the control cultures (i. e. the culture when $MgSO_4 \cdot 7H_2O$ was tested). The initial pH was adjusted to 6.6-7.0.

$MgSO_4 \cdot 7H_2O$ supported good growth, $Na_2S \cdot 9H_2O$, $Na_2S_2O_5$, Na_2SO_4, $NaHSO_3$ and cystine supported fair growth, and cysteine and DL-homocysteine supported poor growth (Fig. 15). Taurine and DL-homocystine supported no growth. This study demonstrated $MgSO_4 \cdot 7H_2O$ to be a superior sulphate source (cf. Fig. 4).

The arginine effects for the growth as a nitrogen source

The preliminary experiment, using arginine as a nitrogen source showed that while allowing for good final yield arginine also caused a long lag-time (cf. Table 8). Moreover, this phenomenon was not due to adaptation, as the results of showing the same growth

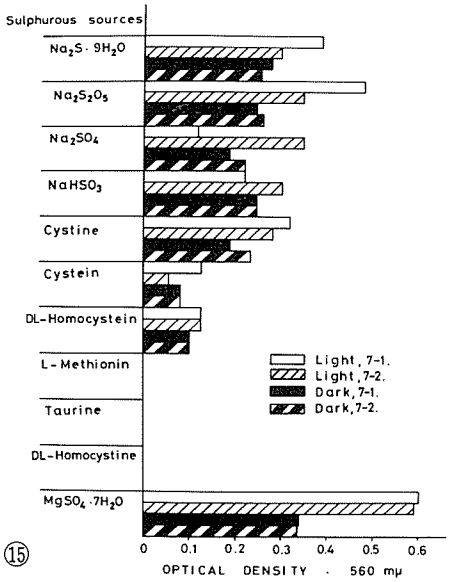


Fig. 15 Effects of sulphurous sources at the concentration of 10 mg% on the growth of *Gonium multicoccum*. Basal medium = M-52 minus $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$, and plus $FeCl_3 \cdot 6H_2O$. Optical densities were measured after 60-hr incubation.

pattern as used the precultured material in arginine or NH_4 medium.

In order to solve this question of the long lag-time in arginine, the following culture experiments were conducted.

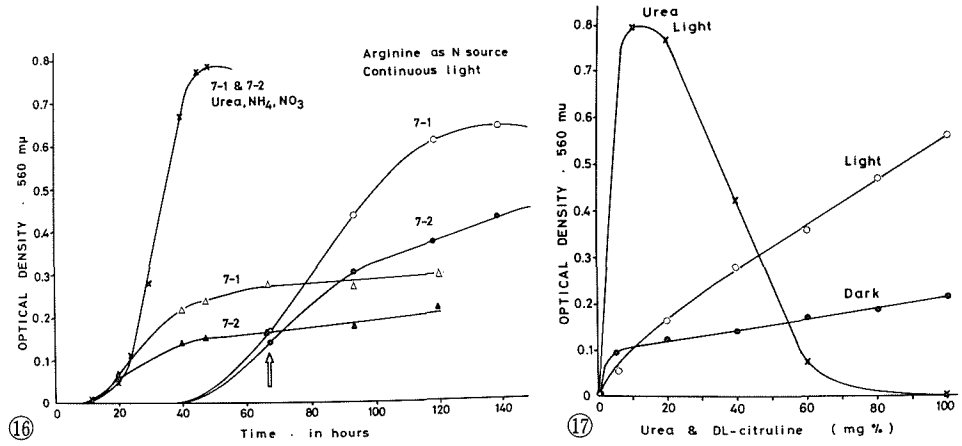
The algae were first cultured in Medium 52 (minus NH_4Cl) with arginine (at the concentration of 40 mg%). After 70 hrs incubation, they reached beginning of the exponential phase, and then were removed from the medium by filtration through HA millipore filter paper. Fresh algae were inoculated into this old medium (conditioned medium) and their growth curve calibrated.

In the old medium (conditioned medium) the long lag-time disappeared and this lag -time was similar to that of NH_4, NO_3 and urea (Fig. 16).

Urea cycle metabolites utilization as nitrogen sources

In addition to urea and arginine (see Table 8 and Fig. 16), other available urea cycle metabolites and related compounds were tested as nitrogen sources. The following nitrogen sources were tested at the two levels (5 mg% and 20 mg%): creatinine, creatine, DL -citrulline, DL-norvaline and L-ornithine.

Preliminary experiments showed that DL-citrulline supported growth only slightly, and the other compounds tested were not utilized. In the next experiment DL-citrulline was provided in varied concentrations and compared with urea as a nitrogen source. Urea was required at concentrations of approximately 20 mg% for the maximum growth. However, DL-citrulline was required at concentrations higher than 100 mg% (Fig. 17), whereas these



Figs. 16-17 Growth of *Gonium multicoccum*: Fig. 16, Effects of arginine (—○—, —●—), urea, NH_4 and NO_3 (—×—) and conditioned medium derived from the arginine medium (—△—, —▲—) after 70-hr incubation (⇦), on the growth of 7-1 and 7-2 strains in continuous light condition; Fig. 17, Effects of urea (—×—) and DL-citrulline (—○—, —●—) concentrations in M-52 minus ammonia. Optical densitites were measured after 60-hr incubation in the light and dark.

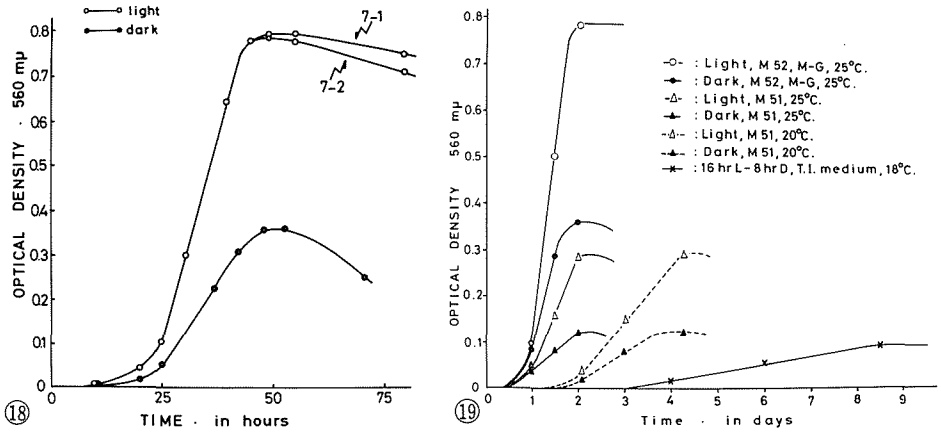


Fig. 18 Growth of *Gonium multicoccum* in Medium G at 25±1℃ and in the light and dark.
Fig. 19 Developmental culture media for the growth of *G. multicoccum*. Growth curves were obtained under various culture conditions and in different media.

concentrations of urea inhibited growth.

Growth curves

As a final test of the findings and interpretations of the preceding nutritional experiments, growth curves were plotted for the two strains in media M-G, M-T. I., M-51 and M-52 (Figs. 18 and 19). Under continuous light in M-G, the two strains reached the maximum growth (O. D. 0.77) within 50-hr culture, and then gradually decreased in O. D. During the initial lag phase and the exponential the two strains grew at the same rate and only after obtaining maximum O. D. they were different. Both strains reached the maximum growth (ca. O. D. 0.36) in the dark within 50 hrs, and they rapidly decreased.

A growth rate reaching 5.8 doublings per day was obtained at 25±1℃ in the continuous light (cf. HOOGENHOUT and AMESZ, 1965). The final yield in Medium G was approximately three times that in Medium 51 (O. D.: 0.28), and eight times that in T. I. medium (O. D.: 0.10, Fig. 19). In summary, the developed medium, M-G provided for a greater yield and a faster growth rate than the other tested media.

3. Growth measurement and growth rate

Different methods of measurement for the growth

Three methods of measurement for the growth of *G. multicoccum* were employed in the present experiment: optical density (O. D.), dry weight (D. W.) and packed cell volume (P. C. V.). Growth curves were plotted for the following four culture conditions; 7-1 and 7-2 strains in the continuous light, 7-1 and 7-2 strains in darkness. Further, the relationship between the three methods of measurement was determined. In this experiment, 50

m*l* of Medium 52 were used in 100 m*l* Erlenmeyer flasks capped with cotton plugs.

Figure 20 shows the three growth curves as determined by the different methods of measurement. The growth curves of 7-1 and 7-2 strains in the light and dark as determined by O. D. and D. W. were identical until the final exponential phase of growth.

The growth curve of 7-1 strain in the light declined more rapidly after reaching maximum growth than did 7-2 strain. The two strains had identical growth curves in the dark.

The growth curves were different in the light and dark for 7-1 and 7-2 strains when P. C. V. was plotted. Further, the P. C. V. growth curves differed from the other two in that they took longer time to reach maximum growth.

Figure 21 shows the correlation between O. D. and D. W. There was essentially a straight line relationships, regardless of strains or light or dark conditions.

Figures 22 and 23 show the correlation between D. W. and P. C. V., and between O. D. and P. C. V. respectively. These results indicated a considerable difference in P. C. V. between 7-1 and 7-2 strains grown in the light, but not in the dark. This difference in P. C. V. between 7-1 and 7-2 strains grown in the light, may be due to a difference in cell expansion.

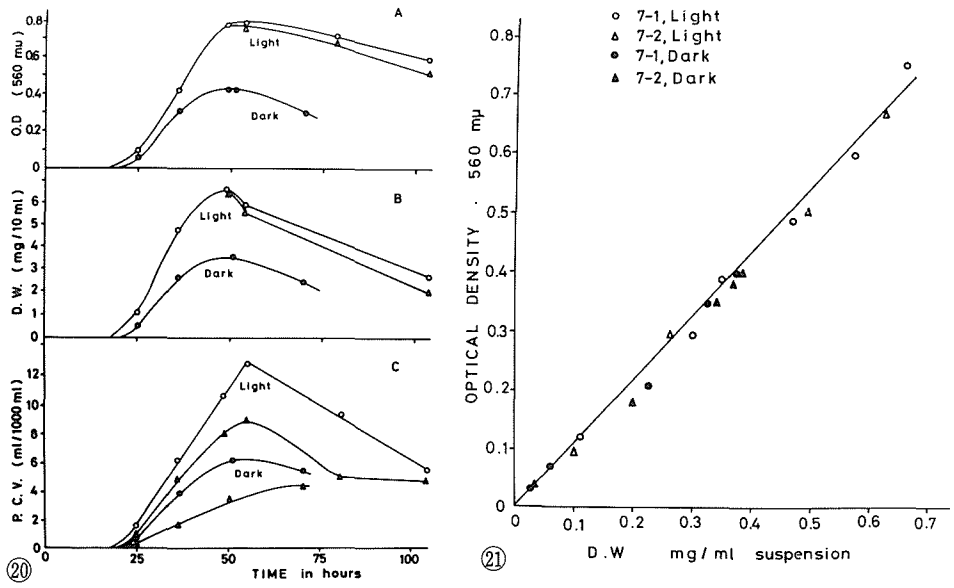


Fig. 20 Growth curves of *Gonium multicoccum* (7-1 strain=—○—, —●—; 7-2 =—△—, —▲—) cultured in 150 m*l* of Medium 52 (in 300 m*l* Erlenmeyer flasks) at 25℃ in the light and dark; Growth was measured by optical density (A), dry weight (B) and packed cell volume (C).

Fig. 21 Correlation between optical density and dry weight at various growth phases of *G. multicoccum*.

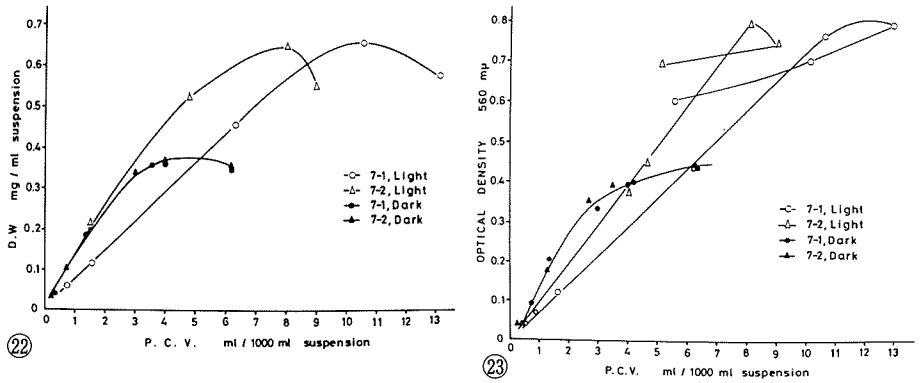


Fig. 22 Correlation between dry weight and packed cell volume at various growth phases of *Gonium multicoccum*.

Fig. 23 Correlation between optical density and packed cell volume at various growth phases of *G. multicoccum*.

The interrelationships of O. D., D. W. and P. C. V. could be used as calibration curves of growth for *G. multicoccum*.

Optical density as a turbidimeter

In this study growth has mainly been measured in terms of optical density using a Baush & Lomb spectrophotometer with a test tube adaptor. Measurements were made at 560 mμ, thus, using the instrument as a turbidimeter. In this way, one avoided measuring growth as a function of chlorophyll concentration.

Figure 24 shows three absorption spectra of *G. multicoccum* 7-1 strain which were axenically grown in Medium 52 in the light at 25℃. Followings are descriptions of how the spectra were obtained.

A) Absorption spectra of intact cells measured with a Hitachi EPS-3T auto-spectrophotometer using an integrating sphere adaptor.

B) Absorption spectra of intact cells measured with a Baush & Lomb Spectronic 20 photometer.

C) Absorption spectra of aceton extracts (boiled for 3 min. at 80℃.) measured with a Hitachi 124 double-beam spectrophotometer.

These results show that chlorophyll did not interfere with O.D. measurements of whole cells made at 560 mμ. This is particularly true for whole cell measurements made with the Baush & Lomb spectrophotometer predominantly used in this study (Fig. 24 B).

Growth rate and temperature shift

Strains 7-1 and 7-2 were subjected to a study of the effects of temperature on their growth rate in continuous light, and compared with other algae.

Figure 25 shows the results of inoculum amount on the growth constant (kg) for the two

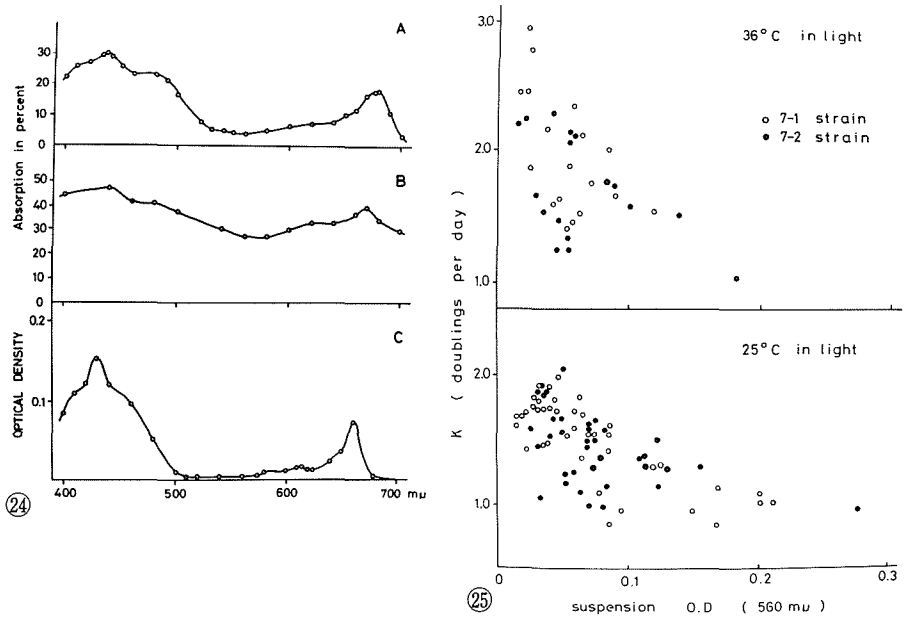


Fig. 24 Absorption spectra of whole cells and acetone extracts from *Gonium multicoccum*. A= whole cell (with integrating sphere adaptor); B=whole cell (Baush & Lomb Spectronic 20 photometer); C=acetone extract.

Fig. 25 Effects of temperature and initial inoculum size on the growth constant (*kg*) at the lag phase of *G. multicoccum* as measured after 30-hr incubation.

strains at two temperatures. These results indicated that the growth constant was influenced by the amount of inoculum, and reached higher with little inoculum suspension (within the limiting region under O. D. 0.05) than with much one. However, the difference of growth constant caused by the distinction of strains could not be found in this experiment. On the other hand, the variation of growth constant could be recognized the extend over fairly wide range of *K*. Each growth constant *K* at certain temperatures was calculated with the average of four or more tubes and two cultures of 7-1 and 7-2 strains from at least three separate experiments.

Figure 26 shows the growth constant as the influence of temperature shift. The maximum growth constant of *G. multicoccum* (from Nepal) was calculated at $K=7.3$ at 36℃. However, strains 7-1 and 7-2 could not grow and survive at 43℃ and over.

Figure 27 shows the two types of growth curves expressed by semi-log plot in the cases of the initial inoculum of O. D.=0.13 and two drops of algal suspension at the same culture conditions of the continuous light at 25℃. This experiment indicated that *G. multicoccum* grown in Medium 52 did not have a measurable lag-time. Thus, when inoculated with two

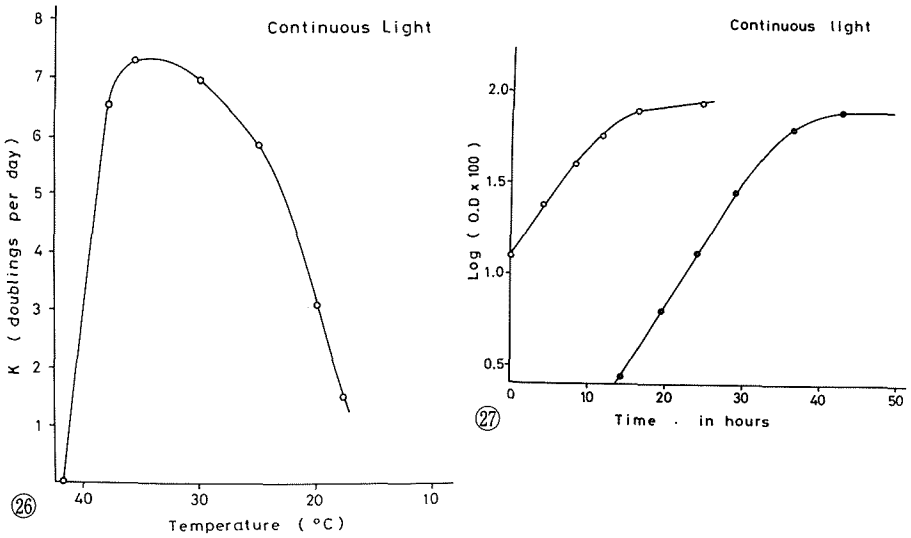


Fig. 26 Effects of temperature on the growth constant (K=doublings per day) of *Gonium multicoccum* in the light. Growth constants were measured and calculated from optical density at the lag phase of growth at each temperature.

Fig. 27 Relationship between lag-time and inoculum size on the growth of *G. multicoccum* at 25℃ in the light. Initial inoculum: —○—, O. D. =0.13 and —●—, two drops of algal suspension.

drops the exact lag-time could not be determined, because the initial optical density of the inoculum could not be measured in a Bauch & Lomb spectrophotometer. Thus, the lag phase characteristic for this alga grown in Medium 52 was greatly shortened by the larger inoculum.

Growth rate on the level of colony development

In this experiment, the developmental processes of selected colonies were observed. The increase in cell number, the growth rate and generation time of colony development were determined.

Only 16-celled colonies of the 7-1 strain were used in this experiment. Ten m*l* of Medium-1 agar medium (1% agar medium, Table 4) were employed. A single 16-celled colony was inoculated on the flat agar surface in a Petri dish (95×18 mm), and cultured in the light at 36℃. Colony development was observed under a microscope and micro-photographs were taken.

Plate II shows the relationship between colony development and culture time. These indicated that one generation time required for one 16-celled colony to produce sixteen, 16-celled colonies was occurred approximately 8 hrs. This gives a calculated K of 12, when expressed in doublings per day.

Further, the following results were obtained from the present experiments. Cell number of colony was not genotypically determined, but was effected by culture conditions. The formation and cell-divisions of daughter colonies were synchronized in culture.

4. Effects of inhibitors for the vegetative growth

Two inhibitors were used in this study. 3-(p-chlorophenyl)-1, 1-dimethyl-urea (CMU) was dissolved in 99% ethyl alcohol. The ethyl alcohol with CMU was used at a final concentration of 0.3% in the experimental medium. 2, 4 Dinitrophenol (DNP) was dissolved in deionized water, and used at different concentrations.

Growth was measured as O. D. with Spectronic 20 1/2 test tube (by Bausch & Lomb) in this experiment after 60 hrs incubation.

The effects of CMU and DNP on the vegetative growth of *G. multicoccum* (7-1 and 7-2 strains) are demonstrated in Table 10. DNP, a potent inhibitor of oxidative phosphorylation, but less effective on photosynthetic phosphorylation (DUYSENS, 1964), had no effect in either the light or dark on growth. On the other hand, CMU, a specific inhibitor of noncyclic photophosphorylation (Photosystem II), inhibited growth in the light at the low concentration of 10^{-7} M. The final yield of vegetative growth in the light was similar to that in the dark.

These results suggested that the photoassimilation of acetate may be involved with non-cyclic photophosphorylation.

Table 10 Effects of photosynthetic inhibitors (CMU & DNP) for the vegetative growth of *G. multicoccum*.

	Growth after 60 hrs (O. D. 560 mμ)	
	Light	Dark
Control M 52	0.67	0.25
99% ethanol 0.15 m*l* in M 52	0.66	0.25
CMU 10^{-7}M	0.20	0.25
DNP 10^{-4}M	0.57	0.27
10^{-5}M	0.63	0.25
10^{-6}M	0.63	0.25
10^{-7}M	0.67	0.25

CMU : 3-(p-chlorophenyl)-1, 1-dimethylurea ; alcohol solution. final conc=0.3%.
DNP : 2, 4-dinitrophenol.
Cultures employed 150 m*l* media in 300 m*l* Erlenmeyer flasks. The growth was measured by O. D. with one-cm cell at 560 mμ with Bauch & Lomb spectrophotometer after 60-hr culture.

5. UV absorption spectra difference between culture media of algae grown in light and dark

The algae were cultured in Medium 52 in the light and dark for 35-hr, 50-hr and 60-hr, and then removed from the medium by centrifugation (2,000-4,000 rpm, followed by 10,000 rpm). UV absorption spectra of the old medium (conditioned medium) were measured with a Hitachi 124 double-beam spectrophotometer (Figure 28.)

There were considerable differences in the UV absorption spectra between the light and dark culture media. The absorption peak in the light culture media was at 212 mμ, but in the dark culture media the same wavelengh was observed under the standard zero point. This minus absorption peak increased slightly above the standard zero point for 60-hr old culture in the dark. The differences of absorption spectra were probably caused by different chemical components of the old media in the light and in the dark. Therefore, this difference of absorption spectra may be due to different endoproducts or metabolic products from the cultures grown in the light and dark.

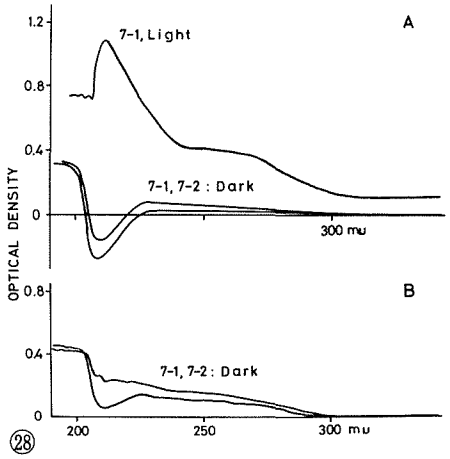


Fig. 28 UV absorption spectra of old culture media from *Gonium multicoccum*. A=50-hr'culture media, 7-1 strain in light, 7-1 and 7-2 in dark; B=60-hr' culture media, 7-1 and 7-2 in dark.

6. Discussion

The following topics will be discussed in this chapter; 1) vitamin nutrition, 2) nitrogen nutrition, 3) carbon sources nutrition, 4) other nutrition and 5) growth rate.

Vitamin nutrition

Within the volvocalean genera, enhancement of growth by thiamine (but only in

combination with B_{12}) has been demonstrated in *Pleodorina* (PRINGSHEIM & PRINGSHEIM, 1959) and in *Astrephomene* (BROOKS, 1965). In the two strains of *Gonium multicoccum* examined, the requirement for an exogenous vitamin source is fulfilled by thiamine. *Gonium multicoccum* is the first member of the volvocalean genera known to require only thiamine. The addition of B_{12} shortens the initial lag phase of growth.

Nitrogen nutrition

PROCTOR (1957) observed that *G. pectorale* assimilates ammonia rather than nitrate. According to STEIN (1966 a), however, growth of *G. pectorale* was similar with either nitrogen source. Both inorganic nitrogen sources affect growth of *G. multicoccum* similarly, but growth is inhibited by a high concentration of ammonia. *G. multicoccum* tolerates at higher nitrate concentrations than many phanktonic algal species (ZOBELL, 1935; CHU, 1942; SPOEHR *et al*. 1949).

Nitrite, at the low concentration of 8 mg% can also serve as an inorganic nitrogen source for *G. multicoccum*, but higher concentrations are inhibitory. This phenomenon has been noted for many algal species (ZOBELL, 1935; LUDWIG, 1938; FOGG *et al*., 1954).

Growth is excellent in urea under both light and dark conditions, and good when arginine is used as the sole N-source. Uric acid, L-glutamine, DL-citrulline and trypticase support some growth in both light and dark conditions. This suggests that *G. multicoccum* contains a wide range of enzymes to permit utilization of diverse sources of N.

Carbon sources nutrition

It is known that the acetate flagellates are generally incapable of utilizing sugars as the carbon source (HUTNER & PROVASOLI, 1951, 1955). Acetate can serve as a carbon source for the growth of many green algae (FOGG & MILLBANK, 1960; DANFORTH, 1962). This character is shared with the strains of *G. multicoccum* used in the present investigation. Efficient utilization of acetate for the growth of *G. multicoccum* in the both light and dark has already been shown by PRINGSHEIM and PRINGSHEIM (1959).

Besides acetate, pyruvate and lactate also support the growth of *G. multicoccum* in the light (Table 9), but they are completely ineffective in the dark. The reason why is not immediately clear.

The metabolic pathways by which acetate carbon is incorporated into cellular carbohydrates have been investigated in the following algae: *Chlamydobotrys stellata* (PRINGSHEIM & PRINGSHEIM, 1959; PRINGSHEIM & WIESSNER, 1960, 1961; WIESSNER, 1962, 1963, 1965, 1966, 1967, 1968, 1969; WIESSNER & KUHL 1962; GAFFRON *et al*., 1963; WIESSNER & GAFFRON, 1964; GOULDING & MERRETT, 1967; MERRETT, 1967), *Chlamydomonas mundana* (EPPLEY & MACIASR, 1962; EPPLEY *et al*., 1963; RUSSEL & GIBBS, 1964, 1966), *Euglena gracilis* (PRINGSHEIM, 1935; OHMANN, 1964), *Chlorella vulgaris* and *Chlorella pyrenoidosa* (SCHLEGEL, 1956, 1959; MERRETT & SYRETT, 1960; SYRETT *et al*., 1963, 1964; GOULDING & MERRETT, 1966; SYRETT, 1966; MERRETT & GOULDING, 1967 a, b).

The experimental organisms *Chlamydobotrys stellata, Chlamydomonas mundana,*

Euglena gracilis and *Chlorella pyrenoidosa* are grown in autotrophic and photo-heterotrophic cultures (acetate as a carbon source), but the former two species can not grow in the dark. On the other hand, as a result of the present experiment, the strains of *G. multicoccum* can be supported by acetate as a carbon source in the light (photo-heterotrophic growth) and in the dark (heterotrophic growth), and moreover they can not grow autotrophically, even in the presence of bubbling CO_2. In this respect, it should be emphasized that these strains, though they contain chlorophyll and other photosynthetic pigments, cannot grow in the light as well as in the dark, in the absence of organic carbon sources. *G. multicoccum* may disclose new relationships between autotrophic, heterotrophic and photo-heterotrophic growth.

Other nutrition

Many species of *Chlorella, Chlamydomonas*, and other algae (ARNON & WESSEL, 1953; KATE & JONES, 1964; KRAUSS & THOMAS, 1954; MACLASR, 1965; MCILRATH & SKOK, 1958; MYERS, 1944; PRATT, 1941; SAGAR & GRANICK, 1953; SOROKIN & KRAUSS, 1958; SOROKIN, 1963; TRAINOR, 1958; WETHERELL, 1958) have been cultured in media containing at least 0.001 M $MgSO_4$. In contrast, the volvocalean algae; *Gonium pectorale* (STEIN, 1966 a), *Volvulina* (CAREFOOT, 1967), *Platydorina* (HARRIS, 1969) and *Volvox* (DARDEN, 1966, PROVASOLI & PINTNER, 1959), have been cultured in a medium containing as low a concentration as 0.00008–0.0003 M of $MgSO_4 \cdot 7H_2O$. In *G. multicoccum*, the two strains grow well at the low concentration of 0.0001 M in the light and 0.00004 M in the dark. They also grow well at a concentration of 0.001 M.

Most algae have been reported to require calcium and phosphate, usually as $CaCl_2$ and KH_2PO_4 or K_2HPO_4, at a concentration of about 0.0001–0.0005 M and 0.001–0.01 M, respectively. *Gonium multicoccum* has similar requirements.

Although Tris is a good buffer for marine algal media (PROVASOLI *et al.*, 1957, PROVASOLI & PINTNER, 1959), *Volvox* (PROVASOLI & PINTNER, 1959) and *Haematococcum* (MCLACHLAN, 1963) are inhibited by it. In contrast, the two strains of *G. multicoccum* are inhibited at low concentrations (0–90 mg%) in the light, but Tris has no effect on growth in the dark. Glycylglycine has the same effect as Tris on the growth of *G. multicoccum*. It may be shown that Tris and glycylglycine have some other effects on growth in the light than pH buffering.

Growth rate

Growth rate K of the two strains of *G. multicoccum* were calculated as 7.3 and 5.8 doublings per day in liquid culture under continuous light at 36℃ and 25℃. Moreover, in an experiment on the increase of cell numbers of one colony during its developmental cycle, 7-1 strain doubled 12 times per day on agar Medium-1 in the light at 36℃. Compared to other algae in liquid culture, higher growth rates than these two strains of *Gonium* have been reported in 5 strains of blue green algae (KRATZ & MYERS, 1955; BAALEN, 1961, 1963; DYER *et al.*, 1961; PEARY & CASTENHOLZ, 1964) and in the green algae *Chlamydomonas*

mundana (MACIASR & EPPLEY, 1963) and *Chlorella pyrenoidosa* strain TX 7-11-05 (SOROKIN, 1960).

According to the list of the algal growth rate by HOOGENHOUT and AMESZ (1965), about 90% of the growth constants for algae range between 0.5 and 5.0. The growth rate of *G. multicoccum* shows one of the most rapid growth rates of algae in liquid culture under these culture conditions. However, the growth rate of $K=12$ for *G. multicoccum* colonies on agar medium is the most rapid growth rate reported for an alga.

The two strains of *G. multicoccum* studied were selected for comparison not because they were necessarily " representative " of the species, but because of their excellent growth and easy culture.

The culture media and techniques developed in this study make available a new experimental volvocalean alga. This alga may provide clues regarding the control of the volvocalean life cycle, reproduction and various physiological/biochemical processes.

II Nutritional Control of Sexuality and Life Cycle of *G. multicoccum*

Gonium is one of the most primitive members of the family Volvocaceae. All cells in a colony have the potential to develop into asexual reproductive cells or isogamous gametes in various culture conditions. Therefore, *Gonium* seemed to be an excellent organism to study the control of sexuality and life cycle.

The sexual process of *Gonium* has been studied morphologically by a number of investigators; ROSTAFINSKI (1875), CHODAT (1894), LEMMERMANN (1898), SCHUSSNIG (1911), KORSHIKOV (1923 b), SCHREIBER (1925), KNIEP (1928), STARR (1955), POCOCK (1955) and STEIN (1958, 1959 and 1966 a). However, these studies have been restricted and nothing is known about the physiological basis of sexual induction. The preliminary studies on the control of gametogenesis has been reported only by STEIN (1966 a) in *G. pectorale*. According to her, gametogenesis in the isogamous genera involves a physiological change with the direct conversion of vegetative cells into gametes, when sexually compatible cultures are brought together under suitable environmental conditions.

SCHREIBER (1925) was the first to grow *Gonium* in culture and to show that sexuality is induced only when the two heterothallic strains of *G. pectorale* are mixed. Since then, the following media for sexual culture experiments have been employed; SCHREIBER's solution (SCHREIBER, 1925; KNIEP, 1928), PRINGSHEIM's soil water medium (PRINGSHEIM, 1946; POCOCK, 1955), PRINGSHEIM's soil water medium with or without the addition of calcium carbonate (STARR, 1955) and modified BEIJERINK's medium (STEIN 1958, 1959; and 1966 a). However, these media contained unknown components and were not adapted for the optimum growth of the algae. The life history and sexuality of *G. multicoccum* have not been studied.

Our knowledge on physiological and biochemical aspects of the reproductive processes of *Gonium* is still incomplete. This is probably due to the limitation of culture technique

suitable for growing a large quantity of the algal material under controlled conditions.

In this chapter, the problems of control of sexuality and life cycle have been investigated, using the media and techniques established in earlier chapters for the culture of *G. multicoccum*.

Sexual reproduction in *Gonium* usually involves two separate processes, sexual differentiation leading to gametogenesis and the actual syngamy to form zygotes. The research reported here concentrated on two interests: A) nutritional control and B) light requirement, for sexuality of *G. multicoccum*. In the study of nutritional control of sexuality, mainly nitrogen sources were surveyed; i. e., is nitrogen deficiency important to gametogenesis as in *Chlamydomonas*. In addition, the requirements of acetate and other nutrients for sexual reproduction have been examined. Is light required for sexual induction as was found for *Chlamydomonas* and *Golenkinia*?

This chapter, then, deals with investigations on morphology, nutritional control of sexual reproduction and life cycle of *G. multicoccum*, particularly, gametogenesis, zygote formation, maturation and germination.

1. Materials and methods

Strains 7-1 and 7-2 of *G. multicoccum* were used in this experiment. Basic culture methods were employed as in the previous chapters. Fifty or 2000 m*l* of medium were used in 100 m*l* or 5000 m*l* Erlenmeyer flasks capped with cotton plugs for laboratory mass-cultures.

Free ammonia was determined, using colorimetrical determination of the absorbance of 400 mμ (optical density) with NESSLER's reagent. Aliquots were pipetted out at appropriate time intervals and after centrifuging off the algal cells the quantity of NH_4 was measured.

2. Results

Morphology of the sexual process

The 7-1 and 7-2 strains of *G. multicoccum* used in this investigation were heterothallic pairs and were the opposite mating type to each other.

Each cell in a colony becomes a gamete. These naked gametes escape from the colony. Initial contact is done by the flagella and then followed by pairing along the length of these organelles. Clumping and fusion of the gametes began a few hours after mixing the sexually compatible cultures and placing in light, and continued for several hours. By the formation of a gradually broadening cytoplasmic bridge at the anterior ends of the fusing gametes these fusing gametes became enclosed in a common membrane. The chloroplasts appeared to fuse within an hour of syngamy and the nuclei fused later. The zygote lost its flagella and either settled to the bottom of the culture vessels or floated on the surface of the liquid.

The zygotes formed a thick wall in proportion to the entire matured zygote. The matured zygotes remaining in light became orange, whereas those placed in darkness,

remained green.

After transferring to fresh media (17–30℃ and in light), the zygote germinated and produced four cells (each termed a gone) united in a colony. During germination, the zygote swelled after rupturing the thick wall. Cytokinesis generally occurs at the end of meiosis after the four nuclei had been formed. The germ colony swam away, leaving the zygote wall. Each cell of the germ colony then underwent four mitotic divisions, producing a typical 16-celled colony, and the four new daughter colonies then separated. The

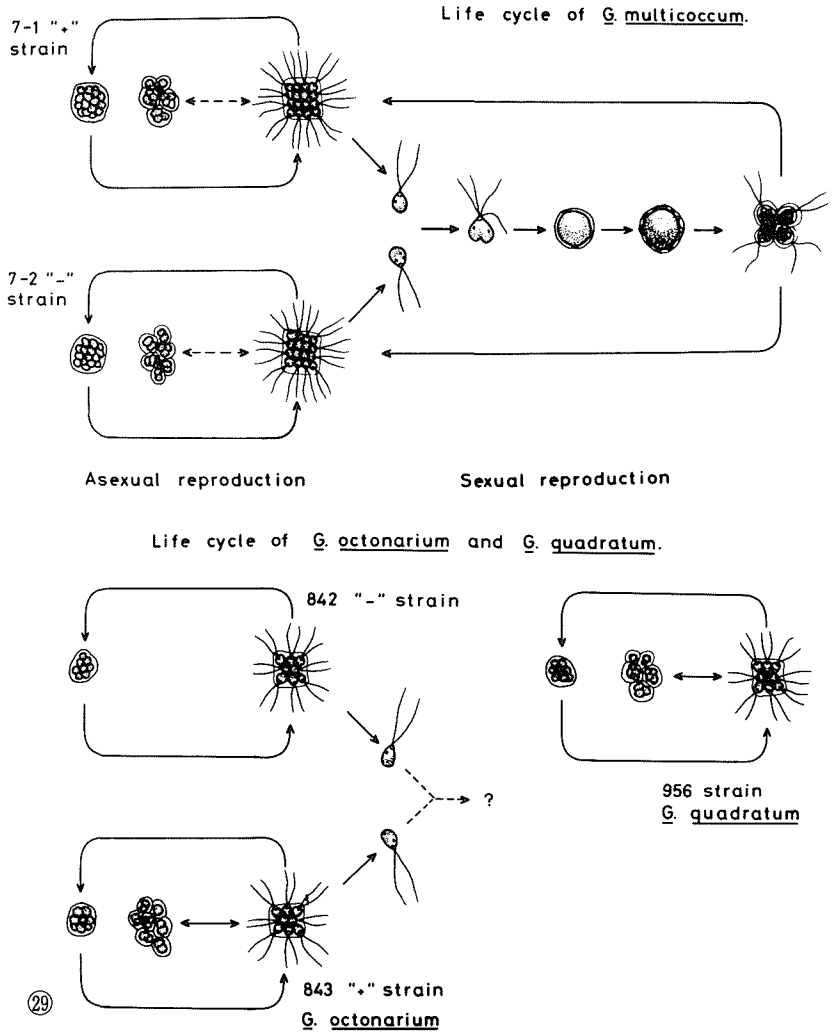


Fig. 29 Life cycle of three species of *Gonium*.

Table 11 Backcross tests of isolated clones from germinated F_1 colonies of *G. multicoccum*.

	F_1 clone numbers												
	1	2	3	4	5	6	7	8	9	10	12	14	15
Parents strains													
7-1, (+)	Z	Z	–	–	Z	Z	–	–	–	–	Z	–	–
7-2, (−)	–	–	Z	Z	–	–	(Z)	Z	Z	Z	–	Z	Z

Test medium for zygote formation was used G-2 medium (see Table 12).
F_1 clones, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 and 15 of *G. multicoccum* were isolated clones from germinated zygotes of 7-1 and 7-2 strains.

chromosomes of colonies were readily stained (by use of Aceto-iron-haematoxylin chloral hydrate method; YABU and TOKIDA, 1966), and it was counted ca. 7 numbers per cell at anaphase (cf. Plate III, F).

The life-cycle of *G. multicoccum* is shown in Fig. 29 and Plates I and III. From either of the 4 gones or the colony produced by each, clonal cultures may be established. To test mating types, cultures arising from the gones were all back-crossed to the parental clonal cultures and designated " plus " or " minus ", depending upon with which parent they mated. An analysis of the gones has shown that one-half are plus and one-half minus (as seen in Table 11).

Preliminary experiment for nutritional control of sexual reproduction

Figure 30 shows the effects of NH_4Cl in the growth of a 7-1 and 7-2 strains mixed culture. There was no difference in either the final yield or the culture time for the maximum growth between the mixed culture (Fig. 30) and separate cultures of the two strains (Fig. 2). Zygote formation was detected by observing the zygotic sediment and microscopic examination. From 2 to 10 days, zygotes were found at NH_4Cl concentrations ranging from 0.1 to

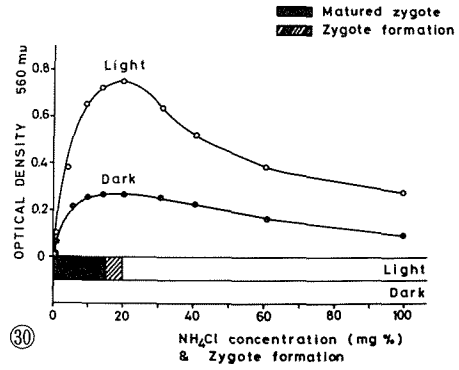


Fig. 30 Effects of NH_4Cl concentrations and light conditions on zygote formation of *Gonium multicoccum*. Optical densities of growth were measured after 60-hr incubation. Zygote formation was observed after 70-hr incubation, and matured zygotes were observed after 10-day in each culture tube.

Table 12 Culture media for sexual reproduction of *G. multicoccum*. (Amounts are per 100 m*l* of medium.)

	G-1	G-2	G-3	G-4
NH_4Cl	—	10 mg	15 mg	10 mg
$MgSO_4 7H_2O$	5 mg	5 mg	5 mg	5 mg
$CaCl_2$	5 mg	5 mg	5 mg	5 mg
KH_2PO_4	15 mg	15 mg	15 mg	15 mg
Na-acetate · $3H_2O$	300 mg	300 mg	300 mg	300 mg
Tris-HCl buffer	1 m*l*	1 m*l*	1 m*l*	—
Metal mix G	1 m*l*	1 m*l*	1 m*l*	1 m*l*
Thiamine	0.02 mg	0.02 mg	0.02 mg	0.02 mg
B_{12}	0.001 μg	0.001 μg	0.001 μg	0.001 μg
pH	6.5	6.5	6.5	6.5

Tris-HCl buffer: one m*l*=Tris-aminomethane, 100 mg.
Metal mix G: SAITO, 1972.
Culture media G-1, G-2, G-3 and G-4 for sexual reproduction were modified from Medium 52 and Medium G.

15 mg% in the light. Matured red zygotes were found at concentrations ranging from 0.1 to 10 mg% in the light (Fig. 30). Zygotes were not be found at higher concentrations of NH_4Cl, or in the dark. In a similar experiment employing $NaNO_3$ zygotes were formed at concentrations ranging from 0.1–15.0 mg% $NaNO_3$ in the light. These results suggested that the growth of 7-1 and 7-2 strains of *G. multicoccum* increased proportionately with nitrogen concentration, and the vegetative colony developed gametes in nitrogen-deficient media in the light.

Thus it would seem that the controlling factors for gametogenesis and zygote formation were nitrogen deficiency and the presence of light and Ca-ion as was observed for *Chlamydomonas*.

On the basis of these results, the life cycle in *G. multicoccum* was controlled by the following media.

(a) Vegetative growth controlling medium: Medium G.

(b) Sexual reproduction controlling media (modified from Medium G, Table 12).
Medium G-1; Nitrogen-free medium.
Medium G-2; NH_4Cl concentration of 10 mg%.
Medium G-3; NH_4Cl concentration of 15 mg%.
Medium G-4; NH_4Cl concentration of 10 mg% and Tris-HCl buffer free.

The relationship between vegetative growth and sexual reproduction

The relationship between vegetative growth and sexual reproduction was explored by testing possible sex inducing factors in mixed cultures of 7-1 and 7-2 strains with Medium G -3. Fig. 31 shows the effects of various culture treatments and culture conditions on sexual

reproduction. At five different growth phases (Fig. 31, arrows denote growth phases) the following experiments were conducted.

(1) Added NH_4Cl; final concentration of 30 mg%.

(2) Added Na_2-EDTA, 1% solution; at the proportion of 0.05 m*l*/10 m*l* medium.

(3) Added 0.2 M citric acid solution; at the proportion of ca. 0.05 m*l*/10 m*l* medium.

(4) Transferred the light cultures to the dark.

Two chelators (Nos. 2, 3 above) would bind and make relatively unavailable some of the micronutrients. This type of deficiency could cause gametogenesis.

Zygote formation (sexual reproduction) occurred only at the stationary phase of the vegetative growth in the light (Fig. 31). In vegetative growth (asexual reproduction), NH_4 was completely assimilated within 50-hr and at the time of the stationary phase in light and dark. Gametogenesis and zygote formation occurred when nitrogen deficiency was reached in the light, but not in the dark. On the other hand, sexual reproduction was not induced by the four treatments enumerated above at each experimental point on the growth curve (Fig. 31, arrows).

The relationship between asexual and sexual reproduction may be explained thusly: The valid controlling factors of gametogenesis were nitrogen deficiency and the presence of light. Under these conditions, mixed colonies of two compatible mating types (7-1 and 7-2 strains) underwent gametogenesis and produced zygotes within 12 hrs.

The difference between light grown colonies and dark grown colonies seemed to originate in NH_4 depletion. Whereas NH_4 was depleted from both light and dark cultures

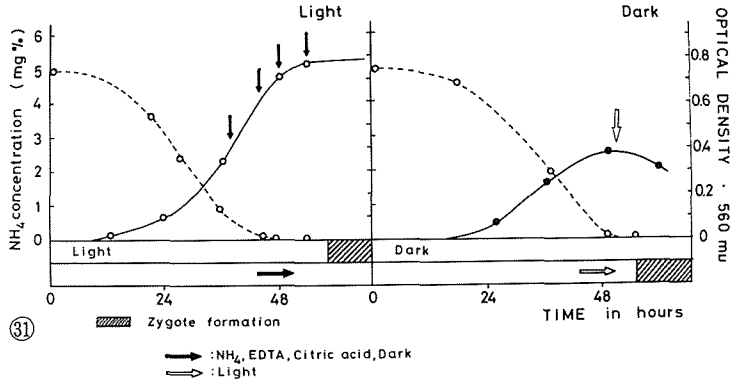


Fig. 31 Effects of nitrogen deficiency, growth phases (noted by arrows) and various limiting factors (NH_4, EDTA, citric acid, light and dark) for zygote formation of *Gonium multicoccum*. NH_4 in medium (···○···) was measured colorimetrically with NESSLER's method. Cultures were grown in 150 m*l* of Medium 52 (in 300 m*l* Erlenmeyer flasks). At each growth phase noted by arrows, NH_4, EDTA, citric acid were added to the media and from light to dark or from dark to light treatments were given (—○—, —●—; growth curves of *G. multicoccum*).

there was, however, only half the final yield in dark as in light. Therefore, there was physiological variation between the light and the dark grown colonies. However, there could not be found the difference of the capacity to change from asexual to sexual reproduction in both colonies.

The controlling factors of gametogenesis and zygote formation

In this experiment, potential sex controlling factors were exactly surveyed in detail in the light and in mixed culture (7-1 and 7-2 strains of *G. multicoccum*). The two strains were separately cultured in Medium G-2 in the dark. The algal colonies cultured for about 50 hrs were used as starting material. To minimize carry-over from old media, the algal colonies were washed three times with glass-distilled water.

Table 13 shows the effects of various nutritional factors on the gametogeneis and zygote formation. As the results of this experiment, the following factors acted on gametogenesis and zygote formation.

(1) Nitrogen-deficiency
(2) Acetate requirement
(3) Ca-ion requirement
(4) Light requirement

These four factors were the most important factors, each of which was equally necessary for the sexual reproduction.

Table 13 Effects of nutritional factors and CMU on the zygote formation of *G. multicoccum*.

	Zygote formation
(Omitted compound from M 52)	
NH_4-free	+
NH_4, Mg-free	+
NH_4, KH_2PO_4-free	+
NH_4, Metal mix G-free	+
NH_4, Vitamin No. 8-free	+
NH_4, Acetate-free	−
NH_4, Ca-free	−
Acetate-free	−
Ca-free	−
(Added following compounds)	
Ethanol, 0.15 m*l* in M 52	+
CMU (1×10^{-7}M)	+
Control M 52	Veg. Growth

CMU: 3-(p-chlorophenyl)-1, 1-dimethylurea.

Zygote formation (+) of *G. multicoccum* was detected microscopically.

Other factors that controlled vegetative growth (K, PO_4, Trace metals-Fe, Mn, Co, Cu, Mo and Zn, 15 kinds of vitamins, Table 5 and This-buffer) were tested, but did not cause the sexual reproduction of *G. multicoccum*.

Nitrogen sources and nitrogen-deficiency

Vegetative growth of *G. multicoccum* was supported by different nitrogen sources (cf. chapter I). Mixed strain cultures were light grown in the following sources of nitrogen: nitrate, nitrite, urea, arginine, L-glutamine, uric acid, tryptone and trypticase. Depletion of any one of these resulted in gametogenesis and zygote formation (Table 14).

Acetate requirement for sexual reproduction

The vegetative growth of *G. multicoccum* was strictly dependent upon acetate as a carbon source (cf. chapter I). Table 13 shows that acetate must be present for sexual reproduction. In sexual inducing situations vegetative growth does not occur and mixed colonies soon undergo gametogenesis. Thus, acetate seems to be utilized as a carbon source in the sexual processes.

In the following experiment, the rate of acetate assimilation was determined by monitoring the pH in the medium. Medium G-4, used in this experiment, did not contain Tris-HCl buffer, thus allowing the pH to change with acetate utilization. The absence of Tris-HCl buffer inhibited growth in the light (cf. Fig. 7) but not in the dark.

Figure 32 shows pH fluctuations during the vegetative and sexual reproduction phases. Within 60 hrs of the vegetative growth in the dark, the pH increased from 6.5 (initial pH) to 8.3 and then gradually increased to pH 8.8 in 4 days. The mixed culture of 7-1 and 7-2

Table 14 Effects of nitrogen and carbon source depletion on sexual reproduction.

Vegetative growth		Zygote formation & maturation		
C-source	N-source	N-supply	N-deficiency	Acetate or pyruvate deficency
Acetate,	NH_4Cl	−	+	−
	$(NH_4)_2HPO_4$	−	+	−
	NH_4NO_3	−	+	−
	$NaNo_3$	−	+	−
	$NaNO_2$	−	+	−
	L-arginine	−	+	−
	L-glutamine	−	+	−
	Urea	−	+	−
	Uric acid	−	+	−
	Trypticase	−	+	−
	Thyptone	−	+	−
Pyruvate,	NH_4Cl	−	+	−

Zygote formation and maturation (+) of *G. multicoccum* were detected microscopically on the basis of changing zygote colour.

strains reached N-deficiency within 60 hrs incubation and pH values were similar to the separate cultures of each strain in the dark. After 60 hrs in the dark the mixed colonies were transferred to light (white light) and under these conditions began to undergo gametogenesis immediately. The pH of this medium increased to pH 8.8 in 2 hrs, and to pH 9.8 in 12 hrs during which zygote formation was initiated. The rapid pH increase indicated rapid utilization of Na-acetate as a carbon and energy source for sexual reproduction.

The experiment demonstrated the importance of acetate to sexuality of *G. multicoccum*. Moreover, acetate could be replaced with pyruvate as a carbon and energy source for the sexual reproduction (cf. Table 14).

Ca-ion requirement for sexual reproduction

In previous experiment, Fig. 31 shows that some metal ions suggested to act in the sexual reproduction, and this process was inhibited with EDTA and citric acids as a chelator. Metal-ions as controlling factors of sexuality were surveyed in a partly elimimated medium from its components of Medium G. As shown in Table 13, Ca-ion is required for gametogenesis and zygote formation. Further, Ca-ion requirement could not be replaced by Mg -ion. Other major and minor mineral components were tested and were not required for sexual reproduction. This suggested that of the nutritionally important divalent cations Ca -ion only was required for sexual reproduction.

Light requirement for sexual reproduction

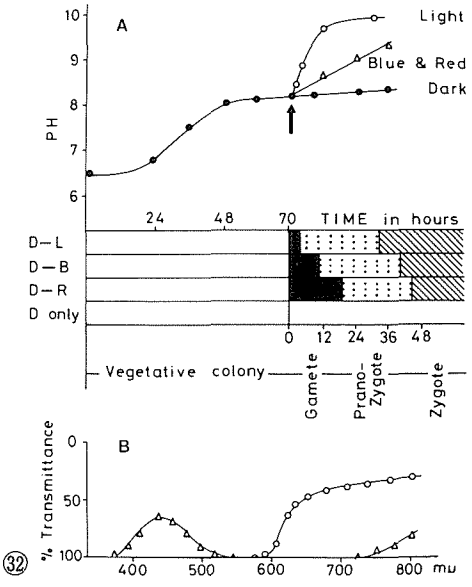


Fig. 32 Effects of light and culture time for sexual processes (gametogenesis, planozygote and zygote formation) of *Gonium multicoccum*, and relationship between pH change and sexual reproduction (A). Transmittance spectra of blue and red filters (B).

Sexual reproduction of *G. multicoccum* was never observed in the dark even if other conditions were optimal (cf. Figs. 30, 31 and 32).

Figure 32 shows the response of mixed colonies first grown for 70 hrs in the dark and then placed in white light (light intensity; 2000–5000 lux). Gametogenesis was initiated immediately after transfer to the light and zygotes were formed within 12 hrs (Plate III). Within 24 hrs the zygotes had settled in the culture vessels. The mixed colonies, grown for 70 hrs in the dark, and then subjected to light flashes or short periods of illumination (ca. 10 min), did not initiate gametogenesis. An experiment similar to the one just described was conducted using red and blue light (Fig. 32). The results indicated that blue was slightly more effective in inducing sexuality than red light. However, this difference may be due to the amount of available energy.

CMU effects on sexual reproduction

Table 13 shows the effects of 10^{-7} M CMU on sexual reproduction. The vegetative growth of *G. multicoccum* in the light was inhibited to the same level as cultures without CMU grown in the dark (cf. chapter I). However, sexual reproduction was not inhibited by the same concentration of CMU in the light.

Zygote maturation

Green aplanozygotes developed into matured zygote which became orange in the light within 7 days (cf. Plate III, IV A–C).

Figure 33 shows the absorption spectrum of acetone extracts of vegetative colonies and matured zygotes, as compared with aceton extracts of cysts of *Haematococcus lacustris*.

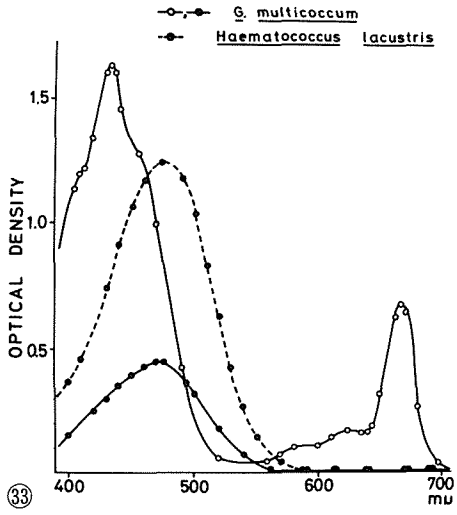


Fig. 33 Absorption spectra of acetone extracts from *Gonium multicoccum* and *Haematococcus lacustris*. Vegetative colony (—○—) and zygote (—●—) of *G. multicoccum*; cyst (---●---) of *H. lacustris*.

Table 15 Effects of nutritional factors on colour variation and the zygote maturation of *G. multicoccum*. (From aplanozygote to matured zygote of life cycle)

	Zygote maturation & colour variation	
(Continuous light)		
Acetate-free	−	Green
Phosphate-free	+	Orange Yellow
Metal mix G-free	+	Orange Yellow
N-free	+	Orange
N, Acetate-free	−	Green
N, Phosphate-free	+	Orange
N, Phosphate, Acetate-free	−	Yellow Green
N, Ca-free	−	Bleaching
N, Mg-free	+	Orange
N, Ca, Mg-free	−	Bleaching
N, Vitamin No. 8-free	+	Orange
Medium 52	+	Orange Yellow
N-free+Trypticase	+	Orange
N-free+ethanol	+	Orange
N-free+ethanol, CMU (10^{-7}M)	−	Green
(Darkness)		
N-free	−	Green

Matured zygotes were observed within 10 days' incubation.
Zygote maturation (+) of *G. multicoccum* was detected by colour change of the zygotes microscopically.

These data indicated that chlorophylls were destroyed in the light during zygote maturation, whereas carotenoids were not.

Table 15 shows the effects of nutrition and light on zygote production and colour. Zygotes maturing in the light became orange in colour. On the other hand, zygotes remained their initial colour (green) when matured in the dark. Moreover, at the acetate -deficiency or in the presence of 10^{-7} M CMU in culture media, zygotes remained green in the light. Omission of Ca-ion or in the presence of ethanol (0.3 m*l*%) in Medium G, G-1, G-2 and G-3, caused the zygotes to lose their pigments and become bleached.

In summary, acetate, light and Ca-ion were important factors for zygote maturation as well as gametogenesis and zygote formation. However, nitrogen was not necessary for zygote maturation.

Zygote germination

The vegetative colonies germinated from matured zygotes in Medium 3, 5, 6 (cf. Table 4) and Medium 52 and G (cf. Table 5) in the light at 18-30℃.

Table 11 shows the back cross tests of germinated colonies derived from clones which were randomly isolated from zygotes of 7-1 and 7-2 strains. The results of this experiment indicated that germinated colonies were approximately equally divided into two mating types, " + " and " − " strains. Zygote germination occurred in the fresh Media 3, 5, 6, 52 and G in the light, but not in the dark.

In strains of F_1, opposite mating types conjugated with each other and formed zygotes. Moreover, colonies of F_2 germinated and gave rise to the two opposite mating types.

The entire life cycle of *G. multicoccum* was observed and controlled axenically in laboratory culture. Figure 34 diagrams the life cycle in *G. multicoccum*. This diagram also shows the relationship between limiting factors (controlling factors) and culture time for the life cycle.

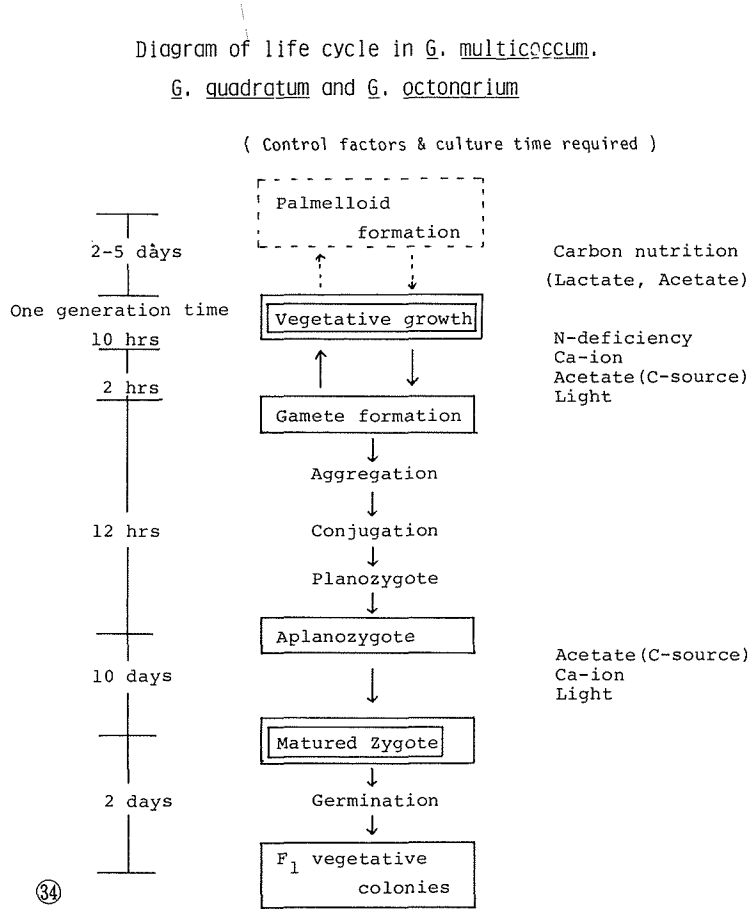


Fig 34 Relationships between limiting factors and culture time on the life cycle of *Gonium multicoccum, G. octonarium* and *G. quadratum*.

3. Discussion

This discussion deals with nutritional control and light requirements of the sexual cycle (gametogenesis, zygote formation, maturation and germination). Discussion on nutritional control will be limited to nitrogen, acetate, Ca-ion and these nutrients as related to light and dark response of *G. multicoccum*.

Nitrogen-deficiency

Nitrogen-deficiency is important in gamete induction in *Chlamydomonas* (SAGER & GRANICK, 1954; TRAINOR, 1958 and 1959; TSUBO, 1957), *Cylindrocystis* (PRINGSHEIM, 1919), *Scenedesmus* (TRAINER & BURG, 1965), *Golenkinia* (ELLIS & MACHLIS, 1968), *Pyronema* (ROBIONSON, 1926), *Pandorina* (WILBOIS, 1958) and *Platydorina* (HARRIS & STARR, 1969).

In the present study, nitrogen-deficiency has been shown to be an important controlling factor of sexuality in *G. multicoccum*. Both 7-1 and 7-2 strains undergo gametogenesis when the nitrogen is deficient in the medium and all other nutrients are supplied at optimum levels. The importance of nitrogen to sexuality is also illustrated by the fact that the rate of sexual induction is in inverse proportion to the level of nitrogen at every stage from vegetative growth of the inoculum to 12 hrs after resuspension in nitrogen-free medium. The age of vegetative growth phase is not related to sexual reproduction. This result agrees with observations on *Golenkinia* (ELLIS & MACHLIS, 1968). In addition, any colonies that utilized any inorganic or organic nitrogen source for vegetative growth, would form gametes when that source was deficient. It is biologically important that nitrogen deficiency triggers gametogenesis in *G. multicoccum* as this process would protect the alga in times of nutritional stress. Once zygotes have been formed, their maturation will be completed in either the presence or absence of nitrogen.

Acetate requirement

It is well known that the algal group, called acetate flagellates, require acetate as a carbon source. However, acetate has not been reported as being important to sexual reproduction. In *G. multicoccum*, acetate was required for gametogenesis, zygote formation, and maturation as well as for vegetative growth. When acetate was omitted from the optimum medium, gametogenesis, zygote formation and maturation did not occur. Acetate is rapidly utilized in gametogenesis, as indicated by pH change. As a carbon source, acetate could be replaced with pyruvate. These results indicated that exogenous energy sources besides light energy were required for the sexuality of *G. multicoccum*. The relationship between acetate utilization and light will be discussed later.

Ca-ion requirement

One of the most interesting aspects of this study is the finding that calcium is required for gametogenesis, zygote formation and maturation. LEWIN (1954) reported that clumping of *Chlamydomonas moewusii* gametes is dependent on the concentration of calcium and is inhibited by washing with distilled water or by adding a chelating agent such as citrate.

This inhibition could be alleviated by high concentrations of magnesium or strontium and completely overcome by increased calcium levels. These procedures do not reverse the mating process once pairing is complete, indicating that calcium is involved in, and required for the agglutination of flagella. Mating is independent of calcium concentrations between 3–30 ppm, although this optimum range is higher if much phosphate is present. WIESE and JONES (1963) confirmed the findings of the earlier study by LEWIN and most of their data are entirely consistent with those obtained by him. The optimal calcium concentration for mating in *C. moewusii* was about 0.0001 M, but EDTA, a chelator, prevented mating but not gamete motility. In *Golenkinia,* ELLIS and MACHLIS (1968) demonstrated that calcium is required in only the male gamete, and may perform the same function in this genus as it does in *Chlamydomonas*. CAREFOOT (1966) demonstrated a similar requirement in *Volvulina steinii*. He used 0.36 M $CaCl_2$ for mating experiments but did not comment on the effects of lower concentrations of this compound. This is almost 200 times the concentration of calcium found to give a maximal response by other investigators and is probably far higher than necessary. The first three studies (LEWIN, 1954; WIESE & JONES, 1963; ELLIS & MACHLIS, 1968) indicate that calcium is involved in flagellar agglutination.

In *Gonium multicoccum*, calcium may also have the same function as it does in *Chlamydomonas*. WIESE and JONES (1963) demonstrated that calcium can be replaced by magnesium, in contrast to LEWIN'S (1954) observations. In *Gonium*, however, magnesium does not effect sexual reproduction and it can not replace calcium. WIESE (1961) showed that in *Chlamydomonas eugametos* gamones derived from the male cells are higher in lipoproteins than those from the female. WIESE and JONES (1963) also suggested that calcium may act by binding certain lipoprotein complexes produced by the male flagella. Thus it is possible, as pointed out by ELLIS and MACHLIS (1968), that calcium interacts with some lipoprotein components of, or produced by, the sperm or male flagella and in some way makes them stickier. However, in the process of zygote maturation, which is not related with flagella, calcium is required in *G. multicoccum*. This suggests that there are different calcium functions for different stages of the life cycle in *G. multicoccum*. There may be two calcium functions for sexual reproduction: one is related to flagellar function (agglutination), and the other is related to the internal metabolism of the cell or zygote of *G. multicoccum*.

Other nutrient requirements

According to ELLIS and MACHLIS (1968), phosphorus deficiency in *Golenkinia* is almost as effective as nitrogen in causing sexual differentiation. In *Gonium multicoccum*, however, phoshorus did not effect sexual reproduction. Also the effect of other nutrients; major and minor mineral components, vitamins and Tris-buffer had no effect on sexuality.

Light requirement

Light was necessary for the gamete induction in *Chlamydomonas* (SMITH, 1948; LEWIN, 1956; FÖRSTER, 1957, 1959; TRAINER, 1958; STIFTER, 1959) and in *Golenkinia* (ELLIS and

MACHLIS, 1968). In the present study, light was essential for gametogenesis and zygote formation of *G. multicoccum*. Nitrogen deficiency did not initiate gametogenesis unless (white) light of at least 1000–4000 lux was present from 2 hrs to 12 hrs. Sexual reproduction in *G. multicoccum* did not occur in the dark in the nitrogen-free medium.

In the present experiment, the effects of two action spectra; red and blue light on sexuality were tested. Blue light was more effective than red for gametogenesis and zygote formation. However, total energy was different for the two light spectra. Thus, the role of wave length remains unknown for *G. multicoccum*.

The mechanisms by which nutrients and illumination bring about sexuality are not well known. Historically, the first serious proposal regarding the role of light was proposed by SAGER and GRANICK (1954). Gametogenesis in *Chlamydomonas reinhardtii* has two requirements: nitrogen deficiency and 4 hr of light. The light can be applied either before or after mixing the two mating types. However, the light requirement can be eliminated by growing the cells in the dark in a medium with low nitrogen and high acetate concentrations. They stated that light acts photosynthetically, providing energy for gametogenesis and carbohydrates to bind nitrogen compounds inhibitory to the sexual process. No action spectrum was given. However, SMITH (1948) examined the action spectrum for sexuality in this species and obtained peaks at 600 mμ and 435 mμ. Moreover, he found that cells illuminated for 2 hrs maintained their sexual capacities for 1 week in the dark. These results show that light may be required for some process other than photosynthesis.

LEWIN (1956), working on *Chlamydomonas moewusii* (a species not dependent on decreased nitrogen for sexual induction), obtained an action spectrum identical to that of photosynthesis for the sexual light requirement and found that phenylurethane inhibits sexuality and photosynthesis to the same degree. He also reported results incompatible with the idea of a simple photosynthetic role for light. LEWIN hypothesized that light is absorbed by the photosynthetic pigments and a hormone synthesized in or near the chloroplast which was active at the flagellar tips. TSUBO (1961) demonstrated that in the same species, a supernatant of the illuminated cells greatly enhanced mating of cells kept in the dark. However, FÖRSTER obtained action spectra for *C. moewusii* (1959) and *C. eugametos* male cells (1957) with peaks of sexual activity at 460 mμ and 590 mμ. STIFTER (1959) confirmed the data for *C. eugametos* male cells and also found that the sexual response is stimulated by red light (680 mμ) given prior to the blue or orange light. This stimulation occurred only in the presence of CO_2, whereas the blue-light effect required oxygen. High CO_2 levels were inhibitory except in the presence of low light intensities. She concluded that there are two light requirements which LEWIN had not adequately separated. One role is photosynthetic as shown by action spectra, the CO_2 requirement and the phenylurethane data obtained by LEWIN. In the other role, light causes a photostimulation of respiration. Evidence for this role comes from the peaks in the blue and orange wavelenghs, an oxygen requirement and the inhibitory effect of high CO_2 levels at certain light intensities. Obviously, according to

this dual light-role hypothesis, respiration as the second light effect is dependent on the prior satisfaction of the first requirement. STIFTER's hypothesis (1959) does not account for the great variation in the length of illumination required for sexuality, the ability of *Chlamydomonas reinhardtii* to retain its sexual potential in the dark for a week (SMITH, 1948), or the effect of light-grown supernatant on dark cells as demonstrated by TSUBO (1961). However, STIFTER's hypothesis fits much of the information regarding *C. moewusii* and *C. eugametos*. In *Golenkinia*, ELLIS and MACHLIS (1968) demonstrated that the followings suggested the possibility of a second function of light in addition to photosynthesis: ethanol, which supports vegetative growth in the dark, could replace light in sexual reproduction; light intensities of approximately 3000 ergs per cm^2 per sec. were adequate for the photosynthetic needs of vegetative cultures but limiting for spermatogenesis; and the process of sexual induction responds to darkness within 3 hrs.

It is not possible to relate these hypotheses to *Gonium multicoccum* because of the limitations of the preliminary experiments on action spectra. Sexuality required at least 2 -hr illumination (ca. 1000-4000 lux). This indicates that photosynthesis is involved. However, the following four facts mitigate against this as being the sole explanation.

(1) Acetate, which supported vegetative growth in the dark, could not replace light in sexual reproduction.

(2) Acetate was required for gametogenesis and zygote formation, and could not replace light in sexual reproduction. Moreover, vegetative growth in the light required acetate as a carbon source (see chapter I).

(3) 10^{-7} M CMU, which inhibited vegetative growth in the light to the same levels in the dark, did not inhibit sexual reproduction.

(4) The pH of the medium for mixed cultures, which gradually increased in Medium G-4 in the dark, rapidly increased in the light during sexual reproduction.

These results cannot be incorporated into a normal photosynthetic scheme in which acetate is required not only for sexual reproduction, but also for asexual reproduction. This is the first report that sexual reproduction of a photo-heterotrophic alga requires acetate or pyruvate as a carbon source. The four above mentioned points suggest that there may be a new function of light in sexual reproduction other than photosynthesis. However, the mechanisms by which light effect sexual reproduction in *G. multicoccum* are not clear.

In *G. multicoccum* a minimum of 2 hrs is necessary for the first appearance of gametes. Assuming that the cells producing gametes within 2 hrs are not genetically different from the others but are simply at a stage allowing them to respond most quickly, and assuming a normal vegetative colony cycle of ca. 16 hrs at 25℃, it follows that cells are responsive to sexual inducing factors at the any phases of their cell cycle. Furthermore, the fact that sexual inhibitors such as nitrogen or darkness are ineffective, if applied after a certain time, indicates that there is an irreversible point in differentiation and that all cells have reached this critical point in about 14 hrs (the aplanozygote phase) after the beginning of the

inductive period of gametogenesis.

Control of sexuality in the green alga *G. multicoccum* is determined by two main factors: the chemically defined Medium G series and the light. Nitrogen deficiency combined with acetate and light, at least partially, through acetate photoassimilation, creates a metabolic state that induces sexuality. The other important factor in gametogenesis, zygote formation and maturation of *G. multicoccum* is calcium.

Gonium multicoccum is photo-heterotrophic and heterotrophic with acetate as a carbon source. This uniqueness of *G. multicoccum* may be of assistance in resolving the mechanism of sexual reproduction.

III A Morphogenetic Substance from *Gonium multicoccum*

During the course of this study on the factors regulating sexual reproduction, a morphogenetic substance which was effective in breaking colonies was found in a cell-free extract from *Gonium multicoccum*. This chapter is concerned with the bioassay and specificity of this substance, which is conveniently designated Colony Breaking Substance; C. B. S.

The relationship between C. B. S. and sexual reproduction is not as yet clear, and also its endogenous physiological function has not yet been resolved. However, C. B. S. has some relationships to the controlling factors of colony dissociation.

1. Materials and methods

Two strains, 7-1 and 7-2 of *G. multicoccum* were used as test organisms. Axenic culture of *G. multicoccum* was conducted at 25±1℃ using Medium 52. Fifty m*l* of the medium were placed in 100m*l* cotton plugged Erlenmeyer flasks. Illumination was provided by a bank of cool white fluorescent lamps at a light intensity of about 4,000 lux at the surface of the flasks.

Algal cells were harvested at each different growth phase by centrifugation (cf. Fig. 35). They were washed 3 times with Pyrex distilled water, suspended in about 10 m*l* of the Medium 52 and ground with at least 1,000 times as much quartz sands as algal material. The homogenates were centrifuged at 10,000 g for 10 min. The supernatant fluid was used as a crude extract of C. B. S. fractions for bioassay and was diluted finally to 50 m*l* Medium 52 per 15 mg dry weight of *G. multicoccum*. This fluid was sterilized using a Millipore HA filter (maximum pore size 0.22 or 0.45 μm).

2. Results and Discussion

Figure 35 shows the times and growth conditions of samples taken for C. B. S. survey. These are as follows:

Sample-1; 7-1 & 7-2 strains grown in light for 36 hr.

S-2; 7-1 & 7-2 strains grown in light for 48 hr.

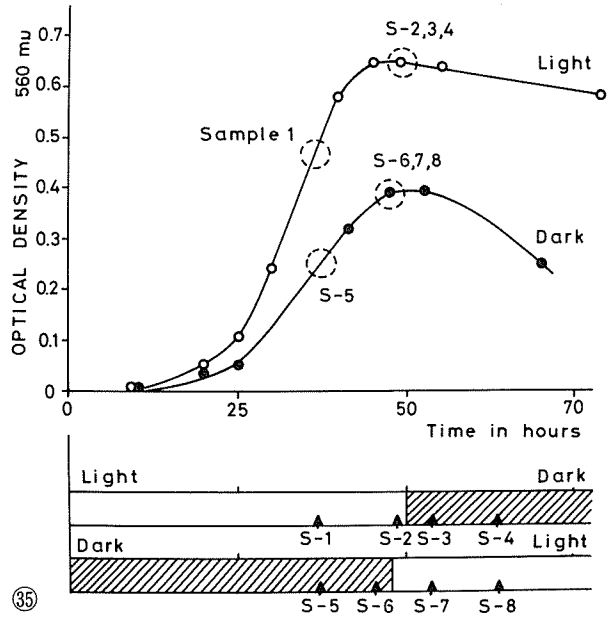


Fig. 35 Survey of cell-free fractions for C. B. S. (colony breaking substance) at four different growth phases of *Gonium multicoccum*.

S-3 ; 7-1 & 7-2 strains grown in light for 48 hr, and then darkened for 5 hr before harvest.
S-4 ; 7-1 & 7-2 strains grown in light for 48 hr, and then darkened for 12 hr before harvest.
S-5 ; 7-1 & 7-2 strains grown in darkness for 38 hr.
S-6 ; 7-1 & 7-2 strains grown in darkness for 45 hr.
S-7 ; 7-1 & 7-2 strains grown in darkness for 45 hr, and then illuminated for 5 hr before harvest.
S-8 ; 7-1 & 7-2 strains grown in darkness for 45 hr, and then illuminated for 12 hr before harvest.

Extract obtained from these 16 different colonies was surveyed for C. B. S. activity. Table 16 shows the effect of C. B. S. activity of each extract for 7-1 and 7-2 strains on normal 16-celled colonies in the light.

In the present experiments, extracts from S-5 (7-2), S-6 (7-2) and S-7 (7-2) showed colony breaking activity on 7-1 and 7-2 strains (Plate IV D-E). The breaking up of these could be detected within 3-hr after addition of extract S-5 (7-2), S-6 (7-2) and S-7(7-2). The extract retained its ability of colony breaking when heated at 40℃ for one hour ; however, the activity was completely lost to heat at 80℃ for 10 mins.

Plate IV D-E show normal colonies in Medium 52 with fractions having no C. B. S. activity, and single cells with fractions having C. B. S. activity.

Table 16 Bioassay for C. B. S. at various growth phases of *G. multicoccum*.

		C. B. S. activity for colonies in light	
		7-1	7-2
Sample 1	(7-1 strain)	−	−
	(7-2 strain)	−	−
S-2	(7-1)	−	−
	(7-2)	−	−
S-3	(7-1)	−	−
	(7-2)	−	−
S-4	(7-1)	−	−
	(7-2)	+	+
S-5	(7-1)	−	−
	(7-2)	+++	+++
S-6	(7-1)	−	−
	(7-2)	+++	+++
S-7	(7-1)	−	−
	(7-2)	++	++
S-8	(7-1)	−	−
	(7-2)	−	−

C. B. S.: colony breaking substance.
S-(1-8) are growth phases (Fig. 36) bioassayed for C. B. S.
(+) indicates C. B. S. activity and (−) no activity.

The biosynthesis of C. B. S. occurred only in cells of 7-2 " − " strain, and in dark culture conditions. The absence of C. B. S. activity in S-8 (7-2) indicates that its biosynthesis may be reversed when moving cells from light to dark conditions. This morphogenetic substance may be related to the dark metabolism of the " − " strain. Possibly this morphogenetic substance has a high molecular weight, and protein-like substance because of its heat-unstability. Chemical structure, physiological function of C. B. S. and the relationship between colony dissociation and C. B. S. will be studied in a continuing investigation.

IV Comparative Studies on the Nutrition of the Genus *Gonium*

Comparative nutritional studies on the genus *Gonium* have been conducted by PRINGSHEIM and PRINGSHEIM only (1959). Morphological observation of palmelloid-stage has been reported in *G. pectorale* (MIGULA, 1890) and *G. sociale* (DANGEARD, 1916). However, details of comparative nutritional studies and palmelloid formation of this algal group are still unexplored.

In this chapter, characteristics of vegetative growth and asexual reproduction on several

species of the genus *Gonium*, were investigated . Particularly, *G. multicoccum, G. octonarium* and *G. quadratum* were investigated regarding growth and the palmelloid formation, which is a type of asexual reproductive differentiation.

1. Materials and methods

Twenty strains of six species of *Gonium ; G. multicoccum, G. quadratum, G. octonarium, G. pectorale, G. sacculiferrum, G. sociale* var. *sacculum* and *G. sociale* var. *sociale* selected for the present investigation are listed in Table 1.

Culture methods were the same as stated in the previous chapters. Table 17 shows culture media used in this study ; Medium P-1, P-2, P-3, P-4, P-5, P-6, P-7, T-1, T-2, T-3, T-4, T-5, T-6 and T-7 ; Medium G, 52, 5 and 6 (cf. Tables 5 and 4).

The following culture conditions were employed :

a : 25℃ in light (ca. 4,000 lux)

b : 25℃ in dark.

c : 25℃ in 16-hr light (ca. 4,000 lux) and 8-hr dark.

d : 18℃ in 14-hr light (ca. 3,000 lux) and 10-hr dark.

e : 14℃ in 14-hr light (ca. 1,000 lux) and 10-hr dark.

f : 14℃ in 10-hr light (ca. 2,000 lux) 14-hr dark.

Table 17 Culture media for comparative studies in the genus *Gonium*.

	P-1	P-2	P-3	P-4	P-5	P-6	P-7	T-1	T-2	T-3	T-4	T-5	T-6	T-7	
KNO_3	20	20	20	20	20	20	20	20	20	20	20	20	20	20	mg
$CaCl_2$	5	5	5	5	5	5	5	5	5	5	5	5	5	5	mg
$MgSO_4$ $7H_2O$	5	5	5	5	5	5	5	5	5	5	5	5	5	5	mg
Metal mix G	1	1	1	1	1	1	1	1	1	1	1	1	1	1	mg
Vitamin mix No. 8	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	mg
KH_2PO_4	17.5	17.5	17.5	17.5	17.5	17.5	17.5	15	15	15	15	15	15	15	mg
K_2HPO_4	7.5	7.5	7.5	7.5	7.5	7.5	7.5	–	–	–	–	–	–	–	mg
Tris-HCl buffer	–	–	–	–	–	–	–	1	1	1	1	1	1	1	mg
$CaCO_3$	5	5	–	–	–	–	–	5	5	–	–	–	–	–	mg
Soil -extract	–	5	5	–	–	–	–	–	5	5	–	–	–	–	mg
Proteose pepton	–	–	–	100	–	–	–	–	–	–	100	–	–	–	mg
Na-acetate • $3H_2O$	–	–	–	–	40	–	–	–	–	–	–	40	–	–	mg
Trypticase	–	–	–	–	40	–	–	–	–	–	–	40	–	–	mg
Yeast extract	–	–	–	–	40	–	–	–	–	–	–	40	–	–	mg
Carbon source mix II	–	–	–	–	–	50	–	–	–	–	–	–	50	–	mg
Glucose	–	–	–	–	–	–	100	–	–	–	–	–	–	100	mg
pH : 6.5–7.0															

Amounts are per 100 m*l* of medium. Metal mix G : see Table 5.
Vitamin mix No. 8 : see Table 5. Carbon source mix II : see Table 3.

2. Results

Vegetative growth characteristics of *Gonium* species

Table 18 shows the vegetative growth characteristics of *Gonium* species grown in various media under the culture conditions of c, d and e (cf. materials and methods in this chapter). The growth of *G. multicoccum, G. octonarium* and *G. quadratum* were different from the other species; *G. pectorale, G. sacculiferrum* and *G. sociale*. The former three species grew in Medium G, 52, 5 and 6 under the continuous light, whereas the latter species did not (Table 19). However, Medium 5 and 6 supported growth of these latter species under the regime of 14-hr light and 10-hr dark at 14℃, or 14-hr light and 10-hr dark at 18℃ (Table 19). Moreover, the former species did not grow in the mineral medium (Medium 52 minus acetate) in the light or in the dark (cf. chapter I). However, the latter could grow in the mineral media.

Table 18 The growth of *Gonium* species in the various kinds of culture media.

Strains No.	Culture media for *Gonium*														
	M 52	P-1	P-2	P-3	P-4	P-5	P-6	P-7	T-1	T-2	T-3	T-4	T-5	T-6	T-7
7-1	+++							−							−
7-2	+++							−							−
8-5	+++							−							−
8-7	+++							−							−
783	+++	+	(+)	(+)	+			−	(+)	(+)	(+)	(+)			−
842	+++	(+)	(+)	(+)	(+)	(+)		−	(+)	(+)	×	×	×		−
843	+++	(+)	(+)					−		(+)		×	×		−
13	−	+	+	+	+		+++	−	(+)	+	+	(+)	+	+++	−
805	−	+	(+)	−	−	−			(+)	(+)	++	×	×		
806	−	(+)								(+)	++		×		
826	−	+	+			+	+++	−	(+)	+	++	+	+	+++	−
827	−	+	+		−	−			(+)	(+)	(+)	×			
956	+++	(+)	(+)	+	+				(+)	(+)	(+)	(+)			
822	−	−	−	−		−			−		(+)				
823	−	−			−	−	−	−	−		−	++	−	(+)	−
935	−	−	−	−	−	−	−	+	−	−	++	−	×	(+)	+
936	−		(+)			(+)	(+)	−				+	++	(+)	+
14	−		+	+	+	+	++	(+)	+	+	+	+	(+)	+	+
15	−	−	(+)	+	+		++	(+)	+	+	(+)	×	×	++	++
197	−	−		(+)			+	−	(+)				++	++	++

Culture media (see Table 17).
Strains number of *Gonium*; see Table 1.
×=bleaching colonies, − =no growth. Increasing amounts of growth as estimated visually, are indicated as (+), +, ++ and +++.

Table 19 Effects of culture media and conditions for the growth in the various strains of *Gonium*.

Species strain's No.		Continuous light M5, M6, & M52	14 hr L-10 hr L M5, M6 & M52
G. multicoccum	7-1	+++	+++
	7-2	+++	+++
	8-5	+++	+++
	8-7	+++	+++
	783	+++	+++
G. octonarium	842	+++	+++
	843	+++	+++
G. pectorale	13	−	+
	805	−	+
	806	−	+
	826	−	++
	827	−	+
G. quadratum	956	+++	+++
G. sacculiferrum	822	−	+
	823	−	+
	935	−	+
G. sociale var. *saccurum*	936	−	++
G. sociale var. *sociale*	14	−	++
	15	−	++
	197	−	++

− =no growth. Increasing amounts of growth, visually estimated, were indicated as +, ++ and +++. Medium 6 and Mdium 5 see Table 4.

As mentioned above, *G. multicoccum, G. octonarium* and *G. quadratum* required organic carbon sources for cell division and colony development, and could grow independent of photosynthesis and photoperiodic conditions. The light action for the growth of these three species differed from normal photosynthesis. The reason is not clear. However, it should be emphasized that the algal strains, though they contain chlorophylls and other photosynthetic pigments, cannot grow in the light or in the dark, in the absence of the organic carbon sources (cf. chapter I).

On the other hand, the growth of *G. pectorale, G. sacculiferrum* and *G. sociale* were dependent on photosynthesis and required the photoperiodic condition for cell division and colonial development. This suggested that there was endogenous biological rhythm for cell division and colony development in these three species. On the basis of their different photoperiodic response and acetate requirements the six species could be divided into two groups (physiological races).

Utilization of carbon sources on the growth of *G. multicoccum*, *G. octonarium* and *G. quadratum*

G. multicoccum, 7-1 and 7-2 strains grew with acetate, pyruvate and lactate as carbon sources (cf. chapter I). In this comparative experiment, the utilization of acetate, glucose, pyruvate and lactate as carbon sources for other strains of *G. multicoccum*, *G. octonarium* and *G. quadratum* were investigated.

Figure 36 shows that eight strains of these three species grew as well as 7-1 and 7-2 strains with acetate in the light and dark. Pyruvate and lactate could replace acetate as a carbon source in the light, but not in the dark (Table 20). Further, glucose did not support growth in either light or dark.

Arginine as a nitrogen source on the growth of *G. multicoccum* and *G. octonarium*

The growth of *G. multicoccum*, using arginine as a nitrogen source, had a long lag-time compared with NH_4, NO_3 or urea (cf. Fig. 16 and Table 8). In this experiment, the growth of *G. octonarium*, using arginine was compared with that of *G. multicoccum*.

Figure 37 shows the effects of arginine on the growth of *G. multicoccum* and *G. octonarium*. The lag-time of *G. octonarium* was not long in arginine, and its growth was

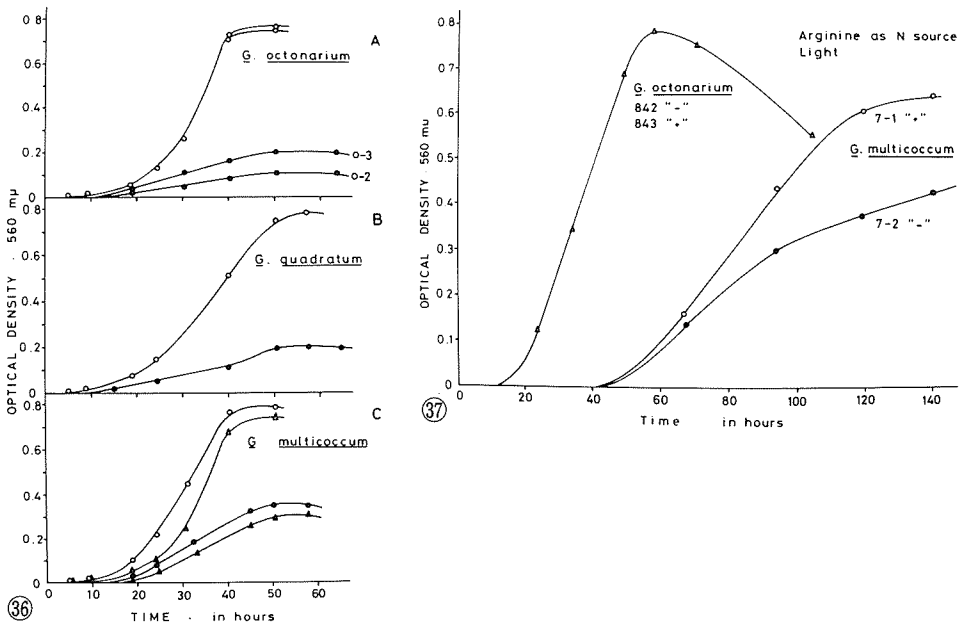


Fig. 36 Growth of three species of *Gonium* cultured in Medium 52 at 25℃. Continuous light, —○—, —△—; continuous dark, —●—, —▲—.

Fig. 37 Effects of arginine as a nitrogen source on the growth of *Gonium multicoccum* (7-1 strain and 7-2) and *G. octonarium* (842 strain and 843).

Table 20 Effects of organic carbon sources on the growth of *G. multicoccum*, *G. quadratum* and *G. octonarium*.

	Acetate		Glucose		Lactate		Pyruvate	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
G. multicoccum								
7-1	+++	++	−	−	++	−	++	−
7-2	+++	++	−	−	++	−	++	−
8-5	+++	++	−	−	++	−	++	−
8-7	+++	++	−	−	++	−	++	−
783	+++	++	−	−	++	−	++	−
G. quadratum								
956	+++	++	−	−	++	−	++	−
G. octonarium								
842	+++	++	−	−	++	−	++	−
843	+++	+	−	−	++	−	++	−

− =no growth. Increasing amounts of growth, visually estimated, were indicated as +, ++ and +++.

supported at the same level as in NH_4, NO_3 or urea. Different enzymatic activity between these two species may explain this different response to arginine as a nitrogen source.

D-lactate utilization

Gonium multicoccum, *G. quadratum* and *G. octonarium* were grown with DL-lactate as a carbon source in the light. Lactate has two chemical structures, D-lactate and L-lactate. In this experiment, differences in utilization of D-lactate and L-lactate as carbon sources were compared between three species of *Gonium* involving eight strains. A concentration of 600 mg% DL-lactate was used.

Figure 38 shows growth curves of these strains when DL-lactate was used as a carbon source in continuous light. As excellent characteristic phenomena in this experiment, DL-lactate was not utilized by any strains in the dark. DL-lactate utilization was dependent upon illumination. Moreover, the growth-curve pattern showed differences between the six strains. First, there was a long lag-time for growth in DL-lactate as compared with acetate, and 10 days were required for the cultures to reach maximum growth, except for strain 956 of *G. quadratum*. Strain 956 had a relatively short lag phase in DL-lactate.

Table 21 shows the growth of the strains at a D-lactate concentration 100 mg% in continuous light. L-lactate, at a concentration of 100 mg% did not support growth of any strain in either the light or dark. Strain 956 of *G. quadratum* grew rapidly when D-lactate was used as a carbon source in the light in contrast to the other strains (cf. Fig. 39).

These strains were precultured in media with DL-lactate or D-lactate for 4 days in the light, then their response to DL-lactate was tested. Their growth pattern remained the same as in the previously described experiment. This indicated that the long lag-time in

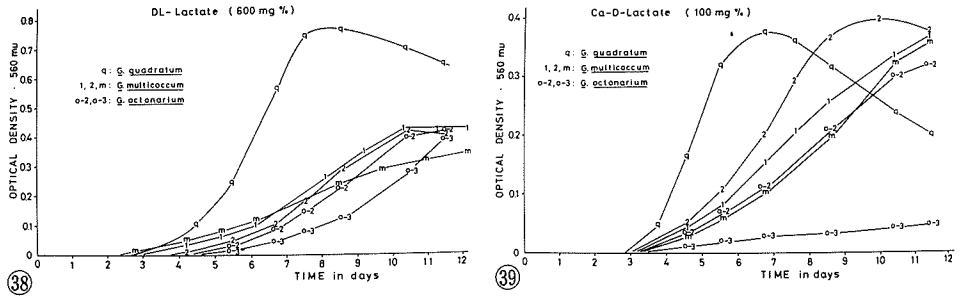


Fig. 38 Growth of *Gonium quadratum* (q=956 strain), *G. multicoccum* (1=7-1 strain, 2=7-2 and m=783) and *G. octonarium* (o-2=842 strain and o-3=843) in DL-lactate (600 mg%) as a carbon source in the light.

Fig. 39 Growth of *G. quadratum, G. multicoccum* and *G. octonarium* in Ca-D-lactate (100 mg%) as a carbon source in the light. Symbols and strains are the same as those of Fig. 38.

Table 21 Effects of D-lactate and L-lactate on the growth of *G. multicoccum, G. quadratum* and *G. octonarium*.

		D-Lactate		L-Lactate	
		Light	Dark	Light	Dark
G. multicoccum	7-1	++	−	−	−
	7-2	++	−	−	−
	783	++	−	−	−
G. quadratum	956	++	−	−	−
G. octonarium	842	++	−	−	−
	843	+	−	−	−

a. Used at optimum concentration (%): D (−)-lactic acid Ca·$4H_2O$, 0.1: L(+)-lactic acid Ca·$4H_2O$, 0.1.

b. − =no growth. Increasing growth, visually estimated, are indicated as +, ++ and +++

lactate resulted from its gradual utilization for growth.

Variation of these strains was investigated in cultures containing 100 mg% D-lactate and 50 mg% L-lactate combined in the same medium. There were no changes in the growth of these strains from the previously described experiments on D-lactate.

Palmelloid formation

Asexual reproductive differentiation —palmelloid formation— was produced in *G. quadratum* and *G. octonarium* by changing culture media and conditions. Table 22 shows the effects of culture media and conditions on palmelloid formation of *Gonium*.

Five of the eight strains tested did not form the palmelloid stage under the experimental conditions. They divided into 8 or 16-celled colonies repeatedly forming new daughter

colonies. One strain of *G. quadratum* studied, formed the palmelloid stage with acetate in Medium 52. In strain 843 of *G. octonarium*, the palmelloid was formed infrequently in Medium 52.

Typical palmelloid formation occurred in *G. quadratum* and strain 842 and 843 of *G. octonarium* with lactate as a carbon source (Table 22). Plate V shows the palmelloid stage of *G. quadratum* developed in a lactate medium. This palmelloid formation in *G. quadratum* was greatest during the lag phase and early exponential growth. During later exponential growth and the stationary phase, normal motile colonies were observed in high ratio to the palmelloid stage.

The palmelloid stage of *G. quadratum* consisted of one, two, three, four or more cells in a gelatinous matrix. These formed large macroscopic clumps (Plate V). These palmelloid clumps were usually produced at the bottom in the culture vessels, however, they were often gathered on the medium surface, forming a thick membrane. This occurred in acetate in the dark.

The palmelloid stage of *G. octonarium* was found throughout in the growth phase with lactate, and vegetative colonies were rarely found. Strain 842 and 843 of *G. octonarium* grew and developed into the palmelloid stage with lactate. Plate V shows the morphology of the palmelloid stage of strain 842 and 843. These strains consisted of one to four or more cells in a gelatinous matrix, and formed the large macroscopic clumps in the same way as *G.*

Table 22 Palmelloid formation of *Gonium* species.

	G. multicoccum					*G. quadratum*	*G. octonarium*	
	7-1	7-2	8-5	8-7	783	956	842	843
Medium 6								
25℃ in light	−	−	−	−	−	−	−	−
25℃ in dark	−	−	−	−	−	−	−	−
25℃ in 16hrs light-8hrs dark	−	−	−	−	−	−	−	−
14℃ in 10hrs light-14hrs dark	−	−	−	−	−	−	−	−
M 52 (acetate)								
25℃ in light	−	−	−	−	−	−	−	−
25℃ in dark	−	−	−	−	−	++	−	+
25℃ in 16hrs light-8hrs dark	−	−	−	−	−	+	−	+
14℃ in 10hrs light-14hrs dark	−	−	−	−	−	(+)	−	+
M 52 (lactate)								
25℃ in light	−	−	−	−	−	+++	++	+++
25℃ in 16hrs light-8hrs dark	−	−	−	−	−	+++	+	+++

− =no palmelloid formation. Increasing of palmelloid formation, visually estimated, are indicated as +, ++ and +++.
Acetate replaced Na-lactate at a concentration of 600 mg% as a carbon source in Medium 52.
(+) palmelloid stages were slightly observed.

quadratum.

3. Discussion

PRINGSHEIM and PRINGSHEIM (1959) have suggested that the genus *Gonium* consists of several nutritional/physiological groups. They separated the species in the following manner.

(a) Auxo-autotrophy ; *G. sociale*
(b) Growth stimulation with acetate ; *G. multicoccum, G. pectorale*
(c) Glucose requirement for growth ; *G. sacculiferrum*
(d) Acetate requirement for growth ; *G. quadratum, G. octonarium*
(e) Mixotrophy ; *G. multicoccum, G. quadratum, G. octonarium*
(f) Growth in darkness ; *G. sacculiferrum, G. octonarium, G. quadratum, G. multicoccum*

As the results of the present experiments on the nutrition and vegetative growth characteristics of *Gonium*, it appears that there are two clearly defined physiological groups in this genus (Table 19).

(A) Facultative photosynthesis, organic carbon source required for growth and capacity for growth under continuous light conditions ; *G. multicoccum, G. octonarium* and *G. quadratum.*
(B) Obligate photosynthesis and a requirement for photoperiodic conditions ; *G. pectorale, G. sacculiferrum* and *G. sociale.*

All species of *Gonium* used in this study contained chlorophylls and other photosynthetic pigments. However, acetate as a carbon source supported growth most efficiently for (A)-type species in both the light and dark. This group has a particular advantage in that growth occurred in Medium G in continuous light or dark.

However, (B)-type species could not grow in Medium G, 5 and 6 in continuous light or dark, and required a photoperiodic cycle.

On the basis of these results, the two groups are separated by growth requirements. There is probably an evolutionary basis to this physiological separation of the genus *Gonium* into two groups.

Regarding the utilization of carbon sources, the eight strains of (A)-type species ; *G. multicoccum, G. quadratum* and *G. octonarium*, could use acetate, pyruvate and lactate in the light, and acetate in the dark. On a nutritional basis these behave as acetate flagellates (HUTNER and PROVASOLI, 1951, 1955 ; LWOFF, 1951 ; DANFORTH, 1962), which are typically incapable of using sugars.

DL-lactate utilization as a carbon source has been investigated in heterotrophic growth of colourless algae ; *Prototheca zopfii* (ANDERSON, 1945), *Chilomonas paramecium* (COSGROVE and SWANSON, 1952 ; HOLZ, 1954) and in heterotrophic growth of marine diatoms (LEWIN and LEWIN, 1960 ; LEWIN, 1963). Some other algae, *Chlorella ellipsoidea*

(SAMEJIMA and MYER, 1958), *Tribonema* (BELCHER and MILLER, 1960) and *Euglena gracilis* Z strain (COOK, 1968), they did not grow in DL-lactate. On the other hand, *Chlamydomonas dysosmos* grew in a mineral medium with lactate as the energy source in darkness (LEWIN, 1954 b). Pale-green algae; *Volvulina steinii* (CAREFOOT, 1967) and *Gonium multicoccum* (cf. chapter I), and marine diatoms (BUNT, 1969) utilize DL-lactate in the light as well as the present *Gonium* strains. The above mentioned studies involving DL-lactate did not investigate the D and L forms separately. In this experiment, the eight strains using lactate only used the D-lactate form and only in the light. This is the first report of algal specificity for D-lactate in the light. It may be shown that D-lactate utilization is a characteristic of photoheterotrophic strains of algae.

The selective utilization of D-lactate may result from permeability limitations or special active transport mechanisms in the cell membrance. In bacteria enzymes specific to D-lactate or L-lactate have been found. Variations in growth between the *Gonium* strains suggest that the mechanism for D-lactate utilization may be in part different for different strains.

The results of the present study indicated that the palmelloid formation is well defined in *G. quadratum* and *G. octonarium*. The palmelloid stage of *Gonium* strains results from and may be an adaptation to changing environmental factors. It may be of particular importance to *G. quadratum* as sexual reproduction in nature for this alga is not known.

Figure 29 shows the life cycles of *G. quadratum* and *G. octonarium*. These two strains 842 and 843 of *G. octonarium* listed as isogametic heterothallic pairs and were of opposite mating types (STARR 1964, See Table 1), however, sexual reproduction was not found in the present study. This result suggests that the sexual ability of *G. octonarium* may be an easily disappeared character during a long period of vegetative growth. The palmelloid stage of *G. octonarium* may be a morphological adaptation of asexual reproduction in the same function as *G. quadratum*.

Summary

Initially, studies were conducted on *G. multicoccum* to establish its culture requirements. Once these were determined, studies were undertaken to explore environmental control of growth and sexual and asexual life cycles. Finally, a comparative nutritional study was conducted on six species of *Gonium*.

The experimental results are summarized below;

1. Nutrient requirements of *G. multicoccum* (7-1 and 7-2 strains) were investigated in detail. Acetate supported, most efficiently, growth in the light and dark. Acetate could be replaced with pyruvate or lactate in the light, but not in the dark. Sugars were ineffective as a carbon source.

2. Thiamine was required for optimum growth of *G. multicoccum*. The addition of B_{12} reduced the thiamine lag-time, but did not change the final yield.

3. As sole nitrogen sources, nitrate, ammonia and urea were equally effective for *G. multicoccum*. Nitrite, arginine, glutamine, uric acid and DL-citrulline supported poor growth. Growth with arginine had a long lag-time. However, in old medium (conditioned medium), this long lag-time disappeared and the growth curve was the same as in NH_4, NO_3 or urea.

4. Tris (hydroxymethyl) aminomethane-HCl buffer and glycylglycine inhibited the maximum growth of *G. multicoccum* in the light at low concentrations (0-90 mg%) but not at high concentrations (90-300 mg%). In contrast, Tris had no effect in the dark.

5. $MgSO_4 \cdot 7H_2O$ was the best sulphur source for the growth of *G. multicoccum*. $Na_2S \cdot 9H_2O$, $Na_2S_2O_5$, Na_2SO_4, $NaHSO_3$ and cystine supported some growth, and cysteine and DL-homocysteine supported very little growth.

6. Effects of other major and minor mineral components were also examined. On the basis of these results, chemically defined media, Medium 52 and Medium G were developed. The two strains of *G. multicoccum* reached the maximum growth (O. D. ca. 0.8) within 50 hrs under conditions of continuous light in Medium G. Both strains reached a maximum growth (O. D. ca. 0.36) in the darkness within 50 hrs in Medium G. As a result of the above studies axenic mass culture of *G. multicoccum* is now possible using Medium 52 and G.

7. Three methods of growth measurement of *G. multicoccum* were investigated and interrelated: Optical density (O. D.), Dry weight (D. W.) and Packed cell volume (P. C. V.). The correlation between O. D. and D. W. was essentially a straight line one, and had the same slope regardless of strain or light or dark growth. However, the correlation between D. W. and P. C. V., or between O. D. and P. C. V. was very different between strains 7-1 and 7-2 grown in the light, but not in the dark.

8. The growth constant (K=doublings per day) was influenced by the amount of initial inoculum. The smaller inoculum caused the greater K (for inocula under O. D. 0.05). The maximum growth constant of *G. multicoccum* was calculated at 36℃ in the experiment on temperature shift ($K=7.3$).

9. Colonies of *G. multicoccum* developed on agar medium had a generation time of 8 hrs at 36℃, and their K was 12. The formation and cell-divisions of daughter colonies were synchronized in culture.

10. CMU (1×10^{-7}) inhibited the final yield of vegetative growth of *G. multicoccum* in the light to the same level as in the dark. CMU was not effective in the dark, and DNP was not effective in the light or in the dark.

11. There was considerable difference of UV absorption spectra between the old culture media from light and dark grown *G. multicoccum*. An absorption peak in the light grown algae was observed at 212 mμ, but in the dark grown algae the same wavelength was observed near to but under the standard point of zero.

12. In *G. multicoccum* strains 7-1, " + " and 7-2, " − ", sexual reproductive cycle was observed and controlled in axenic culture. Sexual reproduction controlling media, Medium

G-1, G-2, G-3 and G-4 were established.

13. In *G. multicoccum*, the most important controlling factors for gametogenesis and zygote formation were nitrogen, Ca-ion, acetate and light. Organic and inorganic nitrogen sources would, upon being depleted from the media, induce gametogenesis. Ca-ion and acetate were indispensable controlling factors in gametogenesis and zygote formation. Acetate seemed to be utilized as a carbon and energy source and was not replaced by light. However, acetate could be replaced with pyruvate. Sexual reproduction never occurred in the dark.

14. In *G. multicoccum*, the most important controlling factors for zygote maturation were acetate, Ca-ion and light. Green aplanozygotes developed into matured orange zygotes. Chlorophylls of aplanozygotes were destroyed in the light during zygote maturation, whereas carotenoids were retained. In culture conditions of acetate-deficiency the zygotes did not change colour even in the light. In the absence of Ca-ion, the zygote lost all pigments.

15. The F_1 vegetative colonies germinated from the matured zygotes of *G. multicoccum* in Medium 3, 5, 6 and G in the light at 18-30℃. Germinated colonies were divided into two mating types, " + " and " − ".

16. A morphogenetic substance which is effective in breaking colonies, designated colony breaking substance (C. B. S.), was found in cell-free extracts of *G. multicoccum*. The biosynthesis of C. B. S. occurred only in strain 7-2, " − ", and particularly in dark cultures.

17. *G. multicoccum, G. octonarium* and *G. quadratum* grew excellently in Medium G, 52, 6 and 5 under the continuous light and in the dark, and required organic carbon sources (acetate, pyruvate or D-lactate) for cell division and colony development. Glucose did not support growth of these species in the light or dark. *G. pectorale, G. sacculiferrum* and *G. sociale* could not grow in Medium G, 52, 5 and 6 in continuous light. However, their growth was supported in Medium 5 and 6 under a regime of 14-hr light and 10-hr dark at 14℃, and 18℃.

18. There was a long growth lag-time in DL-lactate as compared with acetate in *G. multicoccum, G. quadratum* and *G. octonarium*. These three species utilized D-lactate for the growth as a carbon source in the light, but not in the dark. Further, these three species could not utilize L-lactate in the light or dark.

19. Typical palmelloid formation occurred in *G. quadratum* and strain 843, " + " of *G. octonarium* with lactate as a carbon source. The palmelloid stage of *G. quadratum* was observed until the early exponential growth phase, and in *G. octonarium*, it was found throughout in the growth curve in lactate. However, these species did not form the palmelloid stage under various culture conditions in organic Medium 6.

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PLATE I

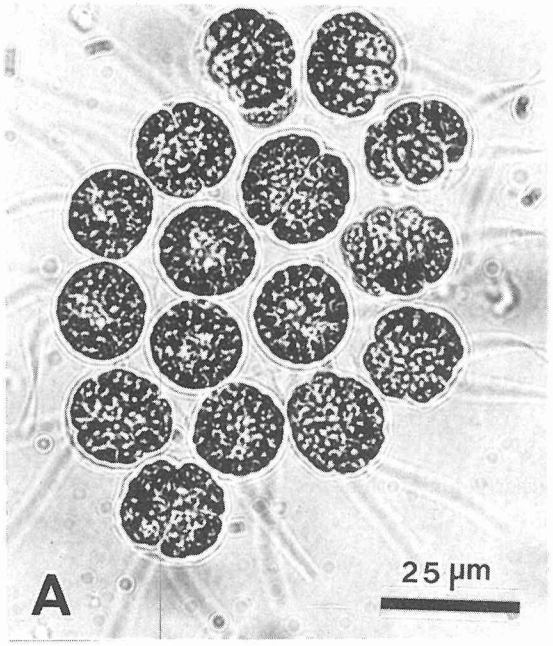
Vegetative colonies of *Gonium multicoccum* (7-1).

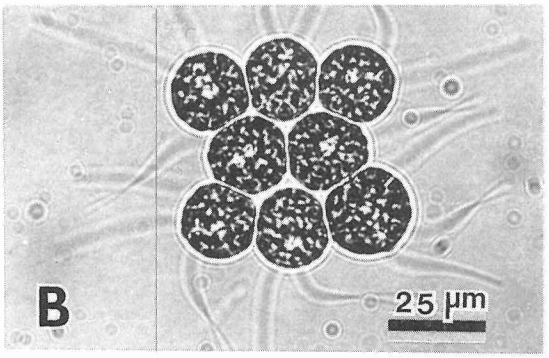
A. Formation of daughter colonies from a matured 16-celled colony.

B. A matured 8-celled colony.

C. A 16-celled colony.

D. Side view of a 16-celled colony.


A
25 µm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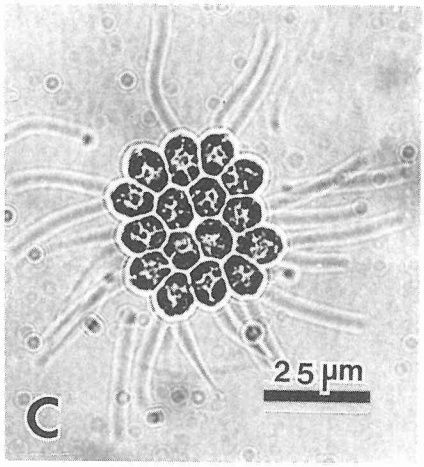
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25 µm

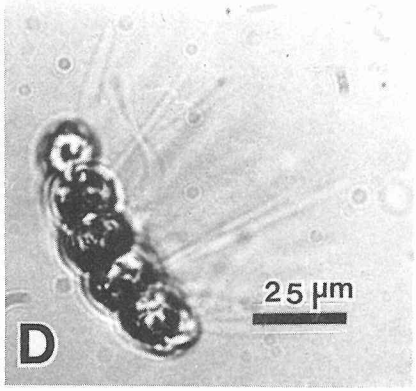

D
25 µm

PLATE II

A series of time-lapse photomicrographs of *Gonium multicoccum* in agar medium. The culture was grown 36 ℃ in the light. The number in parentheses indicates the hours after incubation.

A. A 16-celled colony just inoculated (0).

B. First synchronized daugther colony formation (5).

C-E. Colony development and maturing.

C. Sixteen daugther colonies with 16 cells (6.5).

D. On the same (8.3).

E. Sixteen mature colonies (10).

F. Second synchronized daugther colony formation (13).

G. Colonies after 20 hrs culture.

Scale in A applies also to B-F.

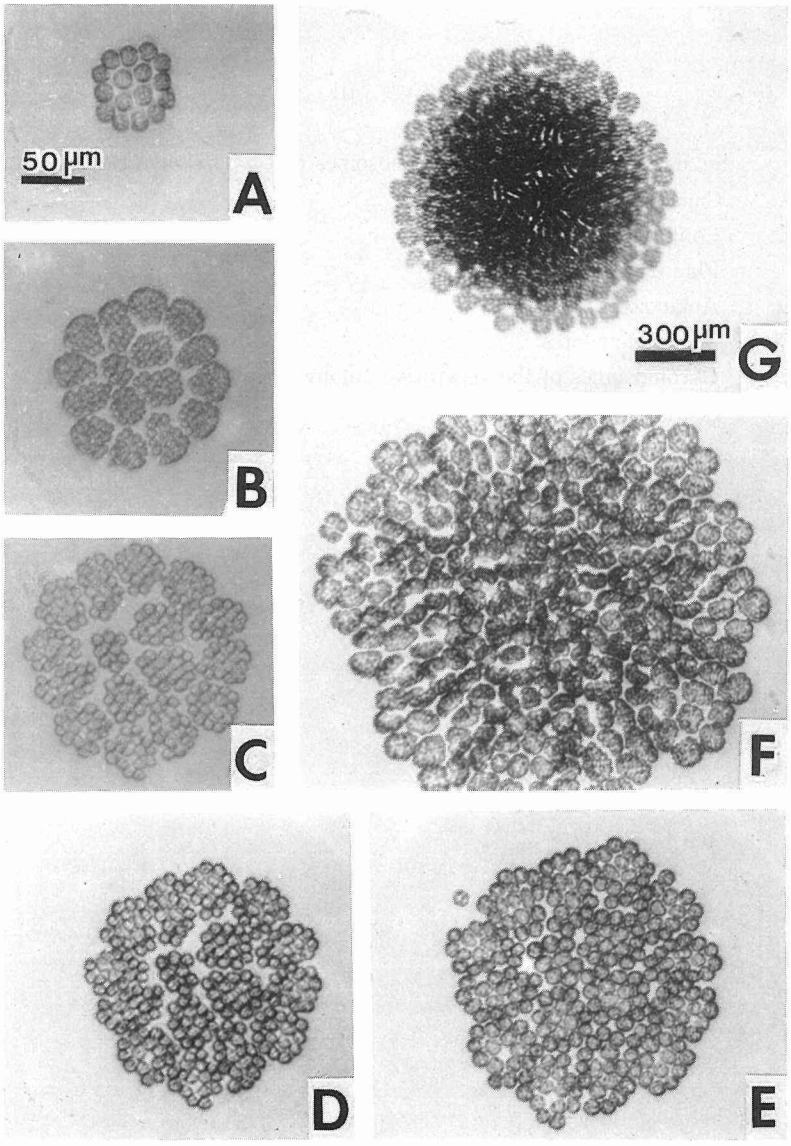

50 µm
A
B
C
D
E
F
300 µm
G

PLATE III

Sexual reproduction and chromosomes of *Gonium multicoccum*.

A. Gametes.
B. Conjugation of gametes.
C. Planozygote.
D. Aplanozygotes.
E. Matured zygotes.
F. Chromosomes of the vegetative colony (haploid 8-celled colony).

Scale in A applies also to B–D.

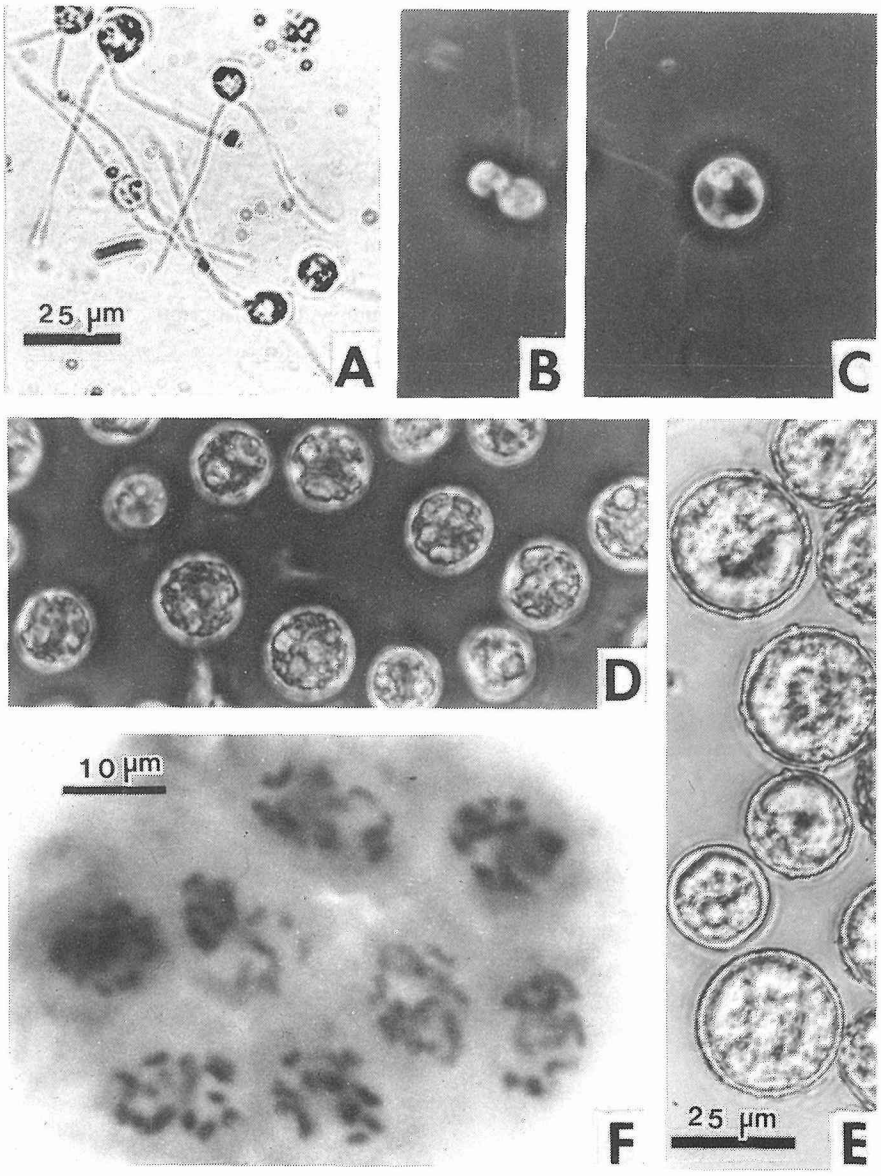

25 µm
A
B
C
D
10 µm
F
25 µm
E

PLATE IV

Synchronization of sexual reproduction in *Gonium multicoccum* (A-C).

A. Vegetative growth in dark (after 50-hr incubation).

B. Gametogenesis and zygote formation (after 2-hr transferred from 50-hr culture in the dark to the light).

C. Matured zygotes (10 days after aplanozygote formation).

Bioassay of C. B. S. (Colony Breaking Substance) in *Gonium multicoccum* (D-E).

D. Normal colonies grown in Medium G.

E. Broken colonies grown in Medium G with S-5 (7-2) fraction (See text).

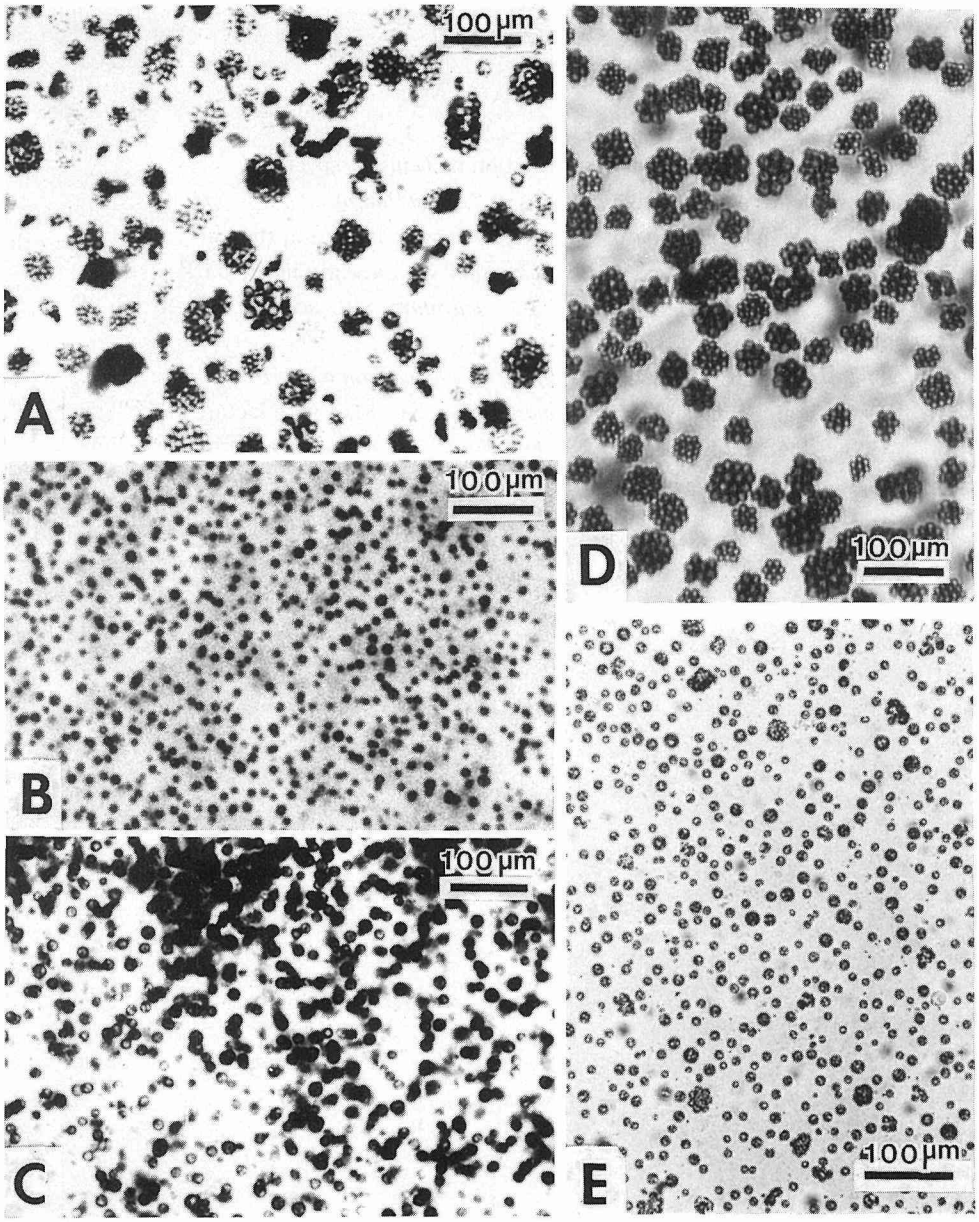

100 µm
A
100 µm
B
100 µm
C
D
100 µm
E
100 µm

PLATE V

Palmelloid formation of *Gonium* species.

A. Normal vegetative colonies of *G. quadratum*.

B. Palmelloid stage of *G. quadratum* with lactate in the light.

C. Palmelloid stage of *G. quadratum* with acetate in the dark.

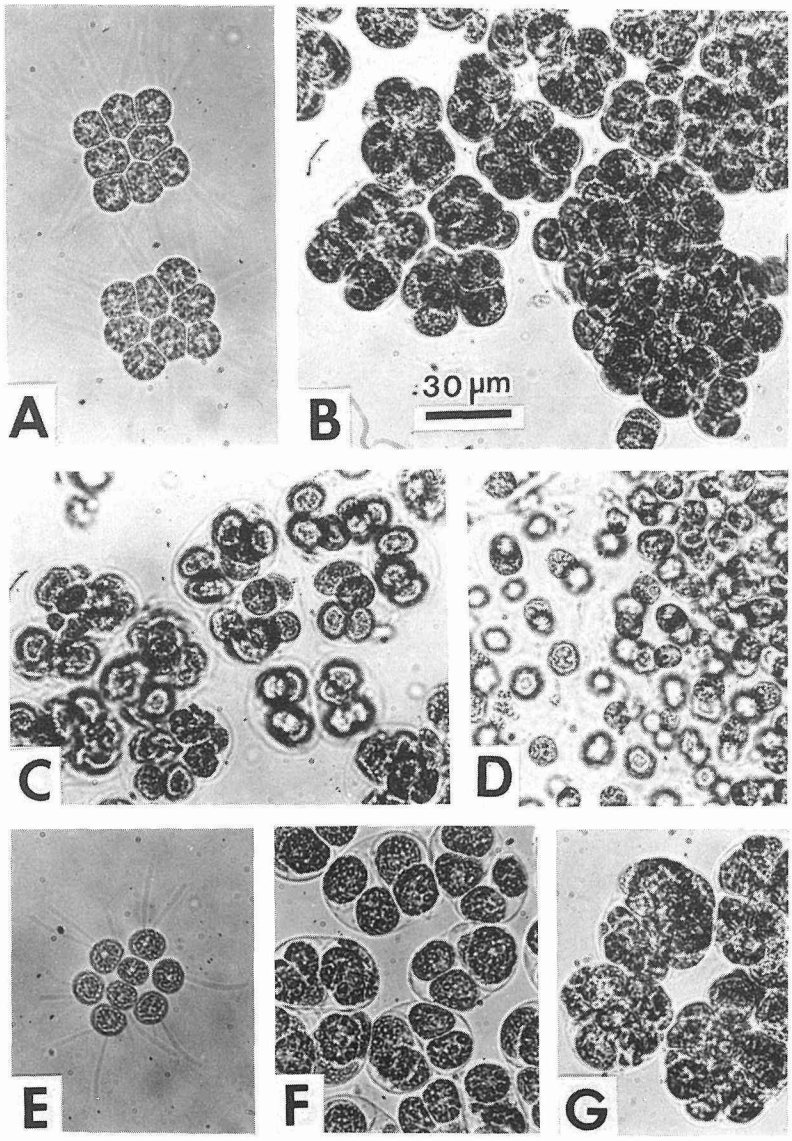
D. Palmelloid-like stage of *G. quadratum* with acetate in a 10 : 14 LD condition.

E. Normal vegetative colony of *G. octonarium* (No. 842).

F. Palmelloid stage of *G. octonarium* (No. 842) with lactate in the light.

G. Palmelloid stage of *G. octonarium* (No. 843) with lactate in the light.

Scale in B applies to all figures.


A
B
30 µm
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D
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G