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Comparative Nitrogen Nutrition of Certain Fresh-Water Algae

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COMPARATIVE NITROGEN NUTRITION OF CERTAIN FRESH-WATER ALGAE

(TITLE)

BY

WILLIAM H. CULP

B.S. in Ed., Eastern Illinois University, 1966

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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I

INTRODUCTION

Systematic research, while still largely classical in its approach inasmuch as strictly morphological criteria are used as the basis for the characterization and classification of living organisms, has made considerable advancements in past years by the increasing use of criteria supplementary to classical morphology. Some such work has been undertaken successfully with morphologically complex forms; however, most significant advancements have been made with microorganisms. It was the bacteriologist who first made it clear that in order to delimit taxa it was necessary to study the organism both morphologically and physiologically in cultures. The taxonomy of other microorganisms, however, is still based primarily upon the morphology and life cycles. Algal taxonomy, still basically classical, has only recently been approached microbiologically.

Before 1946 the algae were ignored almost completely from this new approach mainly because they were considered too difficult to grow in pure culture and most phycologists were not trained sufficiently in chemistry and physiology to attempt such studies. Professor E. G. Pringsheim in his 1946 monograph removed part of this obstacle when he made it clear that algae could be grown in axenic cultures and many of them on chemically defined

media. Due to his work and that of many others, we have today an array of axenic taxa from which valid comparative physiological studies can be made.

This "new taxonomy" appears to have its beginning with the work of Starr in 1955 on the spherical, unicellular, zoospore-producing algae belonging to the Chlorococcaceae. Starr set up some basic morphological criteria for the delimitation of species belonging to this group. These criteria include (1) type of chromatophores, (2) presence or absence of pyrenoids, and (3) type of zoospore. Starr emphasized the study of these organisms over long periods of time in cultures to detect all the morphological conditions needed for positive identification of members of this group. With his new criteria, Starr found many of those algae originally identified as species of Chlorococcum were actually quiescent stages of Carteria, Chlamydomonas, Haematococcus, and others not belonging to the genus Chlorococcum.

Starr summed up the feeling of the use of extra-morphological characteristics for the identification of algae at this time when he stated, "To the practical taxonomists, accustomed to more rapid methods, it may be scant comfort to point out how much better off are phycologists than bacteriologists, in this respect, for only, perhaps, in such genera as Chlorella and Trebouxia, does it seem necessary to resort to physiological taxonomic criteria."

At this time in algal taxonomy Starr might have been right; however, more and more organisms were to be isolated from the soil and air to complicate the picture and make identification and characterization of many more organisms difficult.

Herndon in 1958 extended Starr's criteria to the vegetatively dividing Chlorosphaerales (now called the Chlorosarcinales--see Groover and Bold, 1969.) which have become more and more prominently reported in soil studies.

At this time there was accumulating a large array of unicellular, spherical, zoospore-producing organisms which, because of their great similarities, could not be identified unless studied in cultures over an extended period of time.

The turning point in the "new taxonomy" came in 1960, when Deason and Bold set out to investigate the soil algal flora in regions of Texas. After several months of intensive preliminary work, it became evident to them that their goal was unattainable at this point in the development of soil algal taxonomy. It was obvious to them that it was necessary first to develop and standardize techniques to facilitate the identification of large numbers of organisms. More specifically, it became increasingly apparent that criteria in addition to strictly morphological ones had to be used, if possible, to delimit species. Therefore, the goal of Deason and Bold and many investigators since 1960 has been to develop better

techniques and to discover additional criteria for the delimitation of species of algae applicable to formal descriptions.

Most of this modern experimental phycotaxonomy has come from the laboratories of Bold, Kessler, Krauss, and their associates; they have sought valid extramorphological criteria for the identification and classification of certain chlorophycean taxa, especially but not exclusively those in the Chlorococcaceae and Chlorosarcinaceae (Deason and Bold, 1960; Bold and Parker, 1962; Mattox and Bold, 1962; Bischoff and Bold, 1963; Chantanachat and Bold, 1963; Shihira and Krauss, 1963; Brown and Bold, 1964; Cain, 1965; Smith and Bold, 1966; Brown and McLean, 1969; Groover and Bold, 1969; Smith, 1971).

Bold (1970) summarized some techniques of modern algal taxonomy, pointing out the various supplementary attributes found in his laboratory that have proven valuable as extramorphological criteria. Among these he cites the following: (1) appearance of plant mass (colony) macroscopically and at low magnification, (2) color upon aging in nitrogen-poor media, (3) wall thickness in stable cultures, (4) nutritional studies, especially responses to various nitrogen and carbon sources, (5) enzyme activity, and (6) response to antibiotics.

Nitrogen nutrition, with which this investigation deals, has received considerable attention in the past 20 years and a large volume of data is beginning to develop. Several reviews of algal nitrogen nutrition have been

published (Myers, 1951; Fogg, 1953, 1959; Syrett, 1954, 1962; Krauss, 1958; Hutner, 1964; Kessler, 1964).

The science of inorganic nitrogen nutrition is complex and is one in which much work is currently being done. The literature is voluminous and one is referred to the reviews above for the numerous publications; many of the specific references on inorganic nitrogen nutrition may be found in the discussion which follows.

Numerous organic nitrogen sources are available for use in algal nutritional studies. Of these, urea has received the most attention recently. Numerous reports of algae able to utilize this compound as a sole nitrogen source have been reported (Ludwig, 1938; Allen, 1952; Arnow, et al., 1953; Droop, 1955; Kratz and Myers, 1955; Belcher and Fogg, 1958; Miller and Fogg, 1958; Birdsey and Lynch, 1962; Cain, 1965; Smith and Bold, 1966). Algae unable to utilize urea are apparently less numerous (Allen, 1952; Kratz and Myers, 1955; Syrett, 1962; Cain, 1965; Smith and Bold, 1966).

Urea metabolism in the algae is not fully understood. (See reviews by Syrett, 1954; Reinbothe and Mothes, 1961.) Several theories have been proposed to explain its utilization; the first of these and, perhaps, the most logical, was that urea is directly broken down to CO_2 and NH_3 by urease, as is the case in animal metabolism (Allison, et al., 1954; Bollard, 1959). Urease activity, however, has never been observed in any urea-utilizing alga. Several

other theories have been proposed, none of which have been satisfactorily demonstrated to be of universal occurrence among the algae. The pathway of urea utilization in these organisms, therefore, remains obscure and demands investigation.

Other organic nitrogen sources, such as amino acids, amides, and nitrogenous bases, have been reported to serve as sole nitrogen sources among the algae (See later).

Of the recent increased interest in nitrogen nutrition, few comparative studies have been made and those have dealt with only a few genera. The data which has accumulated on the subject shows that very little attention, with a few noteworthy exceptions (e.g., Shihira and Krauss, 1963; Lewin, 1968), has been paid to the taxonomic implications of the data.

Therefore, it was the purpose of this investigation to compare a number of taxonomically diverse organisms in order to: (1) determine their ability to utilize a number of organic and inorganic nitrogen compounds and, in this way, to further contribute to our knowledge regarding their utilization among the algae, (2) discover, if possible, any patterns of utilization which might help explain the presently enigmatic aspects of urea metabolism, and (3) discover response patterns which might be of taxonomic value.

I I

MATERIALS AND METHODS

The fifty axenic isolates studied in this investigation were purchased from the Culture Collection of Algae at Indiana University (CCIU). A list of these isolates and their isolate numbers are shown in Table 1.

The basal medium, used routinely throughout, was TRIS-buffered inorganic medium (Smith and Wiedeman, 1964) enriched with vitamins (hereafter referred to as TBIMV). The method of preparation and constitution of this medium are shown in Tables 2 and 3. The reasons for the adoption of this medium will be discussed in a later section. It should be noted that KCl has been added to the basal medium to replace the potassium lost when the KNO_3 is replaced by other nitrogen sources. The extra potassium present in the medium when both KNO_3 and KCl are present was deemed permissible since in preliminary work the growth patterns of all of the organisms studied were unaltered by various potassium concentrations.

Droop (1962) pointed out that of all known vitamins, only vitamin B_{12}, thiamine, and biotin have been found to be required by algae. Only two isolates of the Chrysophyta and only four of the Pyrrophyta were reported to have a biotin requirement whereas at least ten isolates of the Chlorophyta required B_{12}; at least seven required thiamine. Thus vitamins B_{12} (cyanocobalamin) and thiamine were added

TABLE 1. Classification of organisms investigated and their isolate numbers.

Classification	CCIU[a]
Cyanophyta	
Anacystis nidulans[b]	625
Chlorophyta	
Volvocales	
Chlamydomonadaceae	
Chlamydomonas reinhardtii (-) Dangeard	90
C. reinhardtii (+) Dangeard	89
C. actinochloris Deason & Bold	964
C. chlamydogama (16-1) Bold	103
C. chlamydogama (16-2) Bold	102
C. eugametos (male) Moewus	9
C. eugametos (female) Moewus	10
C. radiata Deason & Bold	966
Carteria crucifera Korschikoff	432
C. eugametos Mitra	233
C. sp.	2
Volvocaceae	
Gonium pectorale Muller	13
Pandorina morum Bory	18
Eudorina elegans var. *carteri* (Smith) Goldstein	1212
Haematococcaceae	
Haematococcus lacustris (Girod.) Rostaf	16
Stephanosphaera pluvialis Cohn	771
Tetrasporales	
Hormotila blennista	1239
Chlorosarcinales	
Friedmannia israelensis Chantanachat & Bold	1181
Chlorosarcina longispinosa Chantanachat & Bold	1183
C. brevispinosa Chantanachat & Bold	1176
C. stigmatica Deason	962
Tetracystis excentrica Brown et Bold	1456
Chlorosarcinopsis eremi Chantanachat & Bold	1186
C. gelatinosa Chantanachat & Bold	1180
Chlorococcales	
Chlorococcaceae	
Chlorococcum hypnosporum Starr	119
C. echinozygotum Starr	118
Trebouxia anticipata Ahmadjian	904
Neochloris pseudoalveolaris Deason & Bold	975
Protosiphonaceae	
Protosiphon botryoides (Kutz.) Klebs	461

TABLE 1. (Continued)

Classification	CCIU
Oocystaceae	
Chlorella vulgaris Beijerinck	263
C. pyrenoidosa Chick	252
Eremosphaera viridis De Bary	7[c]
Oocystis apiculata W. West	B418
O. marssonii Lemmermann	287
O. sp.	80
Ulotrichales	
Stichococcus mirabilis Lagerheim	316
Hormidium flaccidum A. Br.	322
Ulothrix fimbriata Bold	638
Pleurococcus sp.	433
Zygnematales	
Zygnema circumcarinatum (-) Czurda	43
Z. circumcarinatum (+)	42
Cosmarium botrytis Menegh.	301
Chrysophyta	
Heterococcales	
Botrydiopsis arhiza Borzi	87
B. alpina Vischer	295
B. intercedens Vischer & Pascher	296
Heterotrichales	
Tribonema aequale Pascher	50
Heterosiphonales	
Botrydium becherianum Vischer	158
B. stoloniferium Mitra	156
B. cystosum Vischer	157

[a]Culture Collection, Indiana University

[b]Original description not found. William Kratz made it unialgal; M. B. Allen made it axenic. Tentative identification made by F. Drouet.

[c]Eastern Illinois University culture collection number.

TABLE 2. Preparation of TRIS-buffered inorganic medium with vitamins (TBIMV).[a]

Stock Solutions	Amount
0.1 M KCl	20 ml
0.1 M KNO_3	20 ml
0.1 M Na_2HPO_4	10 ml
0.1 M $MgSO_4 \cdot 7H_2O$	3 ml
0.1 M $CaCl_2 \cdot 2H_2O$	1 ml
0.2 M Tris (hydroxymethylaminomethane) (TRIS)	25 ml

Each of the above is added to approximately 800 ml of distilled water. One ml of each of the following micronutrient stock solutions and supplementary vitamins is then added and a final dilution to 1 liter made.

I.	EDTA	50.00 g	per liter D_{H_2O}[b]
	KOH, 85%	31.00 g	
II.	H_3BO_3	11.42 g	per liter D_{H_2O}
III.	$FeSO_4 \cdot 7H_2O$	4.98 g	per liter acidified H_2O[c]
IV.	$ZnSO_4 \cdot 7H_2O$	8.82 g	per liter acidified H_2O
	$MnCl_2 \cdot 4H_2O$	1.44 g	
	MoO_3	0.71 g	
	$CuSO_4 \cdot 5H_2O$	1.57 g	
	$Co(NO_3)_2 \cdot 6H_2O$	0.49 g	

Supplements:

Cyanocobalamin	50 µg/l
Thiamine HCl	1000 µg/l

[a]The pH of this medium will be approximately 8.8. To adjust the pH to 7.4, HCl was added.

[b]Glass-distilled water

[c]Acidified water: 999 ml distilled water, 1 ml concentrated sulfuric acid (i.e., final concentration 0.03 M H_2SO_4)

TABLE 3. Constitution of TBIMV and BBMV.

Constituent	BBMV		TBIMV	
	mg/liter	µM/liter	mg/liter	µM/liter
KCl	---	---	151	2000
$NaNO_3$	250	2940	---	---
KNO_3	---	---	202	2000
KH_2PO_4	150	1100	---	---
$Na_2HPO_4 \cdot 7H_2O$	---	---	268	1000
K_2HPO_4	100	570	---	---
$MgSO_4 \cdot 7H_2O$	75	300	75	300
$CaCl_2 \cdot 2H_2O$	25	170	15	100
NaCl	25	430	---	---
TRIS	---	---	605	5000
EDTA	50.0	170.0	50.0	170.0
KOH, 85%	31.0	470.0	31.0	470.0
$FeSO_4 \cdot 7H_2O$	4.98	17.8	4.98	17.8
H_3BO_3	11.42	185.0	11.42	185.0
$ZnSO_4 \cdot 7H_2O$	8.82	31.0	8.82	31.0
$MnCl_2 \cdot 7H_2O$	1.44	7.3	1.44	7.3
MoO_3	0.71	4.9	0.71	4.9
$CuSO_4 \cdot 5H_2O$	1.57	6.3	1.57	6.3
$Co(NO_3)_2 \cdot 6H_2O$	0.47	1.7	0.47	1.7
Total Solids	735	6404	1426	11,294
Supplements:				
Cyanocobalamin	50 µg/l			
Thiamine HCl	1000 µg/l			
pH adjusted with HCl to:	6.8		7.4	

to the culture medium to meet the demands of any vitamin-requiring organisms. Biotin was not added since there have been no reports known to the author of it being needed by the isolates under investigation.

When KNO_3 was omitted from the medium to produce a nitrogen-free medium, the abbreviation -N is added (e.g., TBIMV-N). When other nitrogen sources were used, they replaced KNO_3 in the original medium. A list of the nitrogen-containing compounds used and their formulas may be found in the following section (Table 13). The nitrogen sources were supplied to the basal medium to produce a two-millimolar (mM) concentration of nitrogen, and not necessarily 2 mM of the compound itself. Preliminary experimentation indicated that 2.0 mM nitrate-nitrogen was sufficient for most of the organisms under investigation. Four, 10, and 40 millimolar concentrations of nitrogen were used with some of the nitrogen sources to determine whether increased amounts of nitrogen did, indeed, have any effect on the growth patterns.

Growth of the organisms under investigation was also tested on Bold's Basal Medium (Bischoff and Bold, 1963) supplemented with cyanocobalamin and thiamine (hereafter known as BBMV). The constituents of this medium and their corresponding concentrations are shown in Table 3.

Evidence indicates that autoclaving may break down certain constituents or fractions of culture media, thereby leading to inconsistent results (Finkelstein and Lankford,

1957; Mattox and Bold, 1962). Since many of the nitrogen sources used were known to be heat labile, sterilization was accomplished by Seitz filtration.[1] The entire liquid medium was filtered; a Seitz-filtered fraction was never added to a previously autoclaved solution.

The media were dispensed into pre-autoclaved[2] 16 X 150-mm rimless Pyrex test tubes by the use of an automatic pipetting machine.[3] The pipetting machine insured an even, uniform volume of medium from tube to tube and from experiment to experiment. The tubes were capped with plastic closures.

Standard test tube racks did not allow enough light penetration between tubes; therefore, wire racks were made with a ¼-inch spacing between tubes to allow increased light penetration.

Six-week old stock cultures, growing in liquid TBIMV, were used as the source of inocula for all experiments.

Inoculations were carried out under strictly aseptic conditions. Countertops were routinely swabbed

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[1] "Seitz," Hercules Filter Division, The Delaval Separator Company, Poughkeepsie, N. Y. Hercules sterilizing filter sheets were obtained from: Republic Seitz Filter Corp., P. O. Box 229, Milldale, Conn. 06467. Grade ST 3, Size L6 was used.

[2] Fifteen lbs. pressure for 15-20 minutes followed by a slow exhaust of 25 minutes.

[3] Filamatic Vial Filler. National Instrument Company, Baltimore 15, Maryland.

with 70-95% ethyl alcohol prior to transferring. Transfers from stock cultures to the experimental tubes were made by the use of sterile cotton-plugged, disposable Pasteur pipettes equipped with a standard 1-ml rubber bulb. Three drops of inoculum were introduced per tube.

With every series of inoculations, the axenic conditions were routinely checked by inoculating the organism into Bacto-AC broth medium.[4]

A control of TBIMV-N was always run simultaneously with each experiment. Experiments, unless otherwise indicated, were performed in duplicate, subcultured in duplicate, and repeated in duplicate.

The incubation period for most experiments was six weeks. The organisms were grown under carefully controlled environmental conditions, hereafter referred to as standard conditions, which were a culture room maintained at 22°C under "cool white" fluorescent illumination of 350 ft-c intensity on a 24-hour cycle of 16 hours of light and 8 hours of darkness.

A visual estimation of growth was made after four weeks' incubation by suspending the cells with the aid of a Vortex Junior Mixer[5] and then placing them in a lighted test tube viewing box for easy visual comparison. In each experiment an arbitrary value of four was assigned to the

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[4]Difco Laboratories, Detroit, Michigan.

[5]Scientific Industries Inc., Queens Village, N. Y.

growth of the nitrate medium; the growth of that organism in the other media was compared to this standard. A growth reading of two represents half the growth of four; a reading of eight represents twice that growth. Only those differences of two or more units were considered significant. Growth amounts were, therefore, compared from medium to medium for each organism, and not from organism to organism in each medium. This type of comparison was necessary since the organisms under investigation possess their own inherent growth rates which often differ widely from each other. In view of the major objective of this study, i.e., to appraise the ability of a species to utilize a given nitrogen source, this method of visual estimation was deemed valid.

Detailed observations of growth were made during the sixth week period. These observations included, in addition to the above, (1) pH changes, which were recorded by the use of a Coleman Metrion IV pH meter,[6] with a single metering electrode attached to insert directly into the growth tubes for easy readings, and (2) microscopic examination to detect any gross changes in size, shape, number of cells, or any other general cell condition.

For microscopic examination the cultures were again suspended and an aliquot amount placed on a glass slide. To be certain that visual growth estimates (i.e., examination

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[6]Coleman Instruments, Division of Perkin-Elmer Corporation, 42 Madison Street, Maywood, Illinois 60153. Model 28C.

of tube cultures) were a reflection of cell number and not of chlorophyll content, microscopic counts were made by recording the average number of cells observed in ten random fields at 430x magnification. The size of the cells was measured, where of interest, by selecting approximately thirty cells at random and measuring with a standard calibrated ocular micrometer eyepiece attached to a (Bausch and Lomb) compound microscope. Significant differences were occasionally recorded photographically using a Kodak 35 Colorsnap camera attached to the microscope. Macroscopic examinations of growth were also recorded photographically; an example of such a record is given in Figure 1.

I I I

RESULTS AND DISCUSSION

Basal Medium

The literature reveals that many investigations regarding the utilization of specific nitrogen sources by algae have employed poorly buffered media which allow large pH shifts. These pH changes can be detrimental to the growth of the organisms, thereby leading to invalid interpretations. Furthermore, many investigators have used slightly acidic media when, in fact, many algae seem to prefer mildly alkaline conditions (Pringsheim, 1951). Therefore, a medium well-buffered at a slightly alkaline pH is of utility in experiments measuring physiological growth responses in diverse assemblages of algae. The development, by Smith and Wiedeman (1964), of an alkaline growth medium (TBIM) employing the use of an amine buffer, TRIS, seems to fit this need. TRIS, unlike most phosphate buffer systems, is generally considered to be non-utilizable and effective at relatively low concentrations (Smith and Wiedeman, 1964). Reports of TRIS toxicity are known (e.g., McLachlan, 1963; Provasoli and Pintner, 1960), but few such cases have been documented among algae.

Before the decision was made to incorporate TBIMV as the basal medium in this investigation, a preliminary experiment was performed to determine the behavior of the fifty isolates regarding (1) TRIS toxicity, (2) TRIS

utilization, and (3) growth in TBIMV. The isolates were also grown in BBMV to compare growth and pH responses between the two media. Table 4 summarizes the pH changes of the fifty isolates in BBMV and TBIMV. The effectiveness of TRIS in minimizing pH shifts, in contrast to the widely used phosphate buffer system (BBMV) is apparent from the average shift in pH units in each medium.

Table 5 summarizes the growth of the 50 isolates on TBIMV and BBMV. Most of the organisms reached their maximum growth at or before the time of the four-week observation period. In 40 of the 50 isolates, good growth was observed on either TBIMV or BBMV. Five isolates, i.e., Carteria sp., Pandorina morum, Haematococcus lacustris, Stephanosphaera pluvialis, and Eremosphaera viridis, grew only in trace amounts after four weeks in both media. Since they grew very little, comparisons of growth in nitrate to that in other nitrogen sources were practically impossible in these cases. Such tests were necessary, however, in order to determine if, in fact, it was the source of nitrogen which was limiting growth. Other nitrogen sources, such as NH_4Cl, NH_4NO_3, urea, arginine, and asparagine were tested, but in all cases growth was minimal after four weeks. These species must have some requirement which is not satisfied in either BBMV or TBIMV under the conditions used. Of interest was the good growth observed in Haematococcus lacustris and Stephanosphaera pluvialis in 12-week cultures; visual differences in growth were much more

TABLE 4. pH of media after 6 weeks' growth of 50 isolates in BBMV and TBIMV.

	BBMV (Initial pH 6.4)	TBIMV (Initial pH 7.4)
Range of pH at end of growth period	6.8 - 8.0	7.4 - 8.0
Amount of shift, in pH units, at end of growth period	0.4 - 1.6	0.0 - 0.6
Average pH	7.4	7.6
Average shift in pH units	+1.0	+0.2

TABLE 5. Relative growth of 50 isolates in TBIMV and BBMV after 4 weeks of incubation.

# of isolates	TBIMV	BBMV
40	good	good
5	trace	trace
2	trace	none
2	none	none
1	none	good

easily evaluated in the several nitrogen sources than in the 4-week culture. It should be noted, however, that these cultures were exposed to bacterial contamination after the pH of the cultures was measured at 6 weeks. It is, therefore, impossible to say with certainty whether it was the age of the culture or the presence of bacteria which caused the increased growth in these species.

Two species, Cosmarium botrytis and Botrydiopsis arhiza, also showed trace amounts of growth in TBIMV, but did not grow at all in BBMV. This difference might possibly be due to the difference in pH between the two media. Cosmarium botrytis did not show any more favorable growth in other nitrogen sources after 4 weeks, but after 12 weeks it showed good growth, which allowed for accurate visual comparison. Again, however, these tubes were contaminated due to pH studies at 6 weeks.

Two species, Eudorina elegans and Trebouxia anticipata, did not grow in BBMV and TBIMV even after 12 weeks incubation; they were, therefore, dropped from the investigation.

One species, Zygnema circumcarinatum (+), grew well in BBMV but would not grow at all in TBIMV after 4 weeks. Z. circumcarinatum (-) responded much better to growth in TBIMV after 4 weeks. Both species showed good growth in the potentially contaminated 12-week old cultures. This lack of response by Z. circumcarinatum could be due to pH since reports (see Hoshaw, 1968) indicate that this organism grows best in the slightly acid range (i.e., pH 6.5-7.0).

The physiological responses of the organisms toward growth in TBIMV and BBMV might have some taxonomic significance in itself. For example, all of the species of the Chlamydomonadaceae tested grew well in either TBIMV or BBMV except Carteria sp. Of the three species of the Volvocaceae tested, Gonium pectorale grew well in both media, Pandorina morum grew only slightly, and Eudorina elegans did not grow at all. Both isolates of the Haematococcaceae tested, Haematococcus lacustris and Stephanosphaera pluvialis, grew only slightly in either media. All tested isolates belonging to the Chlorosarcinales grew equally well in both media.

Of the chlorococcalean algae tested, only Trebouxia anticipata and Eremosphaera viridis showed a lack of growth in each medium. All ulotrichalean algae tested responded well to TBIMV and BBMV. Of the tested algae belonging to the Zygnematales, none responded well to TBIMV except Zygnema circumcarinatum (-) which showed slight growth. Both strains of Zygnema circumcarinatum grew in BBMV, but Cosmarium botrytis did not. All members of the Chrysophyta tested grew well in both TBIMV and BBMV except Botrydiopsis arhiza. Of course, in order to draw valid taxonomic conclusions, many more isolates should be tested in each of these groups to determine the patterns among all the species; nonetheless, these results suggest possible taxonomic significance, particularly as aids to identification.

TBIMV-N was used routinely as a control medium in each experiment to verify that the stock media contained insufficient nitrogen to support growth. In the initial experiment, this N-free medium was used to determine whether TRIS might itself be utilized by any of the isolates or if any were capable of nitrogen fixation. Wiedeman (1964) has reported the lack of TRIS utilization by a number of green algae. Growth was always negligible in TBIMV-N, with a few noteworthy exceptions. Tribonema aequale repeatedly showed a slight growth in TBIMV-N, even in subcultures, although it was reduced some in the subculturing. Of possible taxonomic significance here is the fact that repeated runs in TBIMV-N showed that Botrydiopsis alpina and B. intercedens also showed growth in the original culture of TBIMV-N; however, subculture data were always zero for these organisms. Botrydiopsis arhiza showed growth two out of six times in the original culture of TBIMV-N. Upon microscopic examination, all the cells of these organisms were typically nitrogen-starved; therefore, they were not utilizing the TRIS as a nitrogen source; nor were they fixing atmospheric nitrogen. These organisms obviously have physiological capabilities allowing them to withstand severe nitrogen deficiencies better than the other species tested.

Due to the favorable growth responses in TBIMV and TBIMV-N media and to the low pH shifts with TBIMV, this medium was incorporated as the basal medium for use throughout this investigation.

AC Broth

In physiological growth response experiments, the organisms must be growing in an axenic condition. It would otherwise be impossible to make valid judgments of their growth or morphological responses since contaminating organisms could alter the nitrogen source and/or the medium. AC Broth was used routinely before each experiment to check the axenic condition of the media and cultures used. AC Broth, a general bacteriological test medium, proved somewhat unreliable in detecting bacterial growth. In some instances inoculated tubes of AC Broth showed no contamination after five days incubation, but the algal media showed obvious contamination either as a cloudy solution, milky residue, or surface fungus growth. In all cases where AC Broth did show contamination, visual examination of the algal culture itself verified the results. Therefore, while AC Broth is adequate in many cases, it is not so in all, and more bacteriological test media should be used routinely before and during each experiment to determine the axenic condition.

Of important interest, physiologically and taxonomically, was the ability of some isolates to utilize AC Broth as a growth medium (Table 6). Of the eight isolates of Chlamydomonas tested, only the male and female strains of Chlamydomonas eugametos utilized AC Broth; however, neither was able to grow in the medium when subcultured. Of interest also was that both species of Chlorococcum tested were able to grow in AC Broth, even when subcultured.

TABLE 6. Growth of certain isolates in AC Broth after the original 6 weeks of incubation and 6 weeks of subculturing.

Organism	KNO_3[a]	Original	Subculture
Chlamydomonas eugametos (male)	4	2	0
C. eugametos (female)	4	3	0
Chlorococcum hypnosporum	4	4	3
C. echinozygotum	4	4	4
Chlorella vulgaris	4	5	5
Pleurococcus sp.	4	4	4
Oocystis marssonii	4	4	-
Chlorosarcinopsis eremi	4	4	-

[a]Control medium = arbitrary growth value of 4.

Pleurococcus sp., whose taxonomic position in the order has been questioned, was the only ulotrichalean alga tested which was able to utilize AC Broth. Chlorella vulgaris utilized AC Broth better than TBIMV. Even in cultures 4 months old, the tubes exhibited a dark green color. Oocystis marssonii and Chlorosarcinopsis eremi grew well in AC Broth, although subculture data were not obtained in these cases. Chlorosarcinopsis eremi has been reported to grow excellently in AC Broth by Chantanachat and Bold (1962). Differences in growth of the 50 isolates in AC Broth again suggest its possible use as an extramorphological taxonomic criterion.

Concentration of KNO_3

Prior to investigating the effects of the various nitrogen sources, it was necessary to determine the concentration of KNO_3 in TBIMV which would be most suitable for the growth of most of the isolates and then supply the other nitrogen sources such that the concentration (of nitrogen) would be equal in each case to this optimum amount. Concentrations of 2, 4, 10, and 40 mM KNO_3 were used in the TBIMV. The results of growth in these media after 6 weeks' incubation is shown in Table 7.

Apparently, high concentrations of KNO_3 inhibit most of the isolates since 23 out of the 37 isolates tested grew less in the 40 mM than in the 2 mM concentration. In 40 mM KNO_3, only 14 grew as well as or better than in

TABLE 7. Growth of 50 axenic isolates after 6 weeks' incubation in TBIMV containing increasing concentrations of KNO_3; concentrations in millimolars per liter.

Organism	Conc. of KNO_3 in mM/l				
	0.0[a]	2.0[b]	4.0	10	40
Anacystis nidulans	0	4	4	4	4
Chlamydomonas reinhardtii (-)	0	4	4	6	6
C. reinhardtii (+)	0	4	4	6	6
C. actinochloris	0	4	4	2	2
C. chlamydogama (16-1)	0	4	4	4	0
C. chlamydogama (16-2)	0	4	4	2	1
C. eugametos (male)	0	4	4	1	0
C. eugametos (female)	0	4	4	2	½
Carteria crucifera	0	4	3	2	½
C. eugametos	0	4	4	4	3
Hormotila blennista	0	4	4	4	4
Friedmannia israelensis	0	4	2	½	½
Chlorosarcina longispinosa	0	4	4	3	1
C. brevispinosa	0	4	3	2	0
C. stigmatica	0	4	4	3	2
Tetracystis excentrica	0	4	4	1	0
Chlorosarcinopsis eremi	0	4	4	4	4
C. gelatinosa	0	4	4	4	4
Chlorococcum hypnosporum	0	4	4	4	2
C. echinozygotum	0	4	4	4	3
Neochloris pseudoalveolaris	0	4	4	4	1
Protosiphon botryoides	0	4	3	0	0
Chlorella vulgaris	0	4	4	4	4
C. pyrenoidosa	0	4	4	4	4
Oocystis apiculata	0	4	3	2	0
O. marssonii	0	4	4	4	4
O. sp.	0	4	4	4	4
Stichococcus mirabilis	0	4	4	4	4
Hormidium flaccidum	0	4	8	4	1
Ulothrix fimbriata	0	4	4	4	0
Pleurococcus sp.	0	4	4	4	4
Botrydiopsis alpina	1	4	5	5	5
B. intercedens	1	4	4	4	4
Tribonema aequale	1	4	4	4	0
Botrydium becherianum	0	4	4	4	0
B. stoloniferium	0	4	4	4	0
B. cystosum	0	4	4	2	0

[a] TBIMV-N control

[b] TBIMV control = arbitrary 4

2 mM KNO_3. Only 3 isolates, Botrydiopsis alpina and both strains of Chlamydomonas reinhardtii, showed increased growth in 40 mM; fourteen isolates did not grow at all at this high concentration. It is apparent from Table 7 that the 2 mM concentration of KNO_3 seems most suitable for most of the isolates; therefore, the other nitrogen sources were supplied in that amount throughout the study. Syrett (1962) reported that species of Chlorella and Scenedesmus respond well to increased concentrations of NO_3^-. Although the results reported here did not indicate increased growth, neither do they indicate decreased growth. This lack of positive response might be due to a deficiency of CO_2 in the culture vessels. Myers (1962) has pointed out that CO_2 is a major factor limiting growth in algal cultures grown in cotton-plugged vessels. The tubes in this investigation were capped with plastic enclosures; however, the CO_2 tension is probably still quite low. The inhibitory effect of relatively high concentrations of KNO_3 is possibly due to a change in the intracellular pH brought about by the increased KNO_3 or, possibly, to osmotic effects.

Morphological changes in the increased concentrations of KNO_3 were limited. All species of Chlamydomonas which showed negative response to increased KNO_3 concentrations showed decreasing amounts of motility and increasing numbers of Palmella stages as the concentrations increased. All species of Chlorosarcina exhibited clumping of the cells at higher concentrations (Figs. 2,3). No significant difference in cell size was noted in any of the isolates.

The nitrogen sources tested will be grouped into the following categories for convenience of discussion: (1) inorganic nitrogen, (2) urea and intermediates in urea biosynthesis, (3) amino acids, and (4) purines and pyrimidines.

Inorganic Nitrogen

The three most common natural forms of inorganic nitrogen exist as molecular, nitrate, and ammonium nitrogen. Among the algae it appears that only those algae belonging to the Cyanophyta are capable of fixing atmospheric nitrogen (Fogg, 1956). None of the isolates used in this investigation was capable of utilizing nitrogen, not even the one blue-green alga, Anacystis nidulans. This report of non-utilization of atmospheric nitrogen by A. nidulans is a confirmation of a previous report by Kratz and Myers (1955). Cells of isolates showed typical symptoms of nitrogen starvation in TBIMV-N (e.g., bleached cells, granular chloroplasts, loss of motility, and irregularly-shaped cells) (Figs. 4,5).

All chlorophyllous algae, with a few noteworthy exceptions, can apparently utilize either ammonium or nitrate when these are supplied in suitable concentrations (Syrett, 1962). NH_4^+ is often used preferentially to NO_3^- when the two are supplied together; however, a given alga may use them with equal facility or even may prefer NO_3^- over NH_4^+ (e.g., Proctor, 1957; Krauss, 1958; Stross, 1963). Before any inorganic nitrogen source can be

biochemically functional (that is, incorporated into amino acids, for example), it appears that it must first be reduced to ammonia. Fewson and Nicholas (1961) proposed a possible reduction sequence for nitrate as follows:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow [NOH] \longrightarrow NH_2OH \longrightarrow NH_3$$

nitrate — nitrite — nitric oxide — nitroxyl — hydroxylamine — ammonia

Since NH_4^+ is already reduced, it would seem logical that it would be utilized more easily than nitrate, which must be reduced before being incorporated into the metabolic system. Nitric oxide is a gas and nitroxyl has never been observed in the free state (Kessler, 1964); therefore, these could not be tested. Hydroxylamine has received only rare attention (See later).

The inorganic sources tested were KNO_3, NH_4Cl, NH_4NO_3, and hydroxylamine (NH_2OH). The results of growth patterns in these nitrogen sources are summarized in Table 8; Table 9 summarizes the pH changes observed in the media after growth.

Of the 47 isolates tested, 35 used NH_4Cl as well as KNO_3. Only one isolate, Botrydium becherianum, appeared to utilize NH_4Cl better than KNO_3. The growth of two isolates recorded as about one-half that observed in KNO_3, 3 recorded only limited growth, and 6 showed no growth. Of possible interest here, taxonomically, is that, of the isolates of the Chlamydomonadaceae tested, only Chlamydomonas radiata and Carteria sp. did not use NH_4Cl. Of the three genera of the Volvocaceae tested, all used NH_4Cl and KNO_3

TABLE 8. Growth and pH changes elicited by 47 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with various nitrogen sources.[a]

Organism	-N[b]	KNO_3[c]	NH_4Cl	NH_4NO_3	NH_2OH
Anacystis nidulans	0 (7.3)	4 (7.6)	4 (6.9)	4 (7.1)	0 (7.4)
Chlamydomonas reinhardtii (-)	0 (7.4)	4 (7.7)	4 (6.4)	4 (7.2)	0 (7.5)
C. reinhardtii (+)	0 (7.5)	4 (7.7)	4 (6.6)	4 (7.0)	0 (7.5)
C. actinochloris	0 (7.5)	4 (7.7)	3 (6.8)	3 (6.8)	0 (7.5)
C. chlamydogama (16-1)	0 (7.5)	4 (7.6)	4 (6.6)	4 (7.0)	0 (7.5)
C. chlamydogama (16-2)	0 (7.5)	4 (7.8)	4 (6.6)	4 (7.2)	0 (7.3)
C. eugametos (male)	0 (7.5)	4 (7.8)	4 (6.2)	4 (7.1)	0 (7.3)
C. eugametos (female)	0 (7.5)	4 (7.8)	4 (6.4)	4 (7.3)	0 (7.3)
C. radiata	0 (7.4)	4 (7.8)	0 (7.3)	2 (7.4)	---
Carteria crucifera	0 (7.5)	4 (8.0)	4 (7.1)	4 (7.6)	½ (7.4)
C. eugametos	0 (7.5)	4 (7.8)	4 (6.9)	4 (7.4)	½ (7.4)
C. sp.	0 (7.5)	4 (7.6)	0 (7.3)	2 (7.4)	0 (7.4)
Gonium pectorale	0 (7.3)	4 (7.7)	4 (6.8)	4 (7.2)	0 (7.4)
Pandorina morum	0 (7.4)	4 (7.5)	1 (7.3)	4 (7.4)	0 (7.5)
Haematococcus lacustris	0 (7.5)	4 (7.5)	1 (7.0)	1 (7.4)	---
Stephanosphaera pluvialis	0 (7.5)	4 (7.5)	4 (7.2)	4 (7.3)	---
Hormotila blennista	0 (7.5)	4 (7.7)	4 (6.5)	4 (6.9)	0 (7.3)
Friedmannia israelensis	0 (7.3)	4 (7.5)	4 (7.1)	4 (7.2)	---
Chlorosarcina longispinosa	0 (7.5)	4 (7.7)	4 (6.7)	3 (7.2)	2 (7.4)
C. brevispinosa	0 (7.3)	4 (7.6)	½ (7.2)	½ (7.2)	1 (7.3)
C. stigmatica	0 (7.2)	4 (7.8)	4 (7.1)	4 (7.1)	0 (7.4)
Tetracystis excentrica	0 (7.4)	4 (7.7)	4 (7.2)	4 (7.2)	---
Chlorosarcinopsis eremi	0 (7.3)	4 (7.9)	4 (6.2)	5 (7.2)	2 (7.2)
C. gelatinosa	0 (7.3)	4 (7.6)	4 (6.6)	5 (7.4)	½ (7.3)
Chlorococcum hypnosporum	0 (7.4)	4 (7.8)	4 (5.9)	4 (7.2)	---
C. echinozygotum	0 (7.3)	4 (7.7)	4 (6.4)	4 (7.2)	---
Neochloris pseudoalveolaris	0 (7.4)	4 (7.5)	4 (7.0)	4 (7.0)	---
Protosiphon botryoides	0 (7.5)	4 (7.6)	4 (7.2)	4 (7.1)	0 (7.3)

TABLE 8. (Continued)

Organism	-N	KNO_3	NH_4Cl	NH_4NO_3	NH_2OH
Chlorella vulgaris	0 (7.5)	4 (7.7)	4 (7.1)	4 (7.2)	---
C. pyrenoidosa	0 (7.4)	4 (7.6)	4 (7.1)	4 (7.2)	---
Coccystis apiculata	0 (7.3)	4 (7.5)	2 (7.1)	2 (7.2)	0 (7.3)
O. marssonii	0 (7.3)	4 (7.5)	4 (6.9)	4 (7.0)	4 (7.4)
O. sp.	0 (7.3)	4 (7.5)	0 (7.2)	0 (7.2)	0 (7.3)
Stichococcus mirabilis	0 (7.3)	4 (7.5)	4 (6.6)	4 (7.0)	0 (7.3)
Hormidium flaccidum	0 (7.5)	4 (7.5)	4 (7.1)	4 (7.1)	2 (7.2)
Ulothrix fimbriata	0 (7.3)	4 (7.5)	4 (7.1)	4 (7.2)	½ (7.2)
Pleurococcus sp.	0 (7.4)	4 (7.5)	3 (7.1)	3 (7.2)	4 (7.2)
Zygnema circumcarinatum (+)	0 (7.4)	4 (7.5)	0 (7.3)	2 (7.3)	---
Z. circumcarinatum (-)	0 (7.2)	4 (7.5)	0 (7.2)	2 (7.3)	---
Cosmarium botrytis	0 (7.2)	4 (7.3)	0 (7.2)	0 (7.2)	---
Botrydiopsis arhiza	0[d] (7.4)	½ (7.5)	½ (7.3)	½ (7.3)	0 (7.3)
B. alpina	0[d] (7.3)	4 (7.3)	4 (7.1)	3 (7.2)	0 (7.3)
B. intercedens	0[d] (7.4)	4 (7.5)	4 (7.2)	4 (7.2)	1 (7.3)
Tribonema aequale	0[d] (7.5)	4 (7.7)	4 (7.2)	4 (7.2)	1 (7.3)
Botrydium becherianum	0 (7.2)	4 (7.6)	6 (5.9)	4 (7.1)	0 (7.3)
B. stoloniferum	0 (7.4)	4 (7.7)	2 (6.6)	2 (7.1)	0 (7.4)
B. cystosum	0 (7.5)	4 (7.6)	1 (7.2)	1 (7.2)	0 (7.4)

[a] Concentration of nitrogen 2.0 mM in each case. Figures in parentheses indicate final pH; the initial pH was 7.3 except NH_2OH, which was 7.4.

[b] TBIMV-N control

[c] TBIMV control = arbitrary 4

[d] See discussion, page 23

TABLE 9. Summary of pH changes in cultures of 47 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with various inorganic nitrogen sources.[a]

	-N	KNO_3	NH_4Cl	NH_4NO_3	NH_2OH
Range of pH at end of growth period	7.2 to 7.5	7.3 to 8.0	5.9 to 7.3	6.8 to 7.6	7.2 to 7.5
Amount of shift in pH units, at end of growth period	-0.1 to +0.2	0.0 to +0.7	0.0 to -1.4	-0.5 to +0.3	-0.2 to +0.1
Average final pH	7.4	7.6	6.7	7.2	7.3
Average shift in pH units	+0.07	+0.3	-0.63	-0.1	-0.12

[a]The initial pH was 7.3 except for NH_2OH, which was 7.4.

equally except Pandorina morum, which used NH_4Cl only slightly. Haematococcus lacustris also used NH_4Cl only slightly. The chlorosarcinalean algae tested all utilized NH_4Cl except Chlorosarcina brevispinosa, which only showed trace signs of growth. Contrary to a previous report (Smith and Bold, 1966), Oocystis sp. showed no growth in NH_4Cl. None of the three zygnematalean isolates tested was able to utilize NH_4Cl. On the other hand, all of the isolates belonging to the Chrysophyta were able to utilize NH_4Cl; however, the species of Botrydium grew to different degrees in it.

Isolates in TBIMV supplied with NH_4Cl showed a shift in pH toward the acid side of neutrality, as would be expected, inasmuch as NH_4^+ probably permeates the cell membrane as NH_4OH (Syrett, 1962). Nitrate is usually absorbed as HNO_3; therefore, the pH of the medium tends toward the alkaline side of neutrality. However, the shifts noted with NO_3^- are usually of lesser magnitude than with NH_4^+; such was the case in the present investigation (See Table 9). TRIS minimized these shifts greatly; the much greater shifts in pH reported by other workers using less satisfactorily buffered media (e.g., Cain, 1965) were not observed here.

Morphological responses to ammonia were minimal. Species of the Volvocales, however, showed decreased motility in NH_4Cl (in contrast to KNO_3) and, in some, Palmella stages were more frequent. Many investigators

have reported size increases with NH_4Cl (See Smith and Bold, 1966). The present investigation elicited some increased sizes (Table 10), but most cells remained unchanged. Botrydium becherianum was the only isolate which showed a definite size decrease in NH_4Cl (Figs. 6,7). Neochloris pseudoalveolaris (Figs. 8,9), Protosiphon botryoides, and Botrydiopsis intercedens all exhibited size increases (Table 10). Besides the isolates shown in Table 10, Chlorella pyrenoidosa and Pleurococcus sp. showed size increases; however, since data for calculations were not available, they are not included in Table 10.

Thirty-six of the 47 isolates tested used NH_4NO_3 equally as well as KNO_3. All of those capable of using KNO_3 and NH_4Cl alone were, predictably, also able to utilize NH_4NO_3. Both isolates of the Chlamydomonadaceae which were unable to utilize NH_4^+, i.e., Chlamydomonas radiata and Carteria sp., utilized NH_4NO_3 only half as well as KNO_3 by itself; since the concentration of nitrate in NH_4NO_3 is only one-half that in KNO_3, the reaction in growth noted in these two isolates in NH_4NO_3 was expected. The value of pH verified that these organisms were using NO_3^- instead of NH_4^+. Of the three species of the Zygnematales tested, two, Zygnema circumcarinatum + and - strains, also utilized NH_4NO_3, thus indicating possible NH_4^+ toxicity. Of the chlorococcalean algae, Oocystis sp. also showed no growth in either NH_4Cl or NH_4NO_3. In practically all cases in which either NH_4^+ or NO_3^- could be utilized when supplied

TABLE 10. Mean size differences of cells (in microns) and standard error, for several organisms grown for 6 weeks in TBIMV containing KNO_3 or NH_4Cl.

Organism	KNO_3	NH_4Cl
Neochloris pseudoalveolaris	11.1 ± 0.38	17.2 ± 1.2
Protosiphon botryoides	12.8 ± 1.2	14.5 ± 0.9
Botrydiopsis intercedens	8.1 ± 0.45	11.0 ± 0.6
Botrydium becherianum	14.0 ± 1.2	11.0 ± 1.1

separately, the pH shifts after growth in NH_4NO_3 indicated that NH_4^+ was preferentially absorbed. Carteria crucifera was the only species whose pH shift indicated that it was definitely preferentially absorbing NO_3^-. Carteria eugametos and Carteria sp. had pH values only slightly above the original pH; this indicates either a preference for NO_3^- or at least not a preference for NH_4^+. It appears taxonomically significant that all species of Carteria tested did not exhibit a preference for NH_4^+. Pandorina morum, Haematococcus lacustris, Stephanosphaera pluvialis, and Chlorosarcinopsis gelatinosa did not show such preferential selection, but rather preferred NO_3^-. Species of Haematococcus and Pandorina have been shown to utilize NO_3^- preferentially (Proctor, 1957).

Hydroxylamine has received very little attention as a possible nitrogen source for algae. Some reasons for this are probably due to reports by some authors (e.g., Ludwig, 1938; Syrett, 1962) that hydroxylamine is toxic; reports have also indicated that hydroxylamine appears to react with some organic compounds leading to their destruction by oxidation (Kessler, 1964). It is also questionable whether this compound exists in living organisms (Kessler, 1964). It has not been detected in algal cultures growing on nitrate (Syrett, 1962). Only one report of utilization of this compound is known to the writer; Hattori (1962) found that Anabaena cylindrica, a N-fixer, could assimilate

hydroxylamine-N. Nonetheless it was of interest to investigate the possibility that hydroxylamine might be used as a nitrogen source by at least some of the several different organisms being considered in the present investigation.

The results of this experiment (see Table 8) proved very interesting, both physiologically and taxonomically. Most organisms showed no growth whatsoever in the presence of this compound; the tubes were completely colorless. Carteria crucifera and C. eugametos showed traces of growth; that there were a few green cells in the bottom of the tubes indicates that the compound was at least not toxic to these isolates. Upon microscopic examination, apparently normal motile cells were present. Chlorosarcina longispinosa, C. brevispinosa, and Chlorosarcinopsis eremi registered fair growth in hydroxylamine and the cells appeared normal upon microscopic examination. Chlorosarcinopsis gelatinosa showed only a trace amount of growth. The taxonomic significance lies in the fact that all four chlorosarcinalean organisms tested were able to show signs of growth in hydroxylamine. Hormidium flaccidum, Ulothrix fimbriata, and Pleurococcus sp., all presumably ulotrichalean algae, also showed signs of growth in hydroxylamine; cells appeared normal upon microscopic examination except Hormidium, which appeared nitrogen-starved. Pleurococcus grew as well in NH_2OH as it did in NH_4Cl or KNO_3. Oocystis marssonii behaved similarly. The above observations constitute, aside from those of Hattori (1962)

regarding Anabaena cylindrica, the first report known to the writer that hydroxylamine is non-toxic to some organisms, and that several are, in fact, able to utilize it as a sole source of nitrogen. This compound appears to deserve more attention as a possible nitrogen source for the algae and, therefore, as an aid to the identification and classification of the algae.

Urea and Intermediates in Urea Metabolism

Urea has been reported to serve as a sole source of nitrogen for a wide variety of algae belonging to several classes (See references below). Urea is often considered superior to both NO_3^- and NH_4^+, since pH changes are minimal and the compound can be used in relatively high concentrations without harm to many organisms (Davis, 1953). The exact mechanism of urea utilization in the algae is not fully understood; however, a number of possible pathways have been suggested (Arnow, et al., 1953; Hattori, 1958, 1960). The first proposed theory was the direct breakdown of urea by urease into CO_2 and NH_3 (Allison, et al., 1954; Bollard, 1959); however, since urease activity has never been observed in the algae, it appears that urea is being incorporated without prior breakdown to NH_3. Another possible pathway was, therefore, proposed, wherein urea was thought to combine with ornithine to form arginine (Walker, 1952; Arnow, et al., 1953; Hattori, 1958; Bollard, 1959). Walker (1952) and Bollard (1959) have suggested

that ornithine might be regenerated from arginine by a reversal of the urea cycle. The direct incorporation of urea into guanine has also been proposed (see Syrett, 1962). Hattori (1960) has proposed that urea is split into CO_2 and an amide group which is immediately attached to an acceptor molecule. Despite the numerous suggestions above, the precise mechanism of urea utilization in algae remains obscure.

Reports of chlorophycean algae with the ability to utilize urea are numerous (Ludwig, 1938; Arnow, et al., 1953; Droop, 1955; Kratz and Myers, 1955; Birdsey and Lynch, 1962; Cain, 1965; Smith and Bold, 1966). Similar reports for algae belonging to the Chrysophyta (Belcher and Fogg, 1958; Miller and Fogg, 1958) and to the Cyanophyta (Kratz and Myers, 1955) have also been made, although these are fewer.

Reports of non-utilization of this compound by algae are much more limited than those of utilization. The papers by Cain (1963) and Smith and Bold (1966) are the only reports known to the author of non-utilization of urea by algae belonging to the Chlorophyta. Reports of algae of the Cyanophyta lacking the ability have also been made (Allen, 1952; Kratz and Myers, 1955).

It was of interest in this investigation to extend those reports of algae with or without the ability to utilize urea and also to test the isolates' ability to use compounds of the proposed "urea cycle," It was of further

interest to verify the reports that relatively high concentrations of urea exhibit, in general, no harm to most organisms (Davis, et al., 1953).

A summary of the results obtained when nitrogen was supplied as urea, citrulline, ornithine, and arginine is given in Table 11.

Twenty-one of the 47 isolates tested were able to use urea as well as KNO_3; 19 could not use urea or showed only trace amounts of growth. Five isolates used urea as well as KNO_3 and 2 used it only slightly. Anacystis nidulans, the only blue-green alga tested, did not use urea; this represents a confirmation of a previous report by Kratz and Myers (1955). Similarly, the non-utilization reported here for the several species of Chlamydomonas and Carteria verifies the work of Cain (1965). Neither of the haematococcacean species tested, Haematococcus lacustris or Stephanosphaera pluvialis, were able to utilize urea; this is the only report known to the author of non-utilization of urea in this group. It will be recalled that these organisms grew only slightly in KNO_3; however, since there was absolutely no growth in urea, these reports of non-utilization of urea seem justified. Of the chlorosarcinalean algae tested, only Chlorosarcina longispinosa was not able to utilize urea; this confirms an earlier report by Groover and Bold (1969). Only Neochloris pseudoalveolaris of the chlorococcalean algae tested did not use urea; this is the first report known to the author

TABLE 11. Growth and pH changes elicited by 47 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with urea, ornithine, citrulline, and arginine.[a]

Organism	KNO_3[b]	Urea	Orn.	Cit.	Arg.
Anacystis nidulans	4 (7.6)	0 (7.4)	0 (7.3)	0 (7.4)	3 (7.6)
Chlamydomonas reinhardtii (-)	4 (7.7)	4 (7.4)	5 (7.4)	5 (7.4)	3 (7.4)
C. reinhardtii (+)	4 (7.7)	4 (7.3)	4 (7.4)	4 (7.4)	3 (7.5)
C. actinochloris	4 (7.7)	0 (7.4)	½ (7.5)	½ (7.6)	3 (7.5)
C. chlamydogama (16-1)	4 (7.6)	0 (7.4)	½ (7.5)	½ (7.5)	2 (7.4)
C. chlamydogama (16-2)	4 (7.8)	0 (7.4)	0 (7.3)	0 (7.3)	2 (7.4)
C. eugametos (male)	4 (7.8)	0 (7.4)	½ (7.3)	½ (7.3)	0 (7.5)
C. eugametos (female)	4 (7.8)	0 (7.5)	0 (7.4)	0 (7.4)	0 (7.5)
C. radiata	4 (7.8)	4 (7.5)	---[c]	---	0 (7.5)
Carteria crucifera	4 (8.0)	1 (7.4)	0 (7.4)	0 (7.5)	0 (7.5)
C. eugametos	4 (7.8)	0 (7.4)	4 (7.3)	0 (7.5)	4 (7.5)
C. sp.	4 (7.6)	0 (7.4)	0 (7.4)	0 (7.4)	0 (7.5)
Gonium pectorale	4 (7.7)	4 (7.4)	4 (7.3)	0 (7.5)	0 (7.7)
Pandorina morum	4 (7.5)	1 (7.4)	4 (7.4)	1 (7.4)	0 (7.7)
Haematococcus lacustris	4 (7.5)	0 (7.4)	---	---	---
Stephanosphaera pluvialis	4 (7.5)	0 (7.4)	---	---	---
Hormotila blennista	4 (7.7)	3 (7.4)	2 (7.1)	1 (7.5)	2 (7.4)
Friedmannia israelensis	4 (7.5)	4 (7.5)	---	---	---
Chlorosarcina longispinosa	4 (7.7)	0 (7.4)	0 (7.4)	0 (7.5)	0 (7.4)
C. brevispinosa	4 (7.6)	4 (7.4)	0 (7.5)	0 (7.5)	0 (7.6)
C. stigmatica	4 (7.8)	2 (7.5)	2 (7.3)	2 (7.5)	0 (7.6)
Tetracystis excentrica	4 (7.7)	4 (7.5)	---	---	0 (7.5)
Chlorosarcinopsis eremi	4 (7.9)	4 (7.4)	0 (7.4)	0 (7.4)	---
C. gelatinosa	4 (7.6)	4 (7.4)	0 (7.4)	0 (7.5)	---
Chlorococcum hypnosporum	4 (7.8)	2 (7.4)	---	---	2 (7.3)
C. echinozygotum	4 (7.7)	3 (7.3)	---	---	0 (7.4)

TABLE 11. (Continued)

Organism	KNO_3	Urea	Orn.	Cit.	Arg.
Neochloris pseudoalveolaris	4 (7.5)	0 (7.4)	---	---	---
Protosiphon botryoides	4 (7.6)	4 (7.4)	0 (7.4)	0 (7.5)	1 (7.5)
Chlorella vulgaris	4 (7.7)	4 (7.5)	---	---	4 (7.5)
C. pyrenoidosa	4 (7.6)	4 (7.4)	---	---	4 (7.4)
Oocystis apiculata	4 (7.5)	2 (7.3)	4 (7.4)	4 (7.4)	0 (7.5)
O. marssonii	4 (7.5)	3 (7.3)	6 (7.4)	8 (7.7)	4 (7.4)
O sp.	4 (7.5)	0 (7.3)	4 (7.4)	3 (7.5)	0 (7.3)
Stichococcus mirabilis	4 (7.5)	3 (7.3)	3 (7.4)	4 (7.4)	4 (7.4)
Hormidium flaccidum	4 (7.5)	4 (7.4)	0 (7.3)	0 (7.3)	4 (7.6)
Ulothrix fimbriata	4 (7.5)	4 (7.3)	0 (7.3)	1 (7.3)	3 (7.6)
Pleurococcus sp.	4 (7.5)	3 (7.3)	6 (7.2)	6 (7.3)	4 (7.4)
Zygnema circumcarinatum (+)	4 (7.5)	0 (7.3)	---	---	0 (7.5)
Z. circumcarinatum (-)	4 (7.5)	0 (7.3)	---	---	0 (7.6)
Cosmarium botrytis	4 (7.3)	0 (7.2)	---	---	0 (7.4)
Botrydiopsis arhiza	4 (7.5)	2 (7.3)	0 (7.3)	0 (7.3)	0 (7.4)
B. alpina	4 (7.3)	2 (7.3)	1 (7.4)	2 (7.4)	2 (7.5)
B. intercedens	4 (7.5)	2 (7.4)	1 (7.4)	1 (7.4)	2 (7.5)
Tribonema aequale	4 (7.7)	4 (7.4)	2 (7.4)	2 (7.4)	2 (7.4)
Botrydium becherianum	4 (7.6)	0 (7.2)	0 (7.4)	0 (7.4)	0 (7.5)
B. stoloniferum	4 (7.7)	½ (7.3)	0 (7.4)	0 (7.4)	0 (7.5)
B. cystosum	4 (7.6)	½ (7.3)	0 (7.4)	0 (7.4)	0 (7.5)

[a] Concentration of nitrogen 2.0 mM in each case. Figures in parentheses indicate final pH; the initial pH was 7.4 except KNO_3, which was 7.3.

[b] TBIMV control = arbitrary 4

[c] Not tested

for this species of Neochloris. However, Neochloris cohaerus has been reported by Groover and Bold (1969) to utilize urea. Further work with the several species of this genus might elicit taxonomically valuable patterns of response to urea. None of the zygnematalean algae tested was able to utilize urea; this is apparently the first report known to the author of non-utilization of urea by this group. More isolates in this group need to be tested to determine other possible non-utilizers of urea. The three Botrydium species tested of the Chrysophyta all lacked the ability to use urea. This again appears to be a first report. Of interest here was that all other members of the Chrysophyta tested were able to utilize urea. All members of the Ulotrichales tested were able to utilize urea; this is the only report known to the author of this group being tested for urea utilization. Mattox and Bold (1962) pointed out a number of supplementary attributes used in the taxonomy of ulotrichalean algae but did not cite urea utilization.

The differences in physiological response toward urea reported here may have great taxonomic value. More systematic investigation of related taxa to determine their behavior toward urea as a sole nitrogen source could lead to definite patterns useful as extramorphological criteria in the identification and classification of the algae.

The pH values for the isolates grown in urea nitrogen are minimal, as was expected. Table 12 summarizes the pH changes when nitrogen was supplied to the isolates

TABLE 12. Summary of pH changes in cultures of 36 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with KNO_3, urea, ornithine, citrulline, and arginine.[a]

	KNO_3	Urea	Orn.	Cit.	Arg.
Range of pH at end of growth period	7.3 to 8.0	7.2 to 7.5	7.1 to 7.5	7.3 to 7.7	7.3 to 7.6
Amount of shift in pH units, at end of growth period	0.0 to +0.7	-0.2 to +0.1	-0.3 to +0.1	-0.1 to +0.3	-0.1 to +0.2
Average final pH	7.6	7.4	7.4	7.4	7.5
Average shift in pH units	+0.3	-0.02	-0.05	+0.01	+0.05

[a] The initial pH was 7.4 except for KNO_3, which was 7.3.

as nitrate, urea, ornithine, citrulline, and arginine. The pH change in urea is almost negligible when compared to KNO_3 or NH_4Cl. This small change is one reason that urea is considered a good growth medium since it does not allow large pH shifts which may lead to culture senescence before the nutrients become deficient.

Except for some differences in size, few significant morphological changes were noted with growth in urea. Botrydium stoloniferium (Figs. 10,11) and B. cystosum, which both grew in only trace amounts in urea, showed increased cell size. Cells of B. cystosum had a mean size of 15.19 μ in urea but only 12.24 μ in KNO_3. B. stoloniferium showed an even greater size increase; cells of this species grown in urea were approximately three times as large as those grown in nitrate. It is apparent that although urea does not support significant cell division in these organisms, the compound is nonetheless functional to the extent that the cells are living, larger, and show no apparent symptoms of nitrogen starvation. Chlamydomonas reinhardtii and Tetracystis excentrica also showed slight size changes. Changes in pH, which may lead to size differences by changing the permeation of particular ions, thus affecting growth, are apparently not the cause of the cell enlargement here since pH changes were practically negligible. An explanation for the increase in size is, therefore, obscure.

Tribonema aequale showed an interesting morphological response to urea. When filaments growing in urea were agitated slightly, they immediately broke up into filaments of only a few cells each; this was especially true at higher concentrations. Filaments in KNO_3, on the other hand, did not break, even after forceful agitation for long periods of time. Likewise, filaments of Ulothrix fimbriata in urea were also only one or two cells long when examined microscopically; filaments in KNO_3 did not break up.

Ornithine was utilized by 15 out of the 34 isolates tested. Two isolates used it slightly and 3 used it half as well as KNO_3; seven isolates used it as well as nitrate; three isolates, Pleurococcus sp., Oocystis marssonii, and Chlamydomonas reinhardtii (-), used it slightly better than nitrate. Of interest here is the fact that these same three isolates also used citrulline better than nitrate and they all could use arginine and urea. In fact, all isolates responded the same way to citrulline as they did to ornithine except for Carteria eugametos and Gonium pectorale, which did not utilize citrulline but did use ornithine. Pandorina morum used ornithine but used citrulline only slightly. The changes in pH in these two media were practically negligible as they were in urea and arginine (see Table 12).

Due to contamination of the arginine medium in one experiment, which led to inconsistent results, arginine utilization for many of the isolates was not reported. If they could have been reported, the number of isolates using

arginine would probably be considerably higher; however, due to the contamination, one would not know whether the organisms were utilizing arginine or some other compound derived from arginine by the contaminants. A number of the isolates shown responded to arginine as they did to ornithine and citrulline. Chlamydomonas actinochloris, C. chlamydogama (16-1), and C. chlamydogama (16-2), which apparently did not use ornithine, citrulline, or urea, could utilize arginine. Carteria eugametos was able to use ornithine and arginine but not citrulline; Chlorosarcina stigmatica could not use arginine but it could ornithine and citrulline.

Because of the relatively small number of isolates tested in each medium, significant patterns of response to urea, ornithine, citrulline, and arginine were impossible to derive. Many more isolates need to be tested to see if definite patterns do, in fact, exist in the assmiliation of urea and the intermediates of urea metabolism.

Concentrations of arginine as high as 40 mM did not affect the growth or morphology of the isolates except where filaments of Tribonema were, as in urea, again easily fragmented in tubes containing higher concentrations. Some other morphological changes were observed, such as in size, cell shape, and motility, but were inconsistent in that they did not appear in duplicate experiments or, in some cases, were even reversed. This lack of uniformity from experiment to experiment was observed with some of the others as well.

It seems possible that this lack of uniformity might be explained on the basis that the nitrogen sources used are in some way affecting the time of events of the growth cycle. Chlorococcum will be used as a hypothetical example of this point. If medium A speeded up the growth cycle somewhat in relationship to medium B, so that cells of Chlorococcum in medium A had just produced zoospores and cells of Chlorococcum in medium B were only beginning zoosporogenesis, it would appear on the particular day of observation that cells of Chlorococcum in medium A were smaller than those in medium B. However, if the same cultures were observed on some other day, the conditions might be reversed. Therefore, when size increases, loss of motility, and differences in cell shapes are reported, one must keep in mind that these may only be manifestations of the time of observation. In order to really understand the morphological changes that take place, one needs to observe the cultures periodically and not just once or twice.

Amino Acids

Several amino acids have been reported to serve as sole sources of nitrogen for the growth of several algae (e.g., Ludwig, 1938; Algeus, 1948, 1950; Miller and Fogg, 1958; Cain, 1965; Smith and Bold, 1966; and others). Glycine appears to be the most readily assimilated amino acid and an excellent nitrogen source for some algae (Belcher and Fogg, 1958). Asparagine and glutamine have also been reported

to serve as a sole source of nitrogen by many algae (Miller and Fogg, 1958; Belmont, 1965).

It was of interest to test the diverse assemblage of algae chosen for this investigation for their ability to utilize a variety of amino acids as sole sources of nitrogen. The amino acids to be tested were chosen by selecting one or two amino acids from each category as defined by Harrow and Mazur (1967). Table 13 summarizes the amino acids used, their chemical formulas, and classification. It should be pointed out that alanine was the monocarboxylic acid chosen for investigation and aspartic acid and glutamic acid were chosen as the acidic or dicarboxylic amino acids, but due to contamination, which was not detected until the end of the 6-week incubation period, results of their growth are invalid and, therefore, will not be reported.

Tables 14 and 15 summarize the results of growth of the isolates when nitrogen was supplied as one of the seven amino acids. Table 16 summarizes the results of pH changes observed in the media after 6 weeks' incubation of the isolates in each amino acid tested. For convenience, the utilization by the isolates of the seven amino acids will be discussed according to their classification as set forth in Table 13.

Aromatic Amino Acids

Phenylalanine did not serve as a good source of nitrogen for most of the isolates. Thirty of the 34

TABLE 13. Nitrogen compounds used as possible sources for 50 isolates.

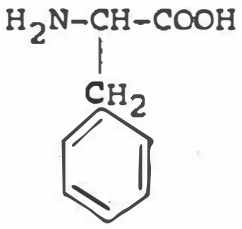
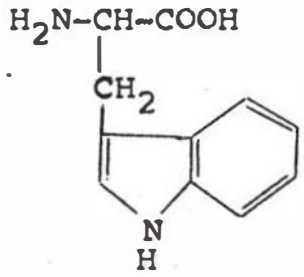
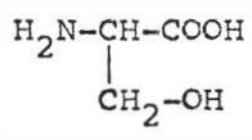
Compound	Formula
I. INORGANIC COMPOUNDS	
Ammonium chloride	NH_4Cl
Ammonium nitrate	NH_4NO_3
Potassium nitrate	KNO_3
Hydroxylamine	NH_2OH
II. ORGANIC COMPOUNDS	
A. Amino acids[a]	
1. Aliphatic monoamino-monocarboxylic acid[b]	
2. Aromatic amino acid	
Phenylalanine (β-phenyl-α-aminopropionic acid)	
Tryptophan (α-amino-β-3-indolpropionic acid)	
3. Hydroxyamino acid	
Serine (β-hydroxy-α-aminopropionic acid)	

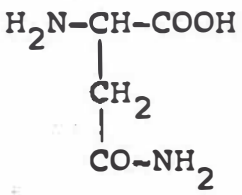
TABLE 13. (Continued)

Compound	Formula

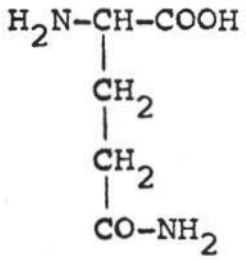
4. Acidic or dicarboxylic acids[c]

5. Acid amide amino acids

Asparagine (aspartic acid amide)

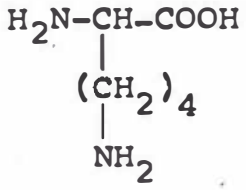


Glutamine (glutamic acid amide)

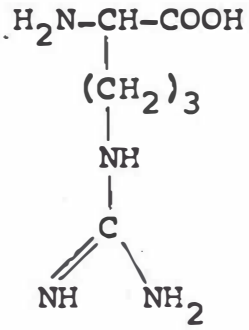


6. Basic amino acids

Lysine (α,ε-diaminocaproic acid)



Arginine (δ-guanidyl-α-aminovaleric acid)



7. Sulfur-containing amino acids

Cystine di-(β-thio-α-aminocaproic acid)

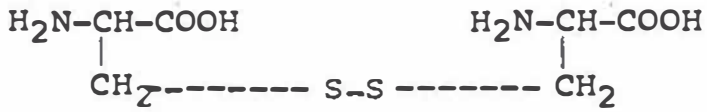


TABLE 13. (Continued)

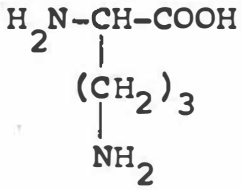
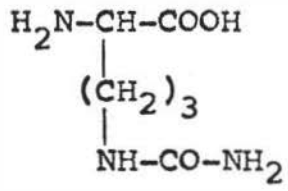
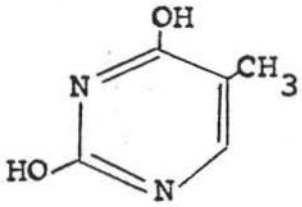
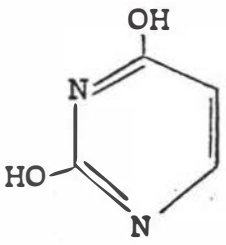
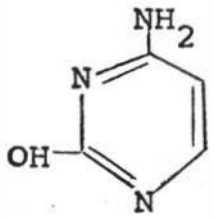
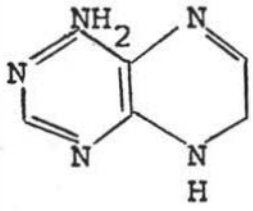
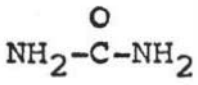
Compound	Formula
8. Secondary amino acids[d]	
B. Intermediates in urea biosynthesis	
Ornithine	
Citrulline	
Arginine[e]	
C. Nitrogenous bases (purines and pyrimidines)	
Thymine	
Uracil (2,6-dioxypyrimidine)	

TABLE 13. (Continued)

Compound	Formula
Cytosine (2-oxy-6-aminopyrimidine)	
Adenine (6-aminopurine)	
D. Urea (Carbamide)	

[a]Classification of these compounds is based upon the system of Harrow and Mazur (1967).

[b]Alanine was chosen as a nitrogen source but, due to contamination of the medium during preparation, it was dropped from the investigation.

[c]Aspartic acid and glutamic acid were chosen but dropped from the investigation due to contamination of the media during preparation.

[d]No amino acids from this group were used.

[e]This amino acid is also presumably involved in urea metabolism in algae.

TABLE 14. Growth and pH changes elicited by 34 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with KNO_3, phenylalanine, tryptophan, and serine.[a]

Organism	KNO_3[b]	Phenylalanine	Tryptophan	Serine
Anacystis nidulans	4 (7.6)	0 (7.5)	2 (7.0)	0 (7.6)
Chlamydomonas reinhardtii (-)	4 (7.7)	1 (7.5)	3 (7.5)	1 (7.7)
C. reinhardtii (+)	4 (7.7)	0 (7.6)	0 (7.5)	0 (7.8)
C. actinochloris	4 (7.7)	0 (7.7)	0 (7.6)	0 (7.9)
C. chlamydogama (16-1)	4 (7.6)	0 (7.6)	0 (7.1)	0 (7.6)
C. chlamydogama (16-2)	4 (7.8)	0 (7.4)	0 (7.0)	0 (7.4)
C. eugametos (male)	4 (7.8)	0 (7.5)	0 (6.9)	0 (7.5)
C. eugametos (female)	4 (7.8)	0 (7.5)	0 (6.9)	0 (7.5)
Carteria crucifera	4 (8.0)	0 (7.5)	0 (7.0)	0 (7.6)
C. eugametos	4 (7.8)	0 (7.6)	0 (7.0)	0 (7.6)
C. sp.	4 (7.6)	0 (7.5)	0 (7.0)	0 (7.6)
Gonium pectorale	4 (7.7)	0 (7.5)	0 (7.0)	0 (7.6)
Pandorina morum	4 (7.5)	0 (7.6)	0 (7.5)	1 (7.7)
Hormotila blennista	4 (7.7)	0 (7.6)	1 (7.0)	0 (7.6)
Chlorosarcina longispinosa	4 (7.7)	0 (7.6)	0 (7.1)	0 (7.6)
C. brevispinosa	4 (7.6)	0 (7.6)	0 (7.0)	0 (7.5)
C. stigmatica	4 (7.8)	2 (7.5)	2 (7.0)	2 (7.5)
Chlorosarcinopsis eremi	4 (7.9)	0 (7.5)	0 (7.0)	0 (7.5)
C. gelatinosa	4 (7.6)	0 (7.5)	0 (7.0)	0 (7.5)
Protosiphon botryoides	4 (7.4)	0 (7.6)	---[c]	0 (7.6)
Oocystis apiculata	4 (7.5)	0 (7.5)	0 (7.0)	0 (7.5)
O. marssonii	4 (7.5)	0 (7.5)	1 (7.0)	0 (7.7)
O. sp.	4 (7.5)	0 (7.5)	1 (7.0)	0 (7.6)

TABLE 14. (Continued)

Organism	KNO_3	Phenylalanine	Tryptophan	Serine
Stichococcus mirabilis	4 (7.5)	0 (7.5)	1 (7.0)	1 (7.6)
Hormidium flaccidum	4 (7.5)	0 (7.4)	2 (6.9)	0 (7.4)
Ulothrix fimbriata	4 (7.5)	0 (7.4)	2 (6.9)	0 (7.5)
Pleurococcus sp.	4 (7.5)	4 (7.3)	2 (6.8)	4 (7.4)
Botrydiopsis arhiza	4 (7.5)	0 (7.2)	0 (6.9)	0 (7.5)
B. alpina	4 (7.3)	0 (7.5)	0 (7.0)	1 (7.5)
B. intercedens	4 (7.5)	0 (7.5)	0 (7.0)	1 (7.5)
Tribonema aequale	4 (7.7)	1 (7.5)	0 (7.0)	1 (7.5)
Botrydium becherianum	4 (7.6)	0 (7.5)	0 (7.2)	0 (7.5)
B. stoloniferum	4 (7.7)	0 (7.5)	0 (7.0)	0 (7.6)
B. cystosum	4 (7.6)	0 (7.5)	0 (7.0)	0 (7.5)

[a]Concentration of nitrogen 2.0 mM in each case. Figures in parentheses indicate final pH; the initial pH was 7.4 except KNO_3, which was 7.3.

[b]TBIMV control = arbitrary 4

[c]Not tested

TABLE 15. Growth and pH changes elicited by 34 axenic isolates after 6 weeks' incubation in TBIMV-N[a] supplemented with KNO_3, asparagine, glutamine, lysine, and cystine.

Organism	KNO_3[b]	Asparagine	Glutamine	Lysine	Cystine
Anacystis nidulans	4 (7.6)	0 (7.4)	5 (7.0)	1 (7.5)	3 (7.5)
Chlamydomonas reinhardtii (-)	4 (7.7)	4 (7.3)	0 (7.1)	3 (7.4)	1 (7.5)
C. reinhardtii (+)	4 (7.7)	2 (7.3)	0 (7.1)	1 (7.6)	0 (7.6)
C. actinochloris	4 (7.7)	3 (7.2)	0 (7.7)	0 (7.7)	1 (7.4)
C. chlamydogama (16-1)	4 (7.6)	0 (7.4)	0 (7.6)	0 (7.7)	0 (7.6)
C. chlamydogama (16-2)	4 (7.8)	0 (7.5)	2 (6.9)	0 (7.4)	0 (7.4)
C. eugametos (male)	4 (7.8)	3 (7.2)	0 (6.9)	0 (7.6)	0 (7.4)
C. eugametos (female)	4 (7.8)	3 (7.3)	0 (6.9)	0 (7.5)	0 (7.4)
Carteria crucifera	4 (8.0)	0 (7.4)	2 (7.0)	0 (7.6)	0 (7.5)
C. eugametos	4 (7.8)	0 (7.4)	2 (7.0)	0 (7.5)	0 (7.4)
C. sp.	4 (7.6)	0 (7.4)	0 (7.1)	0 (7.5)	0 (7.4)
Gonium pectorale	4 (7.7)	0 (7.4)	1 (7.0)	0 (7.5)	0 (7.5)
Pandorina morum	4 (7.5)	1 (7.4)	1 (7.0)	0 (7.5)	0 (7.5)
Hormotila blennista	4 (7.7)	0 (7.4)	1 (7.1)	2 (7.5)	1 (7.4)
Chlorosarcina longispinosa	4 (7.7)	0 (7.4)	1 (7.0)	0 (7.6)	1 (7.5)
C. brevispinosa	4 (7.6)	0 (7.6)	1 (7.0)	0 (7.6)	1 (7.5)
C. stigmatica	4 (7.8)	1 (7.5)	2 (7.0)	2 (7.5)	2 (7.5)
Chlorosarcinopsis eremi	4 (7.9)	0 (7.5)	3 (7.0)	0 (7.5)	0 (7.4)
C. gelatinosa	4 (7.6)	0 (7.4)	1 (7.0)	0 (7.6)	0 (7.5)
Protosiphon botryoides	4 (7.4)	0 (7.5)	0 (7.0)	0 (7.6)	3 (7.6)
Oocystis apiculata	4 (7.5)	1 (7.2)	0 (7.0)	4 (7.5)	4 (7.4)
O. marssonii	4 (7.5)	5 (7.5)	0 (7.0)	0 (7.5)	6 (7.4)
O. sp.	4 (7.5)	0 (6.1)	3 (7.0)	0 (7.5)	2 (7.4)

TABLE 15. (Continued)

Organism	KNO_3	Asparagine	Glutamine	Lysine	Cystine
Stichococcus mirabilis	4 (7.5)	2 (7.2)	6 (7.0)	0 (7.5)	1 (7.5)
Hormidium flaccidum	4 (7.5)	4 (7.3)	2 (6.9)	0 (7.4)	0 (7.4)
Ulothrix fimbriata	4 (7.5)	4 (7.4)	1 (6.9)	0 (7.4)	3 (7.4)
Pleurococcus sp.	4 (7.5)	4 (7.3)	4 (6.9)	6 (7.3)	6 (7.3)
Botrydiopsis arhiza	4 (7.5)	0 (7.3)	0 (6.9)	0 (7.4)	0 (7.3)
B. alpina	4 (7.3)	3 (7.5)	2 (7.3)	1 (7.5)	3 (7.4)
B. intercedens	4 (7.5)	0 (7.3)	3 (7.0)	1 (7.5)	2 (7.5)
Tribonema aequale	4 (7.7)	1 (7.4)	0 (7.0)	2 (7.5)	3 (7.5)
Botrydium becherianum	4 (7.6)	0 (7.3)	1 (7.0)	0 (7.6)	0 (7.4)
B. stoloniferum	4 (7.7)	0 (7.3)	1 (7.0)	0 (7.5)	0 (7.4)
B. cystosum	4 (7.6)	0 (7.3)	1 (7.0)	0 (7.5)	0 (7.4)

[a]Concentration of nitrogen was 2.0 mM in each case. Figures in parentheses indicate final pH; the initial pH was 7.4 except KNO_3 and asparagine, which were 7.3.

[b]TBIMV control = arbitrary 4

TABLE 16. Summary of pH changes in cultures of 34 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with KNO_3, phenylalanine, tryptophan, serine, asparagine, glutamine, lysine, and cystine.[a]

	KNO_3	Phenyl-alanine	Trypto-phan	Serine	Aspara-gine	Gluta-mine	Lysine	Cystine
Range of pH at end of growth period	7.3 to 8.0	7.2 to 7.7	6.8 to 7.5	7.4 to 7.9	7.1 to 7.9	6.9 to 7.4	7.3 to 7.7	7.3 to 7.5
Amount of shift in pH units, at end of growth period	0.0 to +0.7	-0.2 to +0.3	-0.6 to +0.1	0.0 to +0.5	-0.2 to +0.3	-0.5 to 0.0	-0.1 to +0.3	-0.1 to +0.1
Average final pH	7.6	7.5	7.0	7.6	7.3	7.0	7.5	7.4
Average shift in pH units	+0.3	+0.08	-0.38	+0.16	+0.03	-0.4	+0.1	+0.02

[a] The initial pH was 7.4 except for KNO_3 and asparagine, which were 7.3.

isolates tested did not grow in it or did so only in trace amounts. Two isolates grew only slightly, 1 grew half as well as KNO_3, and only one, Pleurococcus sp., used it with the efficiency of KNO_3. Tryptophan, likewise, did not prove to be a good source of nitrogen for the isolates tested. Twenty-three of the 34 tested grew in only trace amounts or not at all. Four isolates grew slightly, 5 about half that of KNO_3, and only one, Chlamydomonas reinhardtii (-), grew as well as in KNO_3. Tryptophan, in general, proved to be better than phenylalanine as a nitrogen source. This could possibly be due to the fact that phenylalanine has only one nitrogen source (an α-amino group) whereas tryptophan has two (an α-amino group and β-3 indol group). Anacystis nidulans, the only alga of the Cyanophyta tested, showed no growth in phenylalanine but showed fair growth in tryptophan. Of the species tested belonging to the Chlamydomonadaceae, only Chlamydomonas reinhardtii (-) showed growth in phenylalanine and tryptophan. Neither species of the Volvocaceae grew in either medium. Hormotila blennista, the only tetrasporalean alga tested, showed limited growth only in tryptophan. Only Chlorosarcina stigmatica of the chlorosarcinalean algae tested showed growth in phenylalanine and tryptophan, but only Pleurococcus sp. grew in phenylalanine. None of the species of the Chrysophyta tested grew in tryptophan, and only Tribonema aequale showed growth in phenylalanine.

The pH shifts in phenylalanine and tryptophan were minimal (see Table 16). Phenylalanine tended slightly toward the acidic whereas tryptophan had a higher shift toward the basic side of neutrality.

Hydroxyamino Acids

Serine was chosen to be tested from this group of amino acids since it contains a propionic acid base as did phenylalanine and tryptophan. Twenty-six of the 34 isolates tested were unable to utilize serine. Six isolates used it slightly, one used it half as well as KNO_3, and one, Pleurococcus sp., used it with the efficiency of KNO_3. Serine appears to be only a slightly better nitrogen source than phenylalanine but not as good as tryptophan. The pH shift was minimal (see Table 16), tending slightly toward the alkaline side of neutrality. As in the phenylalanine and tryptophan media, Chlamydomonas reinhardtii (-) was the only chlamydomonadacean species able to utilize serine. Chlorosarcina stigmatica was the only chlorosarcinalean alga that utilized serine, just as it was the only one to use phenylalanine and tryptophan. Of the ulotrichalean algae tested, only Stichococcus mirabilis and Pleurococcus sp. were able to utilize serine; however, S. mirabilis used it only slightly.

Recognizable patterns of growth appear to exist among phenylalanine, tryptophan, and serine utilization in the algae tested (Table 17). Of the seven isolates exhibiting pattern type I, only one isolate showed growth

TABLE 17. Growth patterns of 14 isolates which showed growth in at least one of the following amino acids: phenylalanine, tryptophan, and serine.

	Growth patterns			Number of isolates
	Phenyl-alanine	Tryptophan	Serine	
I	-[a]	+	-	7
II	+	-	+	2
III	-	-	+	3
IV	-	+	+	1
V	+	+	+	1

[a]Represents either no growth or less growth than +.

in phenylalanine and serine; however, its growth was considerably less in comparison to tryptophan. A possible explanation for the utilization of tryptophan and non-utilization of serine or phenylalanine might be that these algae are unable to utilize the α-amino group present in serine and phenylalanine but are capable of utilizing the β-3 indol group present in tryptophan. Of the two isolates showing pattern type II, one grew in tryptophan but its growth was only half that exhibited by phenylalanine or serine. A possible explanation for this pattern type could be the inability of these algae to use the β-3 indol group of tryptophan; therefore, growth was only half as much since only half as much nitrogen was present in the α-amino group of tryptophan. The other isolate of pattern type II which showed no growth in tryptophan could be explained on the same basis, except in this case there just simply was not enough nitrogen present in the α-amino group for this organism to grow. Pattern types III and IV appear to favor serine over phenylalanine. Since these compounds differ only in the presence of a hydroxyl group in serine, this may have something to do with the α-amino group of this coumpound being utilized better than the α-amino group of phenylalanine. It should be pointed out, however, that the growth was limited in serine and, since this work only represents the original data and not subculture work, this pattern type might not appear as strong in the subculture data. Only one organism (Chlorosarcina

stigmatica) exhibited equal growth in all three amino acids. This is probably a case of equal utilization of the α-amino and β-3 indol group in this organism.

Several taxonomically interesting results were obtained. Of the Chlamydomonas species tested, only Chlamydomonas reinhardtii (-) showed growth in phenylalanine, tryptophan, and serine. Anacystis nidulans, the only blue-green alga tested, was able to utilize only tryptophan. None of the species of Carteria was able to utilize any of these 3 amino acids significantly. Of the chlorosarcinalean algae, only Chlorosarcina stigmatica was able to utilize any of the 3 amino acids and it used all three equally well. All the ulotrichalean genera used tryptophan, but only Pleurococcus sp. used phenylalanine. Pleurococcus sp. and Stichococcus mirabilis were the only ones to use serine. None of the species of Botrydium was able to use any of these amino acids. Of the Botrydiopsis species, only two, Botrydiopsis alpina and B. intercedens, were able to utilize serine, and they grew only slightly.

Acid Amide Amino Acids

Asparagine and glutamine were the two acid amide amino acids used in the investigation. Asparagine proved to be as good a source of nitrogen as KNO_3 for 9 of the 34 isolates tested; two responded half as well, four grew only slightly and 19 grew only a trace or not at all. These compounds proved to be much better sources of nitrogen for most of the isolates than either phenylalanine, tryptophan,

or serine; none was as good, however, as urea. There was apparently no correlation between those organisms using asparagine and those using glutamine. Some used both, some used neither, and some preferred one over the other. More isolates responded to glutamine (22 isolates) than to asparagine (15 isolates).

The changes in pH were again minimal in these media (see Table 16).

Some patterns of taxonomic value were gained from this experiment. Anacystis nidulans was unable to utilize asparagine but it grew excellently in glutamine. All of the Chlamydomonas species could use asparagine except C. chlamydogama (16-1 and 16-2); only C. chlamydogama (16-2) could apparently use glutamine with any great success. None of the Carteria species used asparagine; all but one used glutamine. All of the chlorosarcinalean algae apparently used glutamine to at least a slight extent while none except Chlorosarcina stigmatica used asparagine. The ulotrichalean species tested apparently all used to some extent both asparagine and glutamine. Only Botrydiopsis alpina of the Botrydiopsis species tested used asparagine; B. arhiza was the only one unable to utilize glutamine. Tribonema aequale utilized asparagine slightly but could not utilize glutamine. All the Botrydium species tested used glutamine slightly; however, none was able to utilize asparagine. It appears that

here again physiological tests might have value in taxonomic classification and identification (see later discussion).

It would be of interest to use higher concentrations of these compounds on the isolates since a great number of them showed only slight growth. Since these compounds both have two amino groups, it could be that only one is being utilized; therefore, the 2-mM concentration of nitrogen might not be sufficient for good growth.

Basic Amino Acids

Lysine and arginine were the two amino acids tested in this group. Arginine has been discussed under intermediate compounds in urea biosynthesis; therefore, a discussion of it will not be repeated here except in comparison with lysine.

Only 3 isolates of the 34 tested were able to use lysine as a sole nitrogen source; 3 used it half as well as on KNO_3, 4 used it only slightly, and 24 grew only in trace amounts or not at all. Lysine can be compared more closely to tryptophan in the number of isolates which could utilize it as a sole nitrogen source. Arginine is a much better source of nitrogen for most of the isolates than is lysine. This might possibly be due to the fact that arginine has two more available nitrogen atoms in its structure than does arginine.

Lysine cultures again, due to the great buffering capabilities of TRIS, showed very little change in pH (see Table 16).

Again, taxonomically valuable patterns are apparent. Of the Chlamydomonas species tested, only Chlamydomonas reinhardtii (both + and -) were able to utilize lysine. None of the Carteria species was able to utilize lysine. Chlorosarcina stigmatica was the only chlorosarcinalean species to use this compound; it is noteworthy that this same isolate was the only one of this group to use phenylalanine, tryptophan, and serine. Pleurococcus sp. showed growth in lysine, as it has for all of the amino acids tested. None of the Botrydium species used lysine, and of the Botrydiopsis species tested, only Botrydiopsis arhiza did not use it.

Sulfur-containing Amino Acids

Cystine was arbitrarily chosen as the amino acid to be used from this group. Eight isolates grew well in cystine, 3 half as well as in KNO_3, 6 grew only slightly, and 17 grew in only trace amounts or not at all. Therefore, it appears that cystine compares well with glutamine and asparagine as a fairly good nitrogen source for many of the isolates.

Changes in pH again were minimal in cystine cultures (see Table 16).

Of the volvocalean algae tested only Chlamydomonas reinhardtii (-) and C. actinochloris were able to utilize cystine; however, both utilized it only slightly. Of the chlorosarcinalean algae, all 3 species of Chlorosarcina used cystine slightly; however both species of

Chlorosarcinopsis tested were unable to do so at all. Protosiphon botryoides and the three species of Oocystis tested were able to utilize cystine. Of the ulotrichalean algae tested, only Hormidium flaccidum was unable to utilize cystine; Stichococcus mirabilis used it only slightly, Ulothrix fimbriata used it well, and Pleurococcus sp. used it better than KNO_3. Of the Chrysophyta tested, none of the Botrydium species was able to use cystine; only only Botrydiopsis arhiza of the Botrydiopsis species tested was unable to utilize cystine.

No gross morphological variations were found among the organisms utilizing any of the seven amino acids tested except for the size increase noted with Anacystis nidulans growing in tryptophan (Figs. 13, 14). It should be emphasized that, unfortunately, none of the isolates was subcultured in the 7 amino acids tested and, therefore, these data represent only first-run material.

Purines and Pyrimidines

Reports of purine and pyrimidine utilization by the algae are rare. Cain (1963) has reported a few Chlamydomonas species and Chlamydomonas-like species to use adenine and uric acid. He also reported that none of these were capable of using cytosine, uracil, or thymine as sole nitrogen sources. Birdsey and Lynch (1962) have reported on uric acid and xanthine utilization in a few representative species of

the Chlorophyta; Smith and Bold (1966) have reported uric acid utilization among some members of the Oocystaceae. It was thus of interst to test the ability of the axenic isolates to use these compounds.

In the investigation, one purine (adenine) and 3 pyrimidines (cytosine, thymine, and uracil) were used as sole sources of nitrogen for the isolates. Table 18 represents the growth patterns of those isolates tested in media containing these compounds.

Adenine was the best of these compounds for most of the isolates. Five isolates grew well in adenine, 7 grew half as well as in KNO_3, and 2 grew slightly. Of the Chlamydomonas species tested, only Chlamydomonas reinhardtii (-) and C. chlamydogama (16-2) grew in adenine. C. reinhardtii (-) grew only slightly. Both Carteria crucifera and C. eugametos grew fairly well in adenine; C. sp. was unable to utilize adenine. Again only Chlorosarcina stigmatica of the chlorosarcinalean algae possessed the ability to use adenine. Of the Oocystaceae tested, only Oocystis marssonii used adenine. All of the ulotrichalean algae tested could use adenine; Pleurococcus sp. did so exceptionally well. Of the organisms tested of the Chrysophyta, Botrydiopsis alpina, B. intercedens, Tribonema aequale, and Botrydium stoloniferum used adenine.

Cytosine was not as effective as adenine as a source of nitrogen for the isolates. Of significance is the fact that 8 of the 9 isolates which used cytosine also

TABLE 18. Growth and pH changes elicited by 34 axenic isolates after 6 weeks' incubation in TBIMV-N supplemented with KNO_3, adenine, cytosine, thymine, and uracil.[a]

Organism	KNO_3[b]	Adenine	Cytosine	Thymine	Uracil
Anacystis nidulans	4 (7.6)	0 (7.4)	0 (7.6)	0 (7.6)	0 (7.0)
Chlamydomonas reinhardtii (-)	4 (7.7)	1 (7.5)	1 (7.6)	1 (7.8)	1 (7.2)
C. reinhardtii (+)	4 (7.7)	0 (7.6)	0 (7.7)	1 (7.7)	0 (7.2)
C. actinochloris	4 (7.7)	0 (7.6)	0 (7.7)	0 (7.8)	0 (7.3)
C. chlamydogama (16-1)	4 (7.6)	0 (7.6)	0 (7.7)	0 (7.9)	0 (7.2)
C. chlamydogama (16-2)	4 (7.8)	3 (7.4)	0 (7.5)	0 (7.6)	0 (6.9)
C. eugametos (male)	4 (7.8)	0 (7.4)	0 (7.5)	0 (7.6)	0 (7.1)
C. eugametos (female)	4 (7.8)	0 (7.4)	0 (7.6)	0 (7.6)	0 (7.0)
Carteria crucifera	4 (8.0)	2 (7.5)	0 (7.5)	0 (7.6)	0 (7.0)
C. eugametos	4 (7.8)	2 (7.5)	0 (7.5)	0 (7.6)	0 (7.0)
C. sp.	4 (7.6)	0 (7.4)	0 (7.5)	0 (7.6)	0 (7.1)
Gonium pectorale	4 (7.7)	0 (7.4)	0 (7.6)	0 (7.6)	0 (7.1)
Pandorina morum	4 (7.5)	0 (7.4)	0 (7.7)	0 (7.6)	0 (7.1)
Hormotila blennista	4 (7.7)	0 (7.5)	0 (7.5)	0 (7.6)	0 (7.2)
Chlorosarcina longispinosa	4 (7.7)	0 (7.5)	0 (7.7)	0 (7.7)	0 (7.2)
C. brevispinosa	4 (7.6)	0 (7.5)	0 (7.6)	0 (7.7)	0 (7.2)
C. stigmatica	4 (7.8)	2 (7.5)	2 (7.6)	0 (7.6)	2 (7.2)
Chlorosarcinopsis eremi	4 (7.9)	0 (7.5)	0 (7.6)	0 (7.5)	0 (7.1)
C. gelatinosa	4 (7.6)	0 (7.4)	0 (7.6)	0 (7.5)	0 (7.1)
Protosiphon botryoides	4 (7.4)	0 (7.5)	0 (7.8)	0 (7.7)	0 (7.2)
Oocystis apiculata	4 (7.5)	0 (7.4)	4 (7.6)	4 (7.6)	0 (7.0)
O. marssonii	4 (7.5)	3 (7.5)	0 (7.6)	2 (7.5)	0 (7.1)
O. sp.	4 (7.5)	0 (7.4)	0 (7.5)	0 (7.6)	0 (7.1)

TABLE 18. (Continued)

Organism	KNO_3	Adenine	Cytosine	Thymine	Uracil
Stichococcus mirabilis	4 (7.5)	1 (7.5)	0 (7.6)	0 (7.6)	0 (7.1)
Hormidium flaccidum	4 (7.5)	3 (7.3)	0 (7.4)	0 (7.6)	0 (7.0)
Ulothrix fimbriata	4 (7.5)	2 (7.5)	1 (7.4)	1 (7.5)	3 (7.0)
Pleurococcus sp.	4 (7.5)	6 (7.3)	1 (7.4)	1 (7.5)	0 (7.0)
Botrydiopsis arhiza	4 (7.5)	0 (7.3)	0 (7.4)	0 (7.4)	0 (7.0)
B. alpina	4 (7.3)	4 (7.4)	3 (7.6)	0 (7.6)	1 (7.1)
B. intercedens	4 (7.5)	2 (7.5)	2 (7.6)	1 (7.6)	1 (7.1)
Tribonema aequale	4 (7.7)	2 (7.4)	3 (7.5)	0 (7.6)	1 (7.1)
Botrydium becherianum	4 (7.6)	0 (7.5)	0 (7.5)	0 (7.6)	0 (7.2)
B. stoloniferum	4 (7.7)	2 (7.4)	1 (7.6)	0 (7.6)	0 (7.1)
B. cystosum	4 (7.6)	0 (7.4)	0 (7.5)	0 (7.6)	0 (7.2)

[a]Concentration of nitrogen 2.0 mM in each case. Figures in parentheses indicate final pH; the initual pH was 7.4 except KNO_3, which was 7.3.

[b]TBIMV control = arbitrary 4

were able to use adenine. Only Oocystis apiculata, which used cytosine, did not grow in adenine. Of the ulotrichalean algae, only Ulothrix fimbriata and Pleurococcus sp. used cytosine, but only slightly; the others were unable to utilize it at all. All of the algae of the Chrysophyta tested responded the same way to cytosine as they did to adenine.

Thymine was only used by 7 of the isolates tested. Five used it slightly, one half as well as on KNO_3, and one as well as on KNO_3. This time both strains of Chlamydomonas reinhardtii responded in the same way; both grew slightly in thimine. None of the Carteria species, haematococcacean species, Protosiphon, or chlorosarcinalean algae were able to utilize thymine. Of the ulotrichalean algae, Ulothrix fimbriata and Pleurococcus sp. again used thymine slightly while the others were unable to utilize it at all. Of the algae belonging to the Chrysophyta, only Botrydiopsis intercedens was able to utilize thymine.

Uracil was used by about the same number of isolates as was thymine. All the isolates utilizing uracil even slightly were capable of using adenine. Only Chlamydomonas reinhardtii (+) of the Chlamydomonas species was able to utilize uracil. And, as in the case with cytosine and adenine, only Chlorosarcina stigmatica of the chlorosarcinalean algae was able to utilize uracil. Only Ulothrix fimbriata of the ulotrichalean algae was able to utilize uracil. Of the algae belonging to the Chrysophyta, only

Botrydiopsis alpina, B. intercedens, and Tribonema aequale were able to utilize uracil.

The change in pH values were again minimal in these media (see Table 16).

Gross morphological changes were not observed in any of the isolates growing on the purine and pyrimidine media, except for isolates growing on adenine. When observed microscopically, both Carteria crucifera and C. eugametos characteristically showed very dark green, almost black, cells in adenine. Botrydium stoloniferum increased almost two-fold in size in the adenine medium as it had also done in the urea medium (Figs. 10, 11, 12). The mean size in KNO_3 was 11.1 microns, while in adenine it was 18.8 microns. Why both adenine and urea caused this particular species to show a size increase is certainly not due to pH shifts, since they were minimal in these media. The answer to this morphological variation is, therefore, obscure.

Concluding Remarks

A major objective of this study was to investigate the response of the isolates to a number of inorganic and organic nitrogen sources. The individual responses of the isolates toward each source have been discussed under the appropriate headings in the foregoing discussion. Remarks here will be directed to a comparison of each medium as a sole source of nitrogen for all isolates. Table 19 summarizes the sources according to the number of isolates able to utilize them.

The number of isolates responding to TBIMV has been omitted from Table 19 since all isolates responded well to KNO_3 except Eudorina elegans, Trebouxia anticipata, and Eremosphaera viridis, which were dropped from the investigation (see earlier discussion on basal medium). The nitrogen sources are listed as follows, in decreasing order of utilization: KNO_3, NH_4Cl, urea, glutamine, arginine, ornithine, cystine, asparagine, citrulline, adenine, lysine, tryptophan, cytosine, hydroxylamine, serine, thymine, uracil, and phenylalanine.

Of the inorganic nitrogen sources tested, NO_3^- ranks highest followed by NH_4^+; hydroxylamine, although not a good nitrogen source, surprisingly was better than four of the organic sources tested. Urea and the intermediates of urea biosynthesis all ranked high among the media tested. Urea ranked highest, followed by arginine, ornithine, and citrulline. Of the amino acids tested, glutamine ranked

TABLE 19. Summary of the number of isolates capable of utilizing each of the nitrogen sources tested.

	Inorganic nitrogen		Urea and intermediates of urea biosynthesis				Amino acids							Nitrogenous bases			
	NH_4Cl	NH_2OH	Urea	Ornithine	Citrulline	Arginine	Phenylalanine	Tryptophan	Serine	Asparagine	Glutamine	Lysine	Cystine	Adenine	Cytosine	Thymine	Uracil
Good[a]	35	2	15	10	7	10	1	1	1	9	6	3	8	5	3	1	1
Fair	2	3	4	3	3	7	1	5	1	2	6	3	3	7	2	1	1
Slight	3	3	2	2	4	1	2	4	6	4	9	4	6	2	4	5	4
Total number of isolates exhibiting growth	40	8	21	15	14	18	4	10	8	15	21	10	17	14	9	7	6
Total number of isolates not exhibiting growth	7	26	13	19	20	16	30	24	26	19	13	24	17	20	25	27	28
Total isolates studied	47	34	34	34	34	34	34	34	34	34	34	34	34	34	34	34	34

[a] "Good" is equivalent to a growth reading of 3 or 4; "Fair" is equivalent to 2; "slight" is equivalent to 1.

highest, followed by cystine, asparagine, lysine, tryptophan, serine, and phenylalanine. Adenine ranked highest of the nitrogenous bases tested, followed by cytosine, thymine, and uracil.

The non-utilization of some of the inorganic and organic compounds by the algae investigated is probably due to a number of factors, one of which might be the permeation capabilities of the nitrogen sources. A given alga reported as unable to utilize a specific nitrogen source might in fact be metabolically capable of utilization; if, however, the compound does not enter the cell, it is reported as a non-utilizer. Investigations, both radiographic and chromatographic, need to be performed to determine this possibility.

A second major objective of this investigation was to examine patterns of urea utilization in an effort to elucidate the metabolic pathway of this compound. Urea, ornithine, citrulline, and arginine, when supplied separately to the isolates, did exhibit some patterns of response; however, these patterns did not indicate any suitable correlations to explain the metabolism of urea by the proposed "urea cycle." The writer feels that this methodology might still be of value, however, if data were recorded from isolates growing in media containing various combinations of these nitrogen sources. For example, it might be that isolate "X" will not use urea or ornithine separately, but in combination they might be utilized.

The third objective of this investigation was to elucidate patterns of response which might be of taxonomic significance. That such work is valuable for at least some algae has been shown, for example, by Shihira and Krauss (1965), who developed a physiological key, based solely on nitrogen and carbon utilization, for the identification of 41 species of Chlorella. Employed in the present investigation was a much more diverse group of organisms in an effort to determine whether nutritional patterns useful for the identification and classification of other groups of algae could be applied with equal success. Table 20 shows how patterns in nitrogen nutrition can allow one to identify some species of Chlamydomonas without any regard whatsoever to their morphology. It is, therefore, possible to construct strictly physiological keys to each of these isolates, as was done for Chlorella (Shihira and Krauss, 1965). Table 21 shows how such a key can be constructed, in this case for six isolates belonging to the Chrysophyta.

It should be pointed out that some of the results in Table 20 conflict with those reported by Cain (1965). These conflicts are understandable since the techniques of cultivation were somewhat dissimilar. Cain used BBMV to culture the organisms, leading to large pH shifts in the medium (see earlier discussion on basal medium). He also used small (13 mm) test tubes and initial pH values on the acid side of neutrality; in the present investigation 16 mm test tubes were used and the pH was on the alkaline side

TABLE 20. Patterns of behavior allowing physiological identification of *Chlamydomonas* species tested.

	Can Serve as Sole N Source						
	AC Broth	NH_4Cl	Argi-nine	Aspara-gine	Gluta-mine	Trypto-phan	Lysine
Chlamydomonas reinhardtii (-)	0	+	+	+	0	+	+
C. reinhardtii (+)	0	+	+	+	0	0	+
C. actinochloris	0	+	+	+	0	0	0
C. chlamydogama (16-1)	0	+	+	0	0	0	0
C. chlamydogama (16-2)	0	+	+	0	+	0	0
C. eugametos	+	+	0	+	0	0	0
C. radiata	0	0	0	0	0	0	0

TABLE 21. Physiological key to selected isolates of the Chrysophyta.

1.	Utilizes urea	2
1.	Does not utilize urea	4
	2. Utilizes cystine and adenine	3
	2. Does not utilize cystine and adenine	*Botrydiopsis arhiza*
3.	Utilizes asparagine	*Botrydiopsis alpina*
3.	Does not utilize asparagine .	*Botrydiopsis intercedens*
	4. Utilizes adenine	*Botrydium stoloniferum*
	4. Does not utilize adenine	5
5.	Exhibits excellent growth in NH_4Cl	*Botrydium becherianum*
5.	Exhibits only slight growth in NH_4Cl	*Botrydium cystosum*

of neutrality. Cain's conclusions were not based on subculture data as was largely the case in the present investigation. Subculture data are important since many isolates may not respond initially to nitrogen starvation, i.e., the first culture shows growth; subcultures of these reveal their true responses.

From the above discussion, it should be clear that in order to successfully apply nutritional attributes to taxonomy, precise standards of technique must be followed to help eliminate conflicts of results from laboratory to laboratory. These standards should especially apply to such critical factors as media, environmental conditions, and methods of measuring growth. It would be impossible to make and follow one strict set of standards for every known organism. Smith (1971) has suggested some general standards which will hopefully prevent gross discrepancies in results in most cases. His suggestions are as follows: "(1) the organisms must be isolated into axenic culture in a chemically-defined medium; (2) environmental and cultural conditions must be fully specified and kept as uniform as possible; (3) the nutritional characteristics should be demonstrated for as many isolates of a given taxon as it is reasonably possible to obtain, ideally from widely separated geographic loci; (4) cells from varying nutritional circumstances, including field conditions, should be examined for morphological variations; (5) the results must be duplicated in several experiments;

and (6) isolates used in these investigations should be made available to other scientists."

Physiological keys, such as that shown in Table 21, should not be used alone for identification of the algae but should be used in conjunction with easily determinable morphological variations. Physiological and morphological considerations, when used together, would give, for a set of isolates, a more complete and valid picture of their taxonomy.

The methodology of the present investigation, over all, proved satisfactory for the elucidation of patterns of nitrogen utilization; however, some pitfalls and deficiencies should be pointed out, as follows:

(1) One problem was the inefficiency of AC broth to detect bacterial and fungal contamination; other bacteriological media should be used for this purpose.

(2) There is a need to determine the source of contamination during the preparation of certain media (e.g., media containing urine and coconut milk).

(3) In an investigation of this type, where large numbers of isolates and media are being investigated, one needs numerous culture tubes and racks as well as culture room space. The lack of these facilities caused a reduction of the number of isolates from 47 to 34 in some experiments.

(4) More isolates and more nitrogen sources need to be tested to fill the gap in the pattern types shown. Different combinations of media, as suggested for the

intermediates in urea metabolism, should also be applied to the amino acids and nitrogenous bases.

(5) Subculture data and duplicate experiments should be performed on those experiments where subculture data were not obtained, and specifically, in the case of amino acids, purines and pyrimidines, ornithine, and citrulline.

Some of the results obtained here represent confirmations of work done by previous authors, others represent totally new reports, and others simply suggest that further work needs to be done. The following list of suggestions is included to emphasize that further work should be done in order to confirm, clarify, or extend some of the results obtained in this investigation.

(1) Those species which did not grow well in TBIMV should be grown on a more suitable medium to determine their ability to utilize the various nitrogen sources.

(2) Those isolates which grew better in the cultures at 12 weeks, which were presumably contaminated, should be investigated to determine whether the cause of the growth was due to contamination or to extended time.

(3) The lack of growth of Zygnema circumcarinatum and Cosmarium botrytis in TBIMV needs further study to determine whether TRIS is toxic to the organisms and/or whether some other factor is inhibiting their growth. The non-utilization of urea reported for the zygnematalean algae also point to the need for further investigation of

this entire group to determine the patterns of utilization by other species. Hoshaw (1968) has stated in his review of this group that "the nutritional requirements of the Zygnemataceae have hardly been explored."

(4) The lack of the haematococcacean species and species of Botrydium to utilize urea represents the first such reports known to the writer on these groups. More isolates in these groups should be tested for their ability to utilize urea and other nitrogen sources.

(5) The response of some isolates to growth in NH_2OH represents the only report of NH_2OH utilization known to the writer, with the exception of Anabaena cylindrica (Hattori, 1960). Unfortunately, some of the isolates exhibiting growth in NH_2OH were not subcultured; however, preliminary subculture work for these tends to confirm the original data. More isolates need to be tested for their ability to utilize this compound.

(6) Utilization of nitrite, which unfortunately was not included in this experiment, should be assayed with the isolates used in this investigation.

(7) Nitrogen sources should be supplied to the cultures in various combinations to elicit pattern types which might be beneficial in determining utilization pathways.

(8) Other bacteriological media, in addition to AC broth, need to be explored as possible algal growth media.

(9) The use of millipore filters, as opposed to Seitz filters, for steriliztion should be investigated to determine whether adsorption of ions on the Seitz filters has any effect on the constituents of the media prepared.

(10) The final suggestion would be to test as many more isolates in each group as possible with as many more nitrogen sources as possible and, therefore, to elucidate as many patterns as possible.

I V

SUMMARY

A comparative study of the nitrogen nutrition of 50 taxonomically diverse axenic cultures of fresh-water algae (one from the Cyanophyta, 42 from the Chlorophyta, and 7 from the Chrysophyta) was undertaken in order: (1) to determine their ability to utilize a number of organic and inorganic nitrogen compounds and, in this way, to further contribute to our knowledge regarding their utilization among the algae, (2) to discover, if possible, any patterns of utilization which might help explain the presently enigmatic aspects of algal urea metabolism, and (3) to examine growth response patterns which might be of taxonomic value.

The isolates were grown in an alkaline TRIS-buffered inorganic medium, which reduced pH shifts significantly. The inorganic nitrogen sources tested included potassium nitrate, ammonium chloride, ammonium nitrate, and hydroxylamine. Organic nitrogen sources tested included urea and some intermediates of urea biosynthesis (citrulline, ornithine, and arginine), seven amino acids (phenylalanine, tryptophan, serine, asparagine, glutamine, lysine, and cystine), and four nitrogenous bases (thymine, uracil, cytosine, and adenine).

Of the inorganic nitrogen sources tested KNO_3 was used by more isolates than NH_4Cl; however, when the two were

supplied together as NH_4NO_3, NH_4^+ was preferentially absorbed by most of the isolates. The utilization of hydroxylamine has been previously reported for only one alga. In this investigation, this compound was either utilized or at least was non-toxic to Carteria crucifera, C. eugametos, Chlorosarcina longispinosa, C. brevispinosa, Chlorosarcinopsis eremi, C. gelatinosa, Hormidium flaccidum, Ulothrix fimbriata, and Pleurococcus sp.

Of the organic nitrogen sources, urea served best for most of the isolates. Included are the following new reports of non-utilization of urea among the algae: Haematococcus lacustris, Stephanosphaera pluvialis, Chlorosarcina longispinosa, Neochloris pseudoalveolaris, Zygnema circumcarinatum (+ and - strains), Cosmarium botrytis, Botrydium becherianum, B. stoloniferum, and B. cystosum. The utilization of intermediates of the urea cycle (ornithine, citrulline, and arginine), while eliciting interesting growth response patterns, did not help explain the method of urea metabolism among these algae.

Among the amino acids, glutamine was the best nitrogen source for most isolates, followed by cystine, asparagine, lysine, tryptophan, serine, and phenylalanine. Adenine ranked highest of the nitrogenous bases tested followed by cytosine, thymine, and uracil.

The patterns of nitrogen utilization among the isolates were discussed with regard to their value as extramorphological taxonomic criteria.

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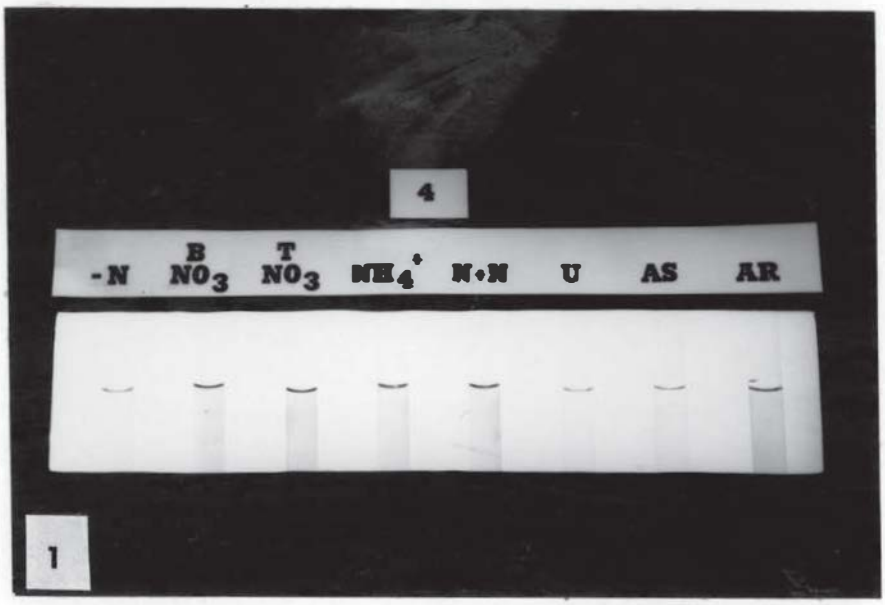
I L L U S T R A T I O N S

Figure 1

Fig. 1 An example of the macroscopic examination of culture tubes to observe growth patterns exhibited in various nitrogen compounds. The illustration represents the growth of Carteria eugametos after 6 weeks' incubation on certain nitrogen sources tested: TBIMV-N (-N), BBMV (B-NO_3), TBIMV (T-NO_3), NH_4Cl (NH_4^+), NH_4NO_3 (N+N), urea (U), asparagine (As), and arginine (Ar). Carteria eugametos was reported as exhibiting no growth in -N, urea, or asparagine, but good growth in BBMV, TBIMV, NH_4Cl, NH_4NO_3, and arginine.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-N
B
NO3
T
NO3
NH4+
N+N
U
AS
AR
1

Figures 2-5

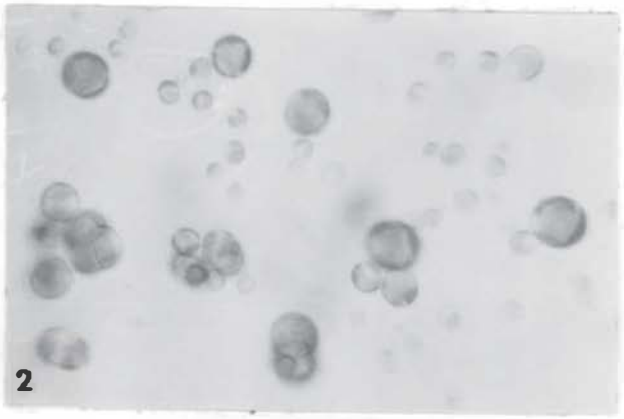
Fig. 2 Cells of *Chlorosarcina longispinosa* as they appear after 6 weeks' growth in TBIMV (2 mM of nitrate nitrogen).

Fig. 3 Cells of *Chlorosarcina longispinosa* as they appear after 6 weeks' growth in TBIMV (40 mM of nitrate nitrogen). Note the clumping of cells.

Fig. 4 Cells of *Oocystis apiculata* as they appear after 6 weeks' growth in TBIMV (2 mM of nitrate nitrogen).

Fig. 5 Cells of *Oocystis apiculata* grown on TBIMV-N. Note typical signs of nitrogen starvation (granular chloroplast, bleached cells, irregular shape).

(All X480)



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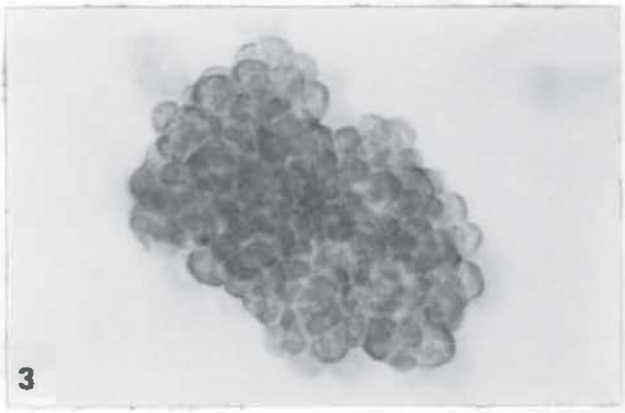

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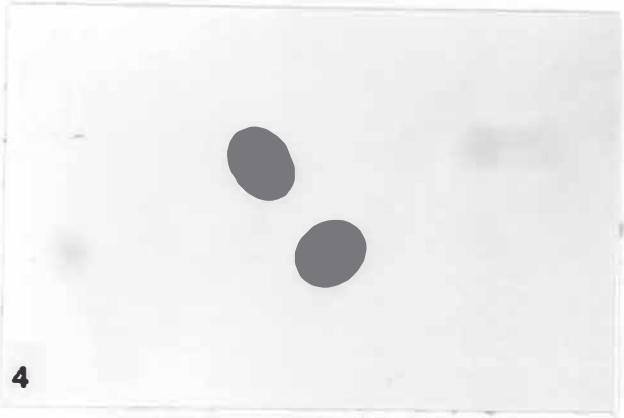

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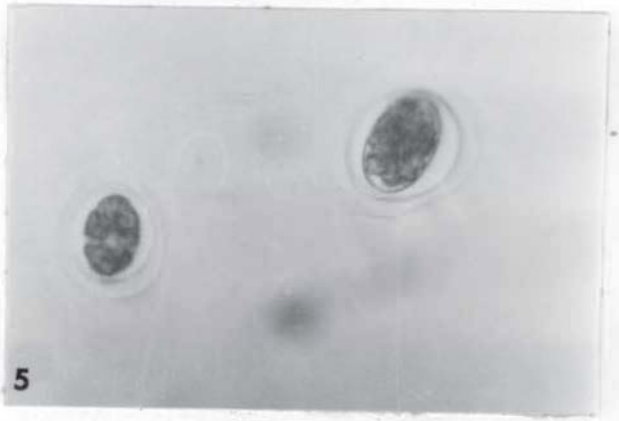

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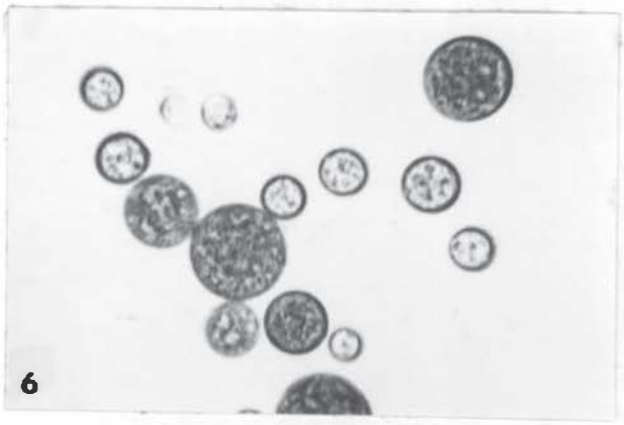
Figures 6-9

Fig. 6 Cells of Botrydium becherianum grown in TBIMV (2 mM of nitrate nitrogen).

Fig. 7 Cells of Botrydium becherianum grown in TBIMV (2 mM of ammonium nitrogen). Note size decrease.

Fig. 8 Cells of Neochloris pseudoalveolaris grown in TBIMV (2 mM of nitrate nitrogen).

Fig. 9 Cells of Neochloris pseudoalveolaris grown in TBIMV (2 mM of ammonium nitrogen). Note size increase.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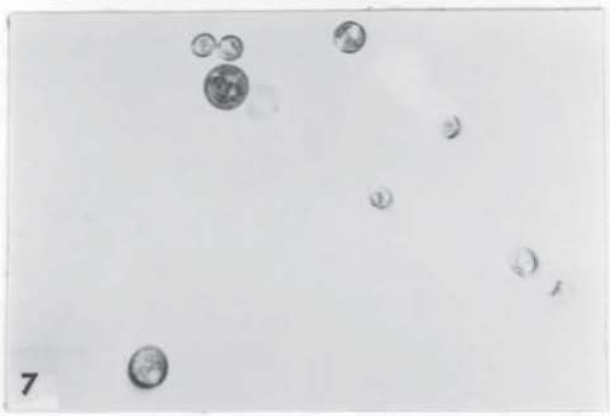
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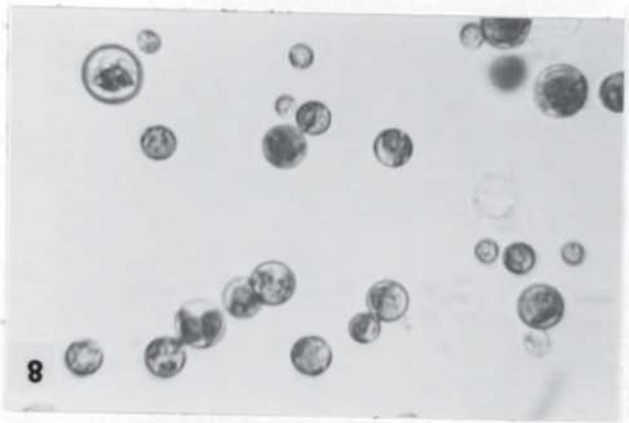
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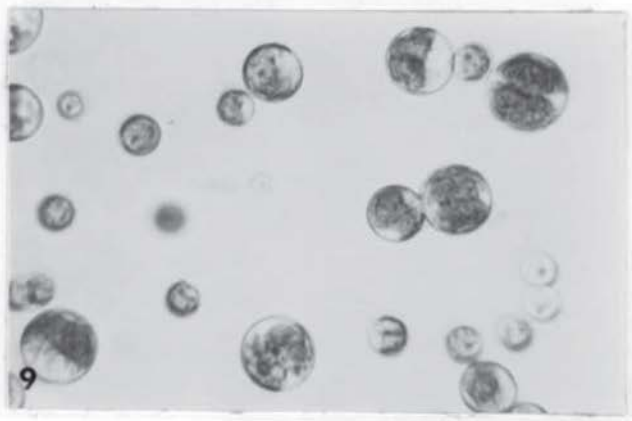
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Figures 10-14

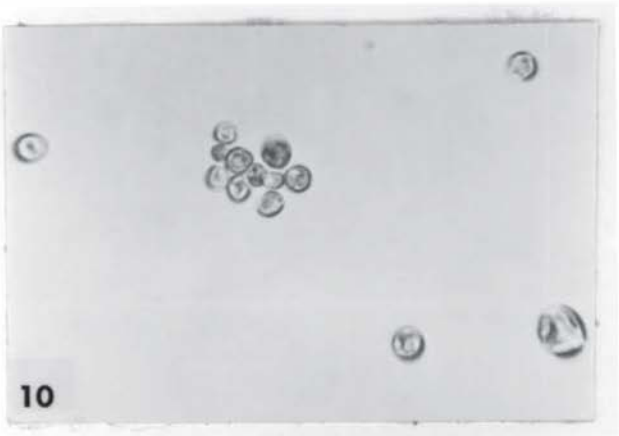
Fig. 10 Cells of Botrydium stoloniferum grown in TBIMV (2 mM of nitrate nitrogen).

Fig. 11 Cells of Botrydium stoloniferum grown in TBIMV (2 mM of urea nitrogen). Note size increase.

Fig. 12 Cells of Botrydium stoloniferum grown in TBIMV (2 mM of adenine nitrogen). Note size increase.

Fig. 13 Cells of Anacystis nidulans grown in TBIMV (2 mM of nitrate nitrogen).

Fig. 14 Cells of Anacystis nidulans grown in TBIMV (2 mM of tryptophan nitrogen). Note increased number of cells producing long filaments.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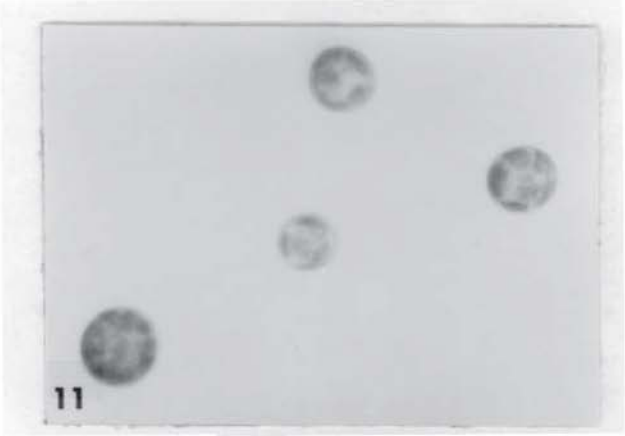
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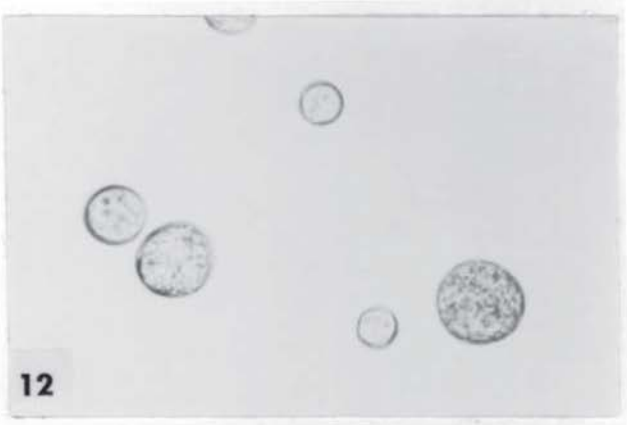
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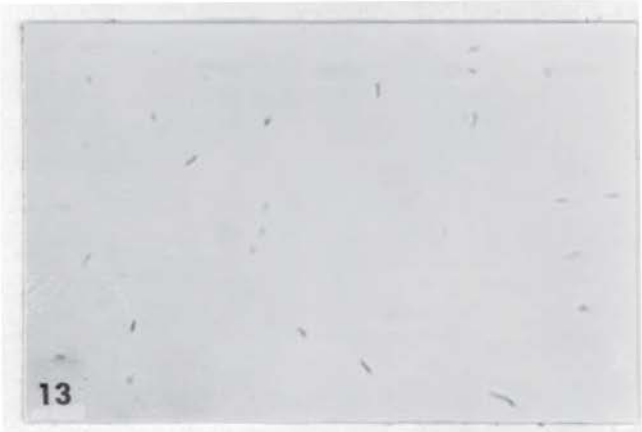
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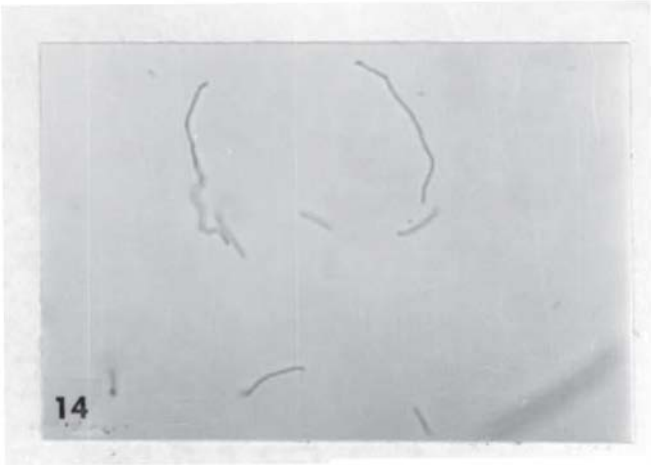
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