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Title: NUTRITIONAL, MORPHOLOGICAL, AND PHYSIOLOGICAL CHARACTERISTICS OF TRENTEPOHLIA (I.U. 1227) IN AXENIC CULTURE ON DEFINED MEDIA

Abstract approved: Redacted for Privacy
Dr. Harry K. Phinney

An unidentified species of Trentepohlia (I.U. 1227) a member of the division Chlorophyta, order Ulotrichales, originally isolated by V. Ahmadjian was maintained in axenic culture on defined media for 19 months. The nutritional requirements, effect of nutrition on morphology, the pigment, lipid and fatty acid composition of this alga, and some of the effects of nutrition and physical regime on the relative composition of these cellular components were examined. Sexual reproduction and development of the germling are reported and described for the first time.

Trentepohlia (I.U. 1227) grew well with both organic and inorganic sources of nitrogen. Comparable growth was obtained with 0.4-0.7 g/l of NH_4Cl and 10 g/l of proteose peptone. Urea did not support as much growth as proteose peptone or NH_4Cl when soil-water extract was omitted from the medium. This organism is a facultative heterotroph,

able to use dextrose, ribose, and arabinose. Growth was enhanced by the addition of these sugars to cultures maintained in the dark and in the light, but optimum growth was obtained in the light on a medium containing sugar.

The ratio of chlorophyll a to chlorophyll b was affected by the light regime and the source of nitrogen, but the total amount of chlorophyll as a percentage of dry weight remained constant under all imposed conditions. The amount of carotenoids in this organism appeared to increase as the cultures aged; and they were determined to be alpha and beta carotene, neurosporine, violaxanthin and lutein.

During the exponential growth phase approximately 17 percent of the dry weight of Trentepohlia (I.U. 1227) was lipids. Triglycerides made up about 90 percent of the total lipid fraction, with fatty acids, sterols, diglycerides, glycolipids, and phospholipids making up the remainder. Oleic acid was the most abundant of the 14 fatty acids isolated, but there were also significant amounts of $C_{14:0}$, $C_{16:1}$, and $C_{16:0}$. The percentage of the various acids did not vary greatly with light regime or nitrogen source. A notable difference in the fatty acid composition was measured when sugar was omitted from the media; the amount of oleic acid decreased by 20 to 35 percent, the percentage of $C_{16:3}$ nearly doubled, and a $C_{14:1}$ fatty acid was observed which had not been present during growth in media containing sugar.

Gametangia developed on the alga when grown on solid media lacking soluble carbon. The isogametes were released when the culture was flooded with sterilized tap water a minimum of 21 days following transfer to the medium without sugar. The fusion of the biflagellate isogametes to form dense spherical zygotes, the subsequent bipolar germination and development of new filaments were observed. If these newly formed filaments were transferred to a medium lacking sugar, they also formed gametangia that released isogametes capable of forming new zygotes developing in the same bipolar fashion. The gametangia were highly variable in size and shape, but were always borne laterally on a single stalk cell. Seven percent of the released swarmers were significantly larger than the mean and were not observed to function as gametes. The function and ultimate fate of the large swarmers have not been determined.

Nutritional, Morphological, and Physiological Characteristics of Trentepohlia (I.U. 1227) in Axenic Culture on Defined Media

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George A. McCoy

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APPROVED:

Redacted for Privacy

Professor of Botany
in charge of major

Redacted for Privacy

Chairman of the Department of Botany
and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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Typed by Deanna L. Cramer for George A. McCoy

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NUTRITIONAL, MORPHOLOGICAL, AND PHYSIOLOGICAL CHARACTERISTICS OF TRENTEPOHLIA (I.U. 1227) IN AXENIC CULTURE ON DEFINED MEDIA

INTRODUCTION

Trentepohlia (Chlorophyta, Ulotrichales) is a genus of green algae with widely distributed species. The genus comprises subaerial species common in tropical and subtropical regions. However, Prescott (1962) reported two species from northern Michigan, and the author has collected this genus growing on trunks of alder on the Olympic Peninsula, on the bark of trees on the Oregon State University campus, covering moist rocks near waterfalls in the Willamette Valley, on rocks subjected to saltwater spray and trunks of trees along the coast of Oregon. Cribb (1958a, 1958b, 1963, 1964, 1968, and 1970) described several members of this genus from Australia growing on trees and rocks in "light rainforests," on trees in open areas, and one epizooic species that grows on a spider. Chapman (1964) indicates that some species are epizooic on gastropods, Trentepohlia is also reported to be a common phycobiont of tropical and subtropical lichens (Ahmadjian, 1967a). Printz (1939) distinguishes 36 species of Trentepohlia, six of which have been reported from the United States.

This thesis describes an examination of Trentepohlia (I.U. 1227) (Starr, 1964) in axenic culture and defined media, including the nutritional requirements, the effect of nutrition on morphology, a description of the pigments and lipid and fatty acid composition of this alga, and the effect of nutrition and physical regime on the relative amounts of these cellular components. The sexual part of the life cycle was examined, albeit incompletely, by manipulating the environmental conditions.

Trentepohlia (I.U. 1227) was selected for this study because it is a phycobiont, was available in unialgal culture, there was a lack of any definitive information on it's nutritional requirements, and descriptions of the life cycle and reproduction in this organism are confusing, ill-defined and often contradictory. The information obtained from an isolated lichen symbiont in culture can be extended to the naturally occurring lichen only with caution. However, much information about the nutritional requirements, and influence of physical regime on the physiology and biochemistry of the symbionts can only be obtained from axenic cultures on defined media (Ahmadjian, 1967b). This thesis is an attempt to define the nutritional requirements of Trentepohlia (I.U. 1227).

There have been several recent reports dealing with the biochemical make-up of algal taxa in culture (Demort et al. (1972); Kleinig (1969); Moore (1975); Parker et al.

(1967); Schlenk et al. (1960); Speehr (1949); Williams and McMillan (1961). It follows that some measure of the variability of the biochemical components of an organism under culture conditions is necessary if a biochemical characterization of a particular taxon is to be meaningful. This work examines some of the factors that affect the fatty acid spectra and the pigments of Trentepohlia (I.U. 1227).

Descriptions of the sexual and asexual reproductive processes in this organism are confused and often contradictory. The nature of the sexual part of the life cycle of Trentepohlia (I.U. 1227) was examined in this study by manipulating isolated cultures in an attempt to define the nature of the sexual reproductive process in this species.

LITERATURE REVIEW

The nutrition of Trentepohlia has been largely ignored to date. However, Trebouxia, which is a common green phycobiont in lichens and is not known to occur as a free-living organism, has been the object of several nutritional studies (Hale, 1967; Ahmadjian, 1967b; Jacobs and Ahmadjian, 1969). These studies have described its heterotrophic nutrition in both light and dark grown cultures and the ability of Trebouxia to utilize both organic and inorganic sources of nitrogen. Much recent work has been done on the specific requirements of green algae (Chlorophyta) for certain organic sources of carbon, on the toxicity of some sugars to the algae, and on the mechanism of glucose uptake in the studies of Gross (1968), Wu, Alston, and Mabry (1968, 1970), Hellebust (1971), Bennett and Hobie (1972), Lylis and Trainor (1973), and Saito (1975). The utilization of organic and inorganic sources of nitrogen by the lichen phycobiont Trebouxia has been discussed by Hale (1967) and Ahmadjian (1967b). Harris (1969) has examined the growth of Platydorina caudata, a free-living planktonic green alga, using organic and inorganic sources of nitrogen.

The research on fats and steroids of the algae has recently been reviewed by Miller (1962). Spoehr and Milner (1949) investigated the changes in the chemical composition of Chlorella spp., expressed in percentage of dry weight of

fats, carbohydrates, and protein, with changing light intensity and temperature. Schlenk et al. (1960), Williams and McMillan (1961), Demort et al. (1972), and Moore (1975) have determined, using laboratory cultures, the fatty acid composition of various species of green algae. Oil globules have been reported from free-living Trentepohlia (Howland, 1929). Uyenco, (1965) noted that in cultures of Trentepohlia isolated from lichens and grown on inorganic media supplemented with soil-water, the lichen symbionts assumed a brighter green color and the yellow and golden-red globules of "haematochrome" decreased perceptibly. Similar oil globules were reported in lichenized Trentepohlia by Ahmadjian (1969).

Little is known about the effect that physical and chemical factors such as light intensity, temperature, and pH have on cellular and morphological processes. Spoehr, and Milner (1949) cautioned that investigators should be careful to separate the cultural effects such as age from the true environmental effects. Fogg (1964) reviewed the effects of environmental conditions on the pattern of metabolism in the algae. Photoperiodism in the algae has been discussed by Round (1968). Round noted that effect of photoperiod in culture has been demonstrated, but the effect in nature is relatively unknown. An increase in the number of mitotic divisions per unit time during dark periods has been documented. Mitosis is inhibited by short

day photoperiods, but stimulated by long day photoperiods. Zoospores in Ulothrix behave as isogametes if subjected to short days (Hygen, 1948). There is one report that the liberation of zoospores (isogametes ?), in Trentepohlia spp. isolated from lichen thalli, could be induced by 16 hour light and 8 hour dark periods (Uyenco, 1965). However, in lichenized algae there is little evidence for a photoperiodic effect, and most phycobionts grow well in complete darkness (Ahmadjian, 1967b).

Descriptions of sexual and asexual reproduction in the genus Trentepohlia in the literature are inconsistent indicating that the processes are either not well understood or vary with the species. Meyer (1909, 1936a, 1936b, and 1937) described detachable windborne sporangia with quadriflagellate zoospores that were liberated when the sporangia were moistened. The gametangium have been described as intercalary or terminal, noticeably different from the sporangia. Printz (1939) indicated that gametangia are lateral or terminal, seldom intercalary, and that gametes are biflagellate and may develop parthenogenetically into filaments. Printz also observed that sporangia are generally intercalary, usually borne singly, but sometimes in pairs. He also indicated that if terminal sporangia occur they are borne on a single stalk cell. Prescott (1962) indicated that zoospores are biflagellate and produced from lateral or terminal hooked or recurved sporangia

and isogametes are produced by modified terminal or intercalary vegetative cells. Chapman (1964) described sessile sporangia with biflagellate swarmers which may be isogametes, stalked detachable sporangia with bi- or quadriflagellate zoospores, funnel shaped sporangia, and aplanospores. Uyenco (1965) was unable to distinguish between gametangia and sporangia in six species of lichenized Trentepohlia in culture, but observed that they were borne laterally or terminally and that cell shape and the size of reproductive and vegetative cells in culture differed in an unpredictable manner from the lichenized cells.

Trentepohlia commonly grows as an orange-red or brown felt-like layer on trees or rocks. Jeremais (1963) determined that the color is due to the carotenoids zeaxanthin and lutein. Chapman (1964) suggests that the carotenoids may be a food reserve accumulated during periods of rapid growth, and become depleted under unfavorable conditions. However, he did not explain why young filaments are green and become increasingly orange-red as they age. Varasova et al. (1965) have determined that pigment composition varied with culture conditions, but was not highly variable between taxa in 20 species of green algae. Alpha and beta carotene, lutein epoxide, lutein, violaxanthin, and neoxanthin are the carotenoids common to all green algae (Kleinig, 1969). Free-living Trentepohlia usually has abundant carotenoids, but in the lichenized state it forms

only small amounts of the carotenoid pigment until the thallus begins to die (Des Abbayes, 1951).

METHODS AND MATERIALS

Culture Media

The principal culture medium used, Trebouxia agar (Starr, 1964) was prepared as follows:

Bristol's solution	850 ml
Soil-water extract	140 ml
Proteose peptone no. 3 (Difco)	10 gm
Glucose	20 gm
Agar	15 gm

Bristol's solution (Bold, 1949) was prepared in the following manner. Six stock solutions, 400 ml in volume, were used. Each stock solution contained one of the following salts in the amount listed.

$NaNO_3$	10 gm
$CaCl_2$	1 gm
$MgSO_4 \cdot 7H_2O$	3 gm
K_2HPO_4	3 gm
KH_2PO_4	7 gm
NaCl	1 gm

Ten ml of each of the above stock solutions were added to 940 ml of glass distilled water. To this solution was added 2 ml of Arnon's trace element solution (Arnon, 1938) and a drop of 1 percent $FeCl_3$ solution.

Trentepohlia (I.U. 1227), a lichen phycobiont isolated from Pyrenula nitidula by Ahmadjian (Starr, 1964), was received from the Indiana University culture collection on Trebouxia medium. It was transferred from the solid medium and maintained in liquid culture during this study except where noted otherwise. It was necessary to eliminate the

two undefined components, soil-water extract and proteose peptone, to obtain a defined medium and assess the nutritional requirements of this alga.

Several modifications of the above medium were employed. In some experiments the soil-water extract, the proteose peptone, or the dextrose were omitted. Agar was omitted to obtain a liquid medium. Various substitutes for proteose peptone were tried, including vitamin-free casein hydroylosate, urea, and NH_4Cl. Nine different sugars were substituted for glucose. When it was necessary to buffer the medium or adjust the pH, 0.1 N NaOH or 0.1 N HCl were used. Dry weights were determined at the end of the culture periods.

Inoculation and Transfer

A sterile wire loop was ordinarily used to make transfers in inoculating new cultures. When studying growth rates, a standard inoculum was prepared by homogenizing a clump of filaments suspended in Bristol's solution in a Sorval Omni-mixer for 3 minutes and introducing an aliquant into each flask.

Measurement of Growth

Determination of dry weight was the principal method for the measurement of growth. The cultures were harvested on tared 0.45 μm Millipore filters which were allowed to

dry for 24 hours in an oven at 70°C. The samples were then transferred to a desiccator and weighed when cool. The mean dry weight of triplicate samples was reported in milligrams.

Pigment Analysis

The cultures of Trentepohlia (I.U. 1227) were harvested then sonicated and extracted three times with hot methanol to a volume of 50 ml. Chlorophyll was determined directly from this extract using a Beckman DB-G spectrophotometer to measure the absorbtion at 650 and 665 nm (Machlachlan and Zalik, 1963). Carotenoids were extracted according to the method described by Williams (1971). The filaments were extracted with hot methanol as for chlorophyll and the extract saponified with 20 ml of 5 N NaOH. The mixture was subsequently partitioned three times with 100 ml of petroleum ether (b.r. = 37° - 45°) and 15 ml of 5 percent NaCl. The extracts were combined, washed with 50 ml of water, partially dried over Na_2SO_4 and evaporated to dryness. The residue was taken up in 2 ml of chloroform, streaked onto a CMC ($CaCO_3$:MgO:$Ca(OH)_2$-59:12:10) thin-layer chromatography plate, preincubated in a saturated atmosphere of the solvent, and developed in two solvent systems, ligroin (b.r. = 100° - 140°C): acetone:chloroform (5:5:4), and 2-propanol: isoctane (1:100). The thin layer plates were placed under anerobic conditions immediately after removal from the

solvent system. Assisted by the fluorescence of the pigment bands under ultraviolet light, the developed bands were scraped from the plates, eluted from the absorbent with acetone and evaporated to dryness. Absorption maxima were determined in petroleum ether and ethanol. HCL in ethanol was used as a test for epoxy groups.

Fatty Acid Analysis

Cultures used in determination of fatty acids were harvested during exponential growth. The alga was scraped from the agar, homogenized in an Omni-mixer in 2:1 chloroform methanol for two minutes (Bligh and Dyer, 1959). After sonication, the extract was filtered through sintered glass funnels and the filtrates were partitioned by the use of 7.5 ml of water. The water-methanol layer was discarded, and the chloroform layer evaporated to dryness under gaseous nitrogen. Five ml of chloroform were added, and the samples were again evaporated to dryness. Three ml each of ethyl ether and five percent hydrogen chloride gas in super dry methanol were added to the samples for methanolysis. The reaction was carried out in a screw cap tube at 80°C for 90 minutes. After cooling, the methyl esters were extracted by adding hexane (1:1). The upper layer was retained for analysis. Extraneous materials was removed on a short silicic acid column. Some of the samples required a further purification by means of thin-layer chromatography

on silica gel G, using dichloromethane as a developing solvent. This step was necessary in order to be certain that all observed peaks were due to methyl esters and not to extraneous material.

Gas liquid chromatography was performed using a 1/8 inch by 6 foot aluminum column containing 15 percent ethylene glycol succinate polymer on 60-80 mesh, acid washed Chromosorb P. An F and M model 700-12 gas chromatograph with dual hydrogen flame detectors was used. The column was maintained at a temperature of 190°C with a helium flow of 30 ml/minute. The percentages by weight of the total fatty acids present in a sample were calculated by the method of Carroll (1961). Identification of the chromatograph peaks were made using a semilog plot of the absolute retention time in centimeters and by comparing the peaks from the sample with the peaks from a standard run at the same time. The degree of unsaturation was confirmed by silver nitrate thin-layer chromatography (Privett et al., 1963). The samples were then hydrogenated and re-chromatographed as a final check on chain length.

Determination of Lipid Components

Lipids were extracted from Trentepohlia (I.U. 1227) grown in complete media using the method described by T. M. Ching (written communication, 1971). Following homogenization in a Sorval Omni-Mixer and grinding in a

mortar with 30 ml of ethanol, 60 ml of ether were added and the sample was filtered. The residue was re-extracted with 50 ml of ethanol:ether (1:2) for 10 minutes. Following filtration, the extract was dryed, then re-dissolved in 20 ml of ether. After washing with 100 ml of water, the extract was again dried in a tared flask and weighed to obtain the total lipids as a percentage of the lipid-free dry weight. The residue was eluted with 2 ml of ether and spotted on a thin-layer silica gel plate with a series of known lipid fractions. It was developed in hexane:ether: acetic acid (75:25:1). The lipid classes were located and identified by means of ultraviolet light, iodine vapor, rhodamine 6-G, spraying the plates with sulfuric acid and charring them, and by the retention time of the spots.

Physical Regime

Cultures were maintained on agar slants in screw cap tubes or in 20 ml of liquid medium in 50 ml flasks with foam plugs (to allow for gas exchange). Stock cultures were kept at room temperature near a north facing window with continuous supplemental fluorescent light. Experimental cultures were maintained at 19°C. Continuous light or a 12 hour photoperiod was employed. Light intensity was approximately 340 foot candles from a combination of fluorescent and incandescent light.

RESULTS

Development of a Defined Medium

Vitamin-free casein hydrolysate was substituted for proteose peptone to determine if proteose peptone was supplying some essential vitamin or was acting as an organic source of nitrogen. The results of this experiment as presented in Table 1 indicate that there was no significant difference in the amount of growth on the two media at the 95 percent significance level. Since the growth obtained was highly variable, in all subsequent experiments the filaments were finely chopped in an Omni-mixer using sterile techniques in an effort to standardize the amount of inoculum.

The increase in dry weight of Trentepohlia (I.U. 1227) was measured when grown on media with various concentrations of NH_4Cl, proteose peptone, and urea to determine if an inorganic source of nitrogen could be substituted for proteose peptone, and to test a defined source of organic nitrogen (Table 2). The pH of all media was adjusted to 6.45, the pH of the Trebouxia medium. Increased amounts of proteose peptone caused a decrease in the amount of growth. The mean dry weight at the end of 60 days incubation was 93.5 mg for the medium with 10 g/l of proteose peptone as opposed to 62.5 mg in the medium with 20 g/l. The amounts

Table 1. Comparison of growth of Trentepohlia (I.U. 1227) in Trebouxia medium containing proteose peptone and vitamin-free casein hydrolysate.

Period of Growth	Vitamin-free casein hydrolysate 10 ml/l		Proteose peptone 20 g/l	
	30 days	60 days	30 days	60 days
dry weight mg	6.3	56.9	10.6	90.5
	13.3	86.8	13.5	73.8
	13.9	94.2	11.1	92.7
	8.2	86.6	5.8	101.1
	17.0	77.8	29.0	81.8
	21.1	58.8	10.1	49.7
Mean	13.3	76.8	13.3	81.6

Table 2. Comparison of NH_4Cl proteose peptone and urea as nitrogen sources for Trentepohlia (I.U. 1227).

	NH_4Cl						Proteose peptone				Urea					
g/l	0.025		0.25		0.5		10		20		2		20		40	
Days	30	60	30	60	30	60	30	60	30	60	30	60	30	60	30	60
Dry weight (mg)	8.3	46.3	8.3	97.7	29.1	97.7	3.6	87.3	2.3	72.6	117.5	107.5	-	0.4	0.8	0.5
	5.3	40.1	11.5	77.3	76.5	77.3	8.6	101.1	4.1	54.0	87.4	126.2	-	5.1	-	0.1
	14.3	43.7	13.0	87.4	34.8	87.4	3.7	96.7	0.3	64.6	126.7	201.3	1.0	2.2	-	-
	5.6	41.2	8.1	96.2	41.0	96.2	4.7	94.2	4.1	50.5	122.9	126.8	0.7	3.0	0.2	-
	14.4	41.5	6.1	92.3	44.0	92.3	0.1	88.2	2.7	70.6	120.5	187.2	0.1	2.3	1.0	-
Mean	9.6	42.5	9.4	90.2	45.1	90.2	4.1	93.5	2.7	62.5	115.0	155.0	0.4	2.6	0.4	0.1

of growth on the medium with 0.25 and 0.5 g/l of NH_4Cl were significantly different at the 95 percent significance level than that in the medium with 10 g/l of proteose peptone. The cultures in media with 2 g/l of urea produced significantly more growth than cultures on any other media, but 20 and 40 g/l of urea inhibited growth.

The cultures in media containing proteose peptone were dark green until approximately 120-130 days following inoculation, at which time they gradually turned the orange-red more typical of the subaerial species observed in their natural habitats. In the urea medium the orange-red pigmentation began to appear in the alga approximately 30 days after inoculation, and after approximately 60 days in NH_4Cl.

A comparison of growth in the media with and without soil-water extract was made (Table 3). An average of 91.6 mg dry weight at the end of 60 days in the complete medium and a mean of 81.4 mg in the Trebouxia medium without soil-water extract was measured. This difference represents a reduction in growth of 11.5 percent, which was significant at the 95 percent significance level. In both media the dark green coloration was maintained until 120 days following inoculation. The rate of increase in dry weight of the alga as a function of time in the culture media was determined in cultures in the complete Trebouxia medium and in the defined medium, which was Trebouxia medium without soil-

Table 3. Dry weight of Trentepohlia (I.U. 1227) grown in Trebouxia medium and Trebouxia medium without soil-water extract.

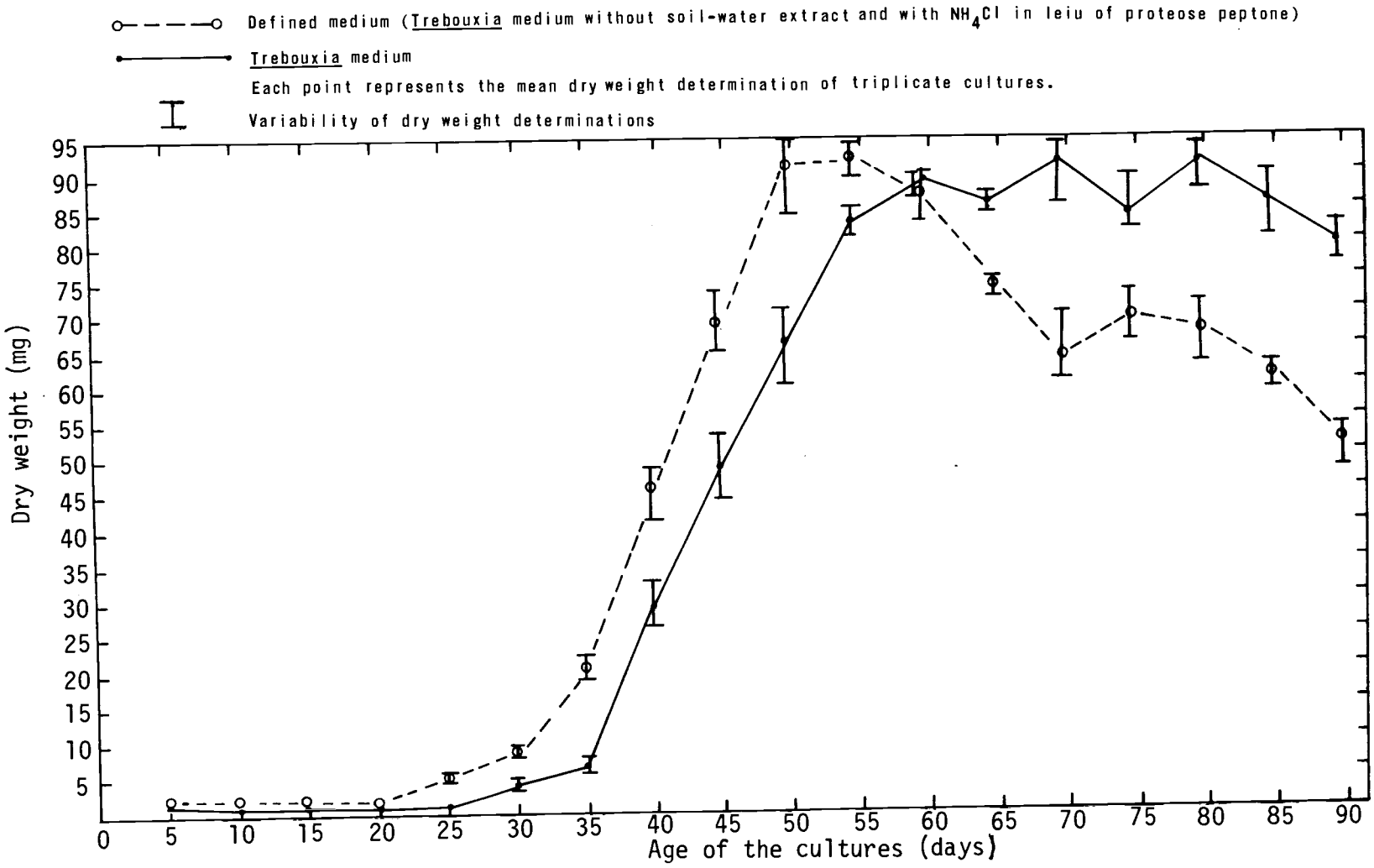
Culture time (days)	Trebouxia medium		Trebouxia medium less soil-water	
	30	60	30	60
Dry weight (mg)	9.6	77.8	7.5	86.7
	8.5	92.5	4.3	72.5
	4.7	94.8	8.8	78.6
	8.3	82.2	4.0	82.0
	4.6	96.4	7.9	74.7
	8.1	103.9	9.7	85.3
	9.7	93.1	9.7	84.8
	9.4	79.8	8.4	93.0
	7.0	85.4	8.9	82.9
	4.8	95.0	9.9	69.7
	5.8	92.7	10.7	79.8
	6.7	101.8	8.4	75.4
	10.2	89.5	8.9	92.0
	11.3	91.9	9.9	81.5
	8.3	96.5	10.7	82.3
Mean	7.8	91.6	8.2	81.4

water extract and with 0.5 g/l NH_4Cl in lieu of proteose peptone (Figure 1). The growth curves are very similar. The cultures in the defined medium reached the inflection point at approximately 60 days. However, a more rapid decrease in dry weight was observed in the cultures in defined medium after 60 days. The alga exhibited marked change in color from dark green with a tinge of red to a pale orange-red in the defined medium between 50 and 75 days. The alga in the Trebouxia medium, however, showed only a slight change in color at 110 days. The pH decreased gradually in the defined medium, reaching 2.0 at about 60 days. In the complete media the pH decreased to 5.0-5.5 at 60 days and to 4.0 to 4.7 at 110 days. Stock cultures were maintained on the defined medium for 19 months by transferring the cultures every 50-60 days.

Nutrition

The substitution of NH_4Cl for proteose peptone and the omission of soil-water extract produced a defined culture medium which would support growth of Trentepohlia (I.U. 1227) within 15 percent of that supported by the Trebouxia medium. Additional experiments were conducted to determine approximately the optimum concentration of NH_4Cl and urea, to determine the relative degree of autotrophy and heterotrophy, and to ascertain some of the sources of organic carbon that Trentepohlia (I.U. 1227) can utilize.

Figure 1.-- Dry weight of Trentepohlia (I.U. 1227) grown in the defined medium and the Trebouxia medium vs age of the culture.



There was no significant difference in the dry weight of the cultures grown in 0.4-7.0 g/l of NH_4Cl (Table 4). However, both below and above this concentration less growth was measured. The largest increase in dry weight of Trentepohlia (I.U. 1227) grown with urea was in those cultures with a concentration of 2 g/l. The dry weights obtained from the cultures containing urea were less than those from the cultures with NH_4Cl. However, in the previous experiment the growth in urea exceeded the growth with NH_4Cl (Table 2). In the first experiment soil-water extract was used but in the latter it was omitted. In the defined medium NH_4Cl promotes growth better than urea and as a consequence, 0.5 g/l of NH_4Cl was used in the culture media in all subsequent experiments.

Ten different sugars were used to determine if Trentepohlia (I.U. 1227) has the ability to use specific sugars (Table 5). Significant growth in the dark occurred only with dextrose, D(-) ribose, and with both steroisomers of arabinose. On media with the other sugars the growth was not significantly different from the cultures in the control medium (defined medium without sugar). The medium with soil-water extract and proteose peptone produced more growth than the defined medium, but less growth than the defined media with dextrose, arabinose, and ribose. In cultures grown in the dark and in the light, on arabinose, ribose, and dextrose measureable increases in dry weight

Table 4. Dry weight of Trentepohlia (I.U. 1227) grown in the defined medium with varied concentrations of NH_4Cl and urea.

Nitrogen Source	Concentration g/l	Dry weight (average of three) mg	
		30 days	60 days
NH_4Cl	0.20	7.9	20.7
NH_4Cl	0.30	7.2	26.5
NH_4Cl	0.40	5.4	51.4
NH_4Cl	0.50	6.3	50.2
NH_4Cl	0.60	5.8	51.8
NH_4Cl	0.70	6.7	50.2
NH_4Cl	0.80	5.7	42.7
NH_4Cl	0.90	8.1	30.2
NH_4Cl	1.00	12.0	32.1
Urea	1.00	13.3	20.5
Urea	2.00	14.3	36.5
Urea	5.00	36.3	3.8
Urea	10.00	7.7	2.5
Proteose peptone (Control)	10.00	2.4	49.6

Table 5. Dry weight of Trentepohlia (I.U. 1227) grown in defined media containing 20 g/l of ten different sugars, no sugar but with soil water extract, no sugar with proteose peptone, and the defined medium without sugar.

Sugar	Dry weight, in mg, at 60 days (average of 5 samples)	
	Constant light	Constant dark
Dextrose	114.6	18.1
L(-) rhamnose	6.8	2.8
Fructose	9.9	2.3
L(+) arabinose	91.3	13.9
D(-) arabinose	96.7	15.0
D(+) xylose	1.8	2.5
D mannose	8.4	2.3
Sucrose	3.4	2.0
D(-) ribose	96.0	12.0
D(+) galactose	6.4	1.2
Soil water extract (no sugar)	17.7	4.5
Proteose peptone (no sugar)	26.3	2.5
Defined medium (no sugar)	5.6	1.5

were recorded. Significant growth also occurred on soil-water extract without sugar, and proteose peptone without sugar, but only in light grown cultures. All cultures grown in the light retained the typical green color except those in the defined medium without sugar, in which the alga was slightly red at 30 days and red-orange with no trace of green at 60 days. Cultures in the dark faded to a pale red-orange in less than 60 days.

Cultures were grown in four different concentrations of dextrose (Table 6). The concentration used in the original Trebouxia medium (20 g/l) promoted the greatest gain in dry weight. Trentepohlia (I.U. 1227) grown in a medium containing 5 g/l of destrose showed more growth than cultures with 1 g/l, and the 40 g/l concentration produced slightly less growth than the 20 g/l, but greater growth than in the lower concentrations.

Pigment Composition

Experiments were conducted to characterize the chlorophyll and carotenoid pigments of Trentepohlia (I.U. 1227) and to determine the effects of changes in the nitrogen source and photoperiod on chlorophyll ratios.

The concentration of chlorophyll a and chlorophyll b were determined in Trentepohlia (I.U. 1227) harvested from the medium containing 0.5 g/l NH_4Cl, 1 g/l urea and 20 g/l proteose peptone. The difference in the concentration of

Table 6. Dry weight, in mg, of Trentepohlia (I.U. 1227) grown in four concentrations of dextrose. The figures represent an average of dry weights of five cultures.

Culture time (days)	Dextrose concentration (g/l)				
	0	1	5	20	40
14	0.8	0.8	1.1	0.8	1.2
28	4.0	4.6	3.2	13.6	4.0
42	2.6	17.2	11.0	55.5	64.6
56	3.4	18.5	66.6	125.1	90.7

total chlorophyll and chlorophyll as a percentage of the dry weight is probably not significant (Table 7). The ratio of chlorophyll a to b, however, differed with the various media used. In Trentepohlia (I.U. 1227) from cultures containing proteose peptone the ratio of chlorophyll a to b was 14.7. The alga in cultures with urea had a lower ratio than that grown in proteose peptone (4.6), but a higher ratio than that with NH_4Cl (2.1). The light regime also had an effect on the ratio of chlorophyll a to b. A 12 hour photoperiod reduced the amount of chlorophyll a to about half that present in the cultures subjected to constant illumination.

A qualitative determination of the carotenoids present in Trentepohlia (I.U. 1227) grown on the complete Trebouxia media was made (Table 8, Figure 2). Alpha and beta carotene, neurosporine, violaxanthin, and lutein were identified. One unidentified pigment was observed on the 2-propanol:isooctane plate.

Lipid and Fatty Acid Composition

A gravimetric determination of the lipid fraction of Trentepohlia (I.U. 1227) grown in the complete medium and harvested during exponential growth indicated that lipids comprised 17.0 percent of the dry weight of the alga. Lipid classes identified from a qualitative determination using thin layer chromatography were fatty acids, diglycerides,

Table 7. Concentration of chlorophyll *a* and *b*, and total chlorophyll as percentage of dry weight of *Trentepohlia* (I.U. 1227) after 30 days, on three different media in constant light and on a 12-hour photoperiod.

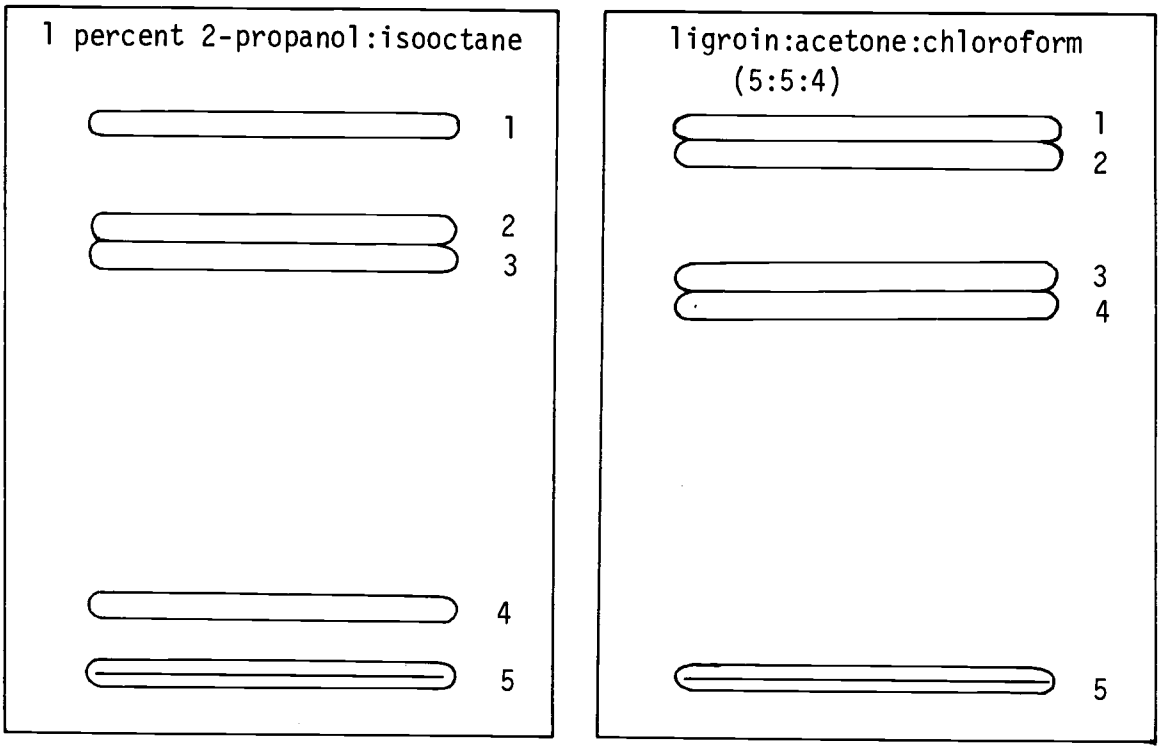
Nitrogen source	Light regime	Dry weight of alga mg	Dry weight of chlorophyll mg			ratio	Dry weight of chlorophyll as percentage of dry weight
			a	*b*	total	*a*/*b*	
NH_4Cl (0.5 g/l)	constant light	1934	10.5	5.0	15.5	2.1	0.80
NH_4Cl (0.5 g/l)	12 hour photoperiod	1450	4.9	5.0	9.9	1.0	0.68
Proteose peptone (20 g/l)	constant light	1972	11.4	0.8	12.3	14.7	0.61
Proteose peptone (20 g/l)	12-hour photoperiod	1014	5.8	1.2	7.0	4.8	0.69
Urea (2 g/l)	constant light	2170	13.3	2.9	16.1	4.6	0.74
Urea (2 g/l)	12-hour photoperiod	1606	8.1	2.5	10.6	3.2	0.66

Table 8. Absorption maxima (nm) of pigments isolated on CMC ($CaCO_3$:MgO:$Ca(OH)_2$) plates. (See Figure 2.)

Band		Petroleum ether*			Ethanol			Ethanol:HCL		
ligroin:acetone:chloroform										
band 1	α- and β-carotene	475	445	428	472	445	420	472	445	420
band 2	neurosporene	465	440	412	---	---	---	---	---	---
band 3	violaxanthin	472	443	425	472	443	420	426	404	---
band 4	lutein	470	442	420	470	445	420	470	445	425
isopropanol in isooctane										
band 1	α-carotene	468	442	420	---	---	---	---	---	---
band 2	β-carotene	472	445	(423)	470	448	(420)	---	---	---
band 3	dark yellow(?)	468	443	(420)	470	442	---	---	---	---
band 4	neurosporene	470	442	418	---	---	---	---	---	---

*b.r. = 37-45°C

Figure 2.-- Schematic of pigments separated on CMC ($CaCO_3$:MgO:$Ca(OH)_2$) plates.



1. α - caroten - light yellow band, fluorescent
2. β carotene - orange band
3. unidentified pigment - dark yellow
4. neurosporene - yellow band
5. origin

1. α - and β - carotene - orange band
2. neurosporene - light orange band fluorescent
3. violaxanthin - yellow band
4. lutein - orange band
5. origin

sterols, glycolipids, phospholipids, and triglycerides. Examination of the chromatograph indicated that triglycerides made up more than 90 percent of the total lipid fraction.

The fatty acid composition of Trentepohlia (I.U. 1227) in various media and under three different light regimes (constant illumination, constant dark, and 12 hour photoperiods) was determined (Table 9). The most abundant fatty acid in Trentepohlia (I.U. 1227) was $C_{18:1}$ in all cultures (38 to 73 percent). A comparison of the alga from the three cultures on the complete medium in three different light regimes suggests little difference in the fatty acid composition as a result of light conditions. However, Trentepohlia (I.U. 1227) from the cultures on the complete medium in a 12 hour photoperiod contained the highest percentage composition of $C_{18:1}$ (73 percent). In Trentepohlia (I.U. 1227) on the same medium, constant illumination produced 67 percent $C_{18:1}$ and darkness 59 percent. The Trentepohlia (I.U. 1227) grown without dextrose had the lowest percentage composition of $C_{18:1}$ (39 and 38 percent) and the highest percentage composition of $C_{16:3}$ (26.6 and 25.1 percent). The $C_{14:1}$ fatty acid was detected only in cultures that did not contain dextrose.

Table 9. Percentage composition of fatty acids from Trentepohlia (I.U. 1227) cultured under varied light regimes and sources of organic nutrients.

Retention time (cm)	Chain length: number of double bonds	Constant illumination				Constant dark	12 hour photoperiod		
		No dextrose proteose peptone	Dextrose NH_3	Dextrose urea	Dextrose proteose peptone	Dextrose proteose peptone	No dextrose urea	Dextrose NH_3	Dextrose proteose peptone
1.35	14:0	4.6	11.1	3.1	4.9	2.1	2.2	4.5	3.0
1.50	14:1	2.3	--	--	--	--	1.2	--	--
1.85	16:0	12.6	8.5	11.5	9.0	13.1	14.8	9.0	6.7
2.05	16:1	7.9	6.0	11.8	8.0	11.8	8.1	7.2	6.4
2.15	16:2	--	--	--	--	--	2.4	--	--
2.50	16:3	26.6	10.8	5.4	5.5	5.8	25.1	9.9	7.6
2.85	18:0	3.1	1.0	3.3	3.7	5.3	5.0	1.4	0.9
3.45	18:1	39.1	55.1	62.9	67.3	59.3	37.8	64.4	73.1
4.10	18:2	2.8	2.4	1.3	1.4	2.3	--	2.7	1.9
4.50	18:2(3?)	0.9	1.5	0.7	0.3	0.5	2.3	0.9	--
5.30	18:3	--	--	--	--	--	0.8	--	--
5.75	20:0	--	0.6	--	--	--	--	--	--
6.05	20:0	--	0.7	--	--	--	--	--	--
6.90	20:1	--	2.4	--	--	--	--	--	--

Morphology in Culture

Trentepohlia (I.U. 1227) is composed of irregularly branched intertwined filaments. In liquid culture it forms spherical clumps, the size of which can be decreased by intermittant or continuous agitation. On solid media pulvinate tufts are formed. The macroscopic appearance of these tufts changes with the moisture content of the culture medium. In very humid tubes with condensed moisture on the agar or the glass inside the tubes, the tufts appear to be very smooth, presumably a result of the filaments being packed tightly together. In a drier environment the filaments grow out from the tufts giving them a tomentose appearance.

The cells are cylindrical to slightly swollen, 10-25 μm wide and 30-70 μm long. There appear to be numerous discoid chloroplasts but the number and shape of these chloroplasts is usually obscured by the presence of carotenoids. The cell wall is thick and lamellose. Observations with polarized light revealed an inner cellulose layer and an outer isotropic layer which has been reported to be pectin (West and Hood, 1911).

The development of gametangia occurred only on filaments grown on a solid medium without a source of available organic carbon. Incipient gametangia were observed at 14 days, but the release of gametes was never obtained in less than 21 days, at which time about 5 percent of the

gametangia would release isogametes. At 30 days 63 percent of the gametangia released swarmers when flooded with sterilized tap water. An aliquant of the tap water with isogametes was then transferred to a petri dish containing the defined media for observation of germination.

The gametangia were lageniform, 30-100 μm in diameter (Plate I, Figure A). Gametangia were observed with one, two and three necks with a pore or operculum through which isogametes were released (Plate I, Figures A, B, and C). The motile cells are pyriform, 2 μm wide by 5 μm long, and have two anterior flagella. Usually all the isogametes were released simultaneously. They appeared to be extruded in a gelatinous matrix bounded by a membrance (Plate II, Figures A and B). The membrane eventually ruptured, allowing dispersal of the isogametes. On occasion isogametes were observed escaping singly, indicating that the membrane ruptured inside the cell before being extruded. The isogametes remained motile in the tap water for approximately 8 hours at room temperature. In distilled water the isogametes were motile for only a few minutes, before plasmolysis occurred.

The fusion of isogametes often began to occur within a few minutes of their release. Gametes were observed joining at the anterior ends, the flagella became entangled, motility ceased, and in a period of less than two hours the two isogametes would fuse and gradually round up into a

Plate I

Figure

A Lageniform gametangium containing isogametes typical of Trentepohlia (I.U. 1227).

B Gametangium of Trentepohlia (I.U. 1227) with two necks. The one on the left is out of focus. The plug is evident in the operculum on the right.

C Three lobed gametangium of Trentepohlia (I.U. 1227) with two opercula. Isogametes have not been released.

D Irregularly shaped gametangium of Trentepohlia (I.U. 1227) with one operculum on the upper lobe. In this photograph the plug is intact.

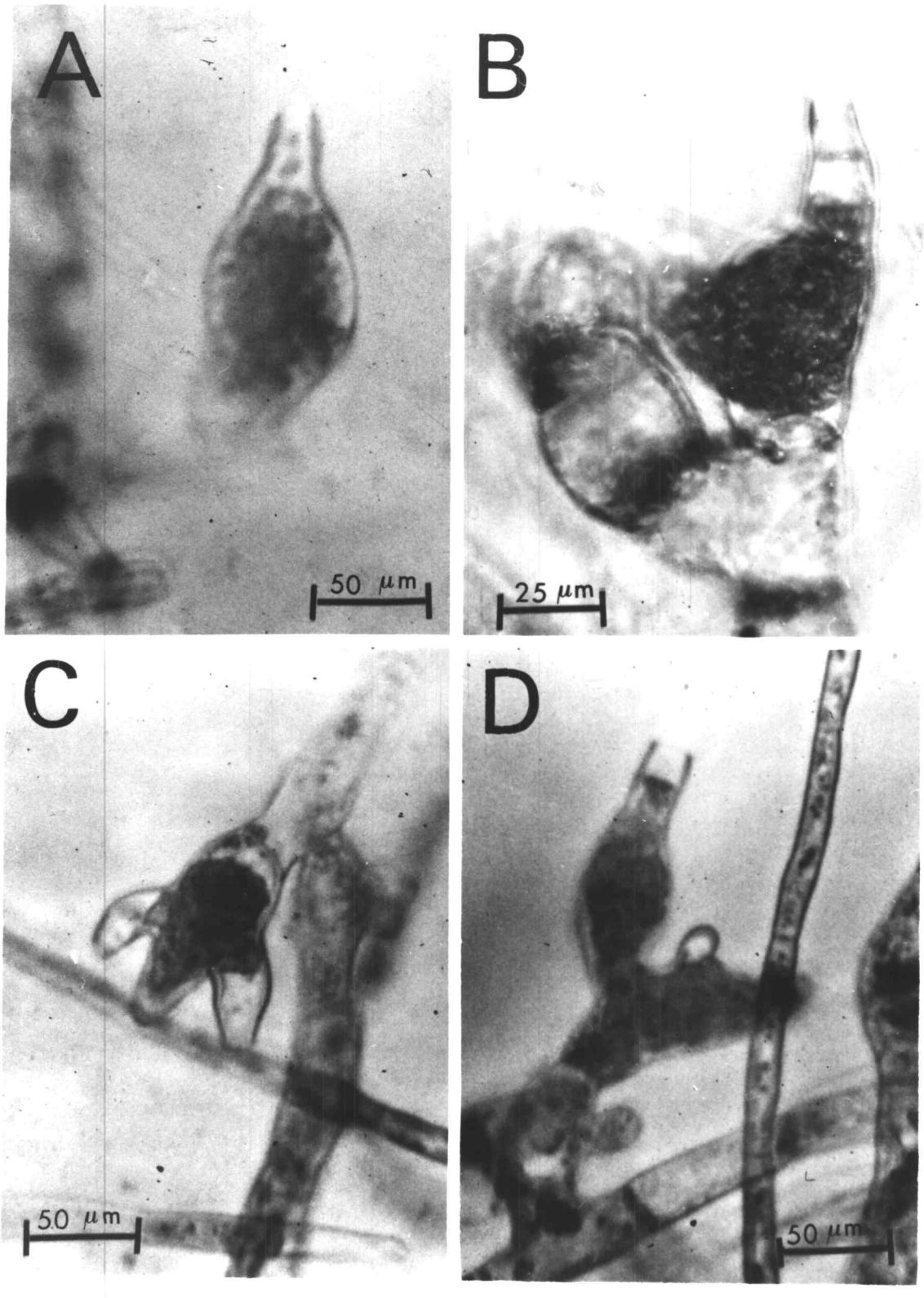

A
50 µm
B
25 µm
C
50 µm
D
50 µm

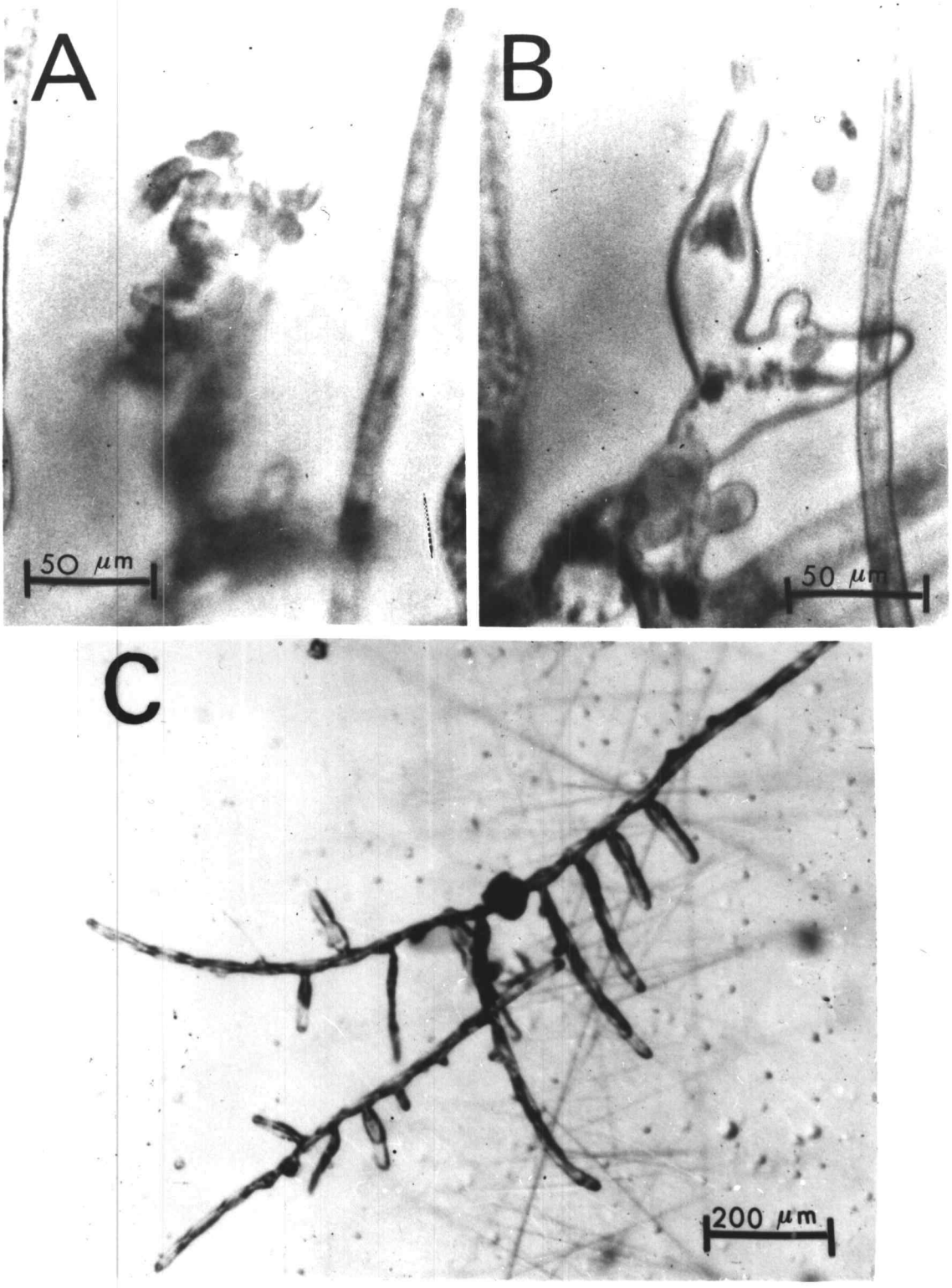
Plate II

Figure

A The same gametangium as in Plate 1-D, the isogametes have just been released.

B The isogametes have ruptured the membrane and dispersed except for a few still within the gametangium.

C Bipolar germination from a zygote of Trentepohlia (I.U. 1227) showing the branching of the filaments.


A
50 µm
B
50 µm
C
200 µm

spherical zygote. Several larger swarmers 4-6 by 10 μm were observed escaping from the same gametangium as the smaller isogametes. These larger cells comprised about 7 percent of the total number of motile cells, but they were not observed to fuse with the smaller gametes or to develop into a zygote.

The position of the coupled isogametes on the agar was marked on the slide after they ceased to be motile. Sixteen to twenty hours later a spherical cell about 10 μm in diameter was observed in this position. This cell grew larger and darker, reaching 40-80 μm in diameter on the 8th to 10th day after plasmogamy. Frequent observations were made of the zygote as it became darker and larger with time. Sometime between the 8th and 10th day, filaments 5-7 cells in length could be seen projecting from opposite sides of the zygote. The filaments from the zygote continued to grow and branch and eventually developed into clones indistinguishable from the original filaments (Plate II, Figure C). Observation of the actual initial bipolar eruption of the filaments from the zygote was never achieved despite repeated and frequent observation. It was determined that this developmental process takes less than two hours, and it appears likely that this process occurs very quickly, perhaps in only a few minutes. If the germination process took much longer than this it probably would have been observed.

Swarmers were also transferred in an aliquant of sterilized tap water to an agar slant tube. Tubes inoculated in this manner produced clumps of filaments that were morphologically indistinguishable from the original ones. When fragments of these clumps were transferred to a slant tube free of any organic carbon source, gametangia were produced. The swarmers from these gametangia were observed fusing, and the zygote with the subsequent bipolar germination developed from the fused isogametes.

DISCUSSION

Growth on the Defined Medium

The defined medium consisted of Bristol's solution with Arnon's trace element solution, 0.5 g/l of NH_4Cl, and 20 g/l of dextrose added. Although the cultures of Trentepohlia (I.U. 1227) were maintained on this medium for 19 months, growth was only approximately 85 percent of that obtained on the original Trebouxia medium and it was necessary to transfer the cultures every 50 to 60 days rather than at 110 day intervals. The green pigmentation characteristic of the cultures in the exponential growth phase began to fade at about 50 days or at the beginning of the static phase. Fat globules began to form at this time and increased in number during the static growing phase. This change in pigmentation, the accumulation of fat globules and the slight decrease in dry weight during the static phase all suggest senescence.

Hale (1967) reported that most strains of Trebouxia are sensitive to changes in nitrogen source, and Spoehr and Milner (1949) determined that the percentage composition of lipids, carbohydrates, and proteins change as cultures of Chlorella spp. age, and that lipid content increases under conditions of nitrogen starvation. It has been found that algae in nitrogen deficient media accumulate carotenoids

and lipids, and that in algae growing exponentially in culture there is little accumulation of reserve products (Fogg, 1967). This would seem to suggest that the nitrogen from NH_4Cl is assimilated and used more quickly than nitrogen from proteose peptone or urea. Ahmadjian (1967b) noted that in a lichenized state Trentepohlia does not form much carotenoid pigment. In contrast Uyenco (1965) observed the lipid globules with the associated carotenoid pigments in the lichenized alga but the alga in culture lacked these globules and was described as bright green. Since the carotenoids in Trentepohlia (I.U. 1227) seem to appear in conjunction with fat globules, the possibility is suggested that the accumulation of both components is triggered by nitrogen starvation, and that perhaps the mycobiont or associated organisms may fix nitrogen, supplying the phycobiont with nitrogen. Uyenco (1965) noted that several lichen thallii in the genus Coenogonium have an inner layer of Trentepohlia but in addition have cephalodia containing clones of blue-green algae. Since nitrogen fixation has been demonstrated in several genera of blue-green algae, the cephalodia may be a source of nitrogen.

The cultures of Trentepohlia (I.U. 1227) in the defined medium began to senesce after 50 days, but the alga in the Trebouxia medium maintain their biomass (as measured by dry weight), and show less development of the carotenoid pigments and fat globules during the static growth phase

(Figure 1). In addition to the more rapid utilization of nutrients in the defined medium, the more rapid decline in the defined medium may be due to it being a poorly buffered medium, suggesting that proteose peptone or soil-water extract or both may function as a buffer, or supply some undetermined micronutrient, or it may indicate that proteose petone supplies nitrogen to the alga at a slower rate than NH_4Cl.

It is not possible to generalize on the utilization of nitrogen compounds by lichenized algae, since most of the nutritional studies of phycobionts have been done with strains of Trebouxia. However, Ahmadjian (1967b) noted that proteose peptone may also act as a carbohydrate source. In Trentepohlia (I.U. 1227) growth is more pronounced with a source of organic nitrogen only if a carbon source is also added. This report appears to be the first nutritional investigation of a member of the genus Trentepohlia and the first time a member of this genus has been grown in axenic culture on a defined medium.

Nutritional Characteristics

Ammonium chloride is a better substitute for proteose peptone than is urea (Table 5). The cultures of Trentepohlia (I.U. 1227) in urea turned orange-red in less than a week, while the cultures in the medium with NH_4Cl did not turn orange-red until approximately 50 days after transfer.

However, Ahmadjian (1967b) reported that urea can be stored by lichen fungi and when growth conditions are favorable it can be used as a source of nitrogen by both symbionts. The conclusions drawn from these data are that Trentepohlia (I.U. 1227) grows better when there is an abundant supply of exogenous nitrogen, and unlike Trebouxia, it is capable of using either an inorganic or an organic source of nitrogen equally well. Other phycobionts such as Coccomyxa, Chlorella, Hyalococcus, and Stichoccus show no clear preference for organic rather than inorganic sources of nitrogen (Ahmadjian, 1967b).

Trentepohlia (I.U. 1227) is a facultative heterotroph, exhibiting more growth when sugar and light are available (Table 5). The amount of growth in a medium with sugar and light is greater than the sum of the growth in a sugar medium in the dark and in a medium without sugar in the light. Facultative heterotrophy is common among green lichen phycobionts and lichen phycobionts commonly grow well in complete darkness (Ahmadjian, 1967b). A similar phenomenon was noted in Trebouxia where growth was stimulated by an exogenous carbon source. Light was not essential but growth of Trebouxia was greater with sugar in the light. It is not known whether the increased growth in the presence of a carbon source in the light is due to an increased rate of sugar uptake from the medium or the added effect of CO_2 assimilation (Ahmadjian, 1967b). Bennett and

Hobbie (1972) found that light had no effect on the total uptake of glucose in Chlamydomonas but did reduce the percentage of $^{14}CO_2$ evolved from 61 percent of the total taken up in the dark to 0 percent at 220 foot-candles. They stated that this decrease could be due to either preferential use of the $^{14}CO_2$ in photosynthesis or of the photosynthate in cellular respiration.

Hellebust (1971) found that dextrose uptake by the diatom Cyclotella was controlled by a dark induction and light inactivation of the glucose transport system. Lylis and Trainor (1973) reported that Cyclotella meneghiniana grew heterotrophically in darkness on dextrose. In continuous light of an intensity greater than 300 foot-candles, growth was not enhanced by dextrose, but under diurnal conditions 12-14 hours of darkness were required for dextrose to enhance growth. Saito (1975) found that in three strains of Gonium, growth was supported by D- and L- lactate in continuous light; however, in darkness these strains could utilize the L- lactate but not the D- lactate. No explanation is offered for the increased growth of Trentepohlia (I.U. 1227) with a carbon source in the light, but it seems probable that both sources of energy must be present to activate all metabolic pathways in this organism.

Trentepohlia (I.U. 1227) grew well only on arabinose, dextrose, and ribose. On six other sugars little or no significant additional growth occurred when compared with

the control (defined medium without sugar). No explanation for this specificity for a carbon source is known. However, specificity for carbon sources among the algae has been reported by other investigators. Lylis and Trainor (1973) found that Cyclotella meneghiniana grew heterotrophically in darkness on dextrose, but 20 other carbohydrates they tested did not promote growth. Saito (1975) found different amounts of growth in Gonium spp. with different stereoisomers of the same sugar. Ahmadjian (1967b) observed that in Trebouxia dextrose and fructose promoted the most growth; less growth was noted with galactose, sucrose, maltose, and manitol.

If proteose peptone acts as a carbon source for Trentepohlia (I.U. 1227), as has been reported for other genera by Ahmadjian (1967b), then it only acts as a carbon source in the light, for no growth was measured in the dark grown cultures with proteose peptone. This suggests that proteose peptone acts only as a nitrogen source.

Inhibition of growth by carbohydrates has been reported by Wu, Alston and Mabry (1968, 1970) who found that the green alga Chlorococcum echinozygotum was inhibited by an autoclaved solution of xylose at a concentration of 6 g/l. They demonstrated that when xylose was autoclaved it was broken down into several derivatives, one of which, xylulose, was the inhibitory agent. It was found that it inhibited CO_2 uptake after it enters the cells of the

organism, where it is converted to xylulose 1,5-diphosphate which acts as a competitive inhibitor of carboxydismutase. Gross (1968) found that D-mannose and related derivatives blocked autotrophic and heterotrophic growth of several species of Chlorella. One of the sugars tested in this study was xylose. It is possible that xylose and other sugars do not only fail to promote growth but inhibit growth in Trentepohlia (I.U. 1227). This possible inhibitory action was not investigated in this study.

Pigment Content

The pigment content of Trentepohlia (I.U. 1227) varies with both the light regime and the culture medium (Table 7). Brown and Richardson (1968) have found that the amount of chlorophyll produced by several species of algae in culture varies directly with the light intensity. The hypothesis that the amount of chlorophyll produced varies directly with the amount of light energy received by the organism, whether from higher intensity illumination or longer length of exposure needs to be examined. In this study the total amount of chlorophyll as a percentage of dry weight did not appear to be different in constant illumination, under a 12 hour photoperiod, or in different culture media, however the total amount of chlorophyll produced was greater in the cultures in constant illumination than in the cultures in a 12 hour photoperiod.

Variations in pigment composition with cultural conditions have been noted by Varasova et al. (1965). Varasova examined the pigment composition of 20 species of 3 genera of protococcal algae and found that the general pigment composition as measured by dry weight varied for all 3 genera within the same limits and was not highly variable for a single species. The influence of pH and darkness was also investigated. It was found that the more highly oxidized pigments showed the greatest stability under adverse conditions, and were more slowly destroyed than the less oxidized pigments.

A qualitative determination of the carotenoids in Trentepohlia (I.U. 1227) yielded alpha and beta carotene, neurosporene, violaxanthin, lutein and an unidentified pigment band which may be an isomeric form of beta carotene. The variability of the percentage composition of these pigments in culture was not investigated in this study, but visual observation of the cultures suggest that carotenoids become more abundant under adverse conditions when the nutrients in the medium becomes depleted or the organism begins to dry out. E.A. George (written communication, 1970) also observed that on aging and drying out Trentepohlia spp. become the orange-red typical of natural free-living species.

Des Abbayes (1951) noted that in a lichenized state Trentepohlia forms only a small amount of beta carotene.

This suggests that physiologically the phycobiont is exposed to environmental conditions much like those in the culture medium during the exponential growth phase, where there is an abundant supply of nutrients. Ahmadjian (1967b) observed that Trebouxia forms little carotenoid pigment on an organic medium but dense carotenoids are observed in a mineral medium. Ahmadjian suggests two explanations. Either the demand for organic compounds by the mycobiont or the phycobiont is great enough to interfere with the normal metabolic processes involved in pigment formation, or the fungus exerts an inhibitory action on the phycobionts capacity for pigment synthesis. The former seems more likely considering the reduction in pigmentation on mineral media. Uyenco (1965) did not grow Trentepohlia on a strictly mineral medium, but inferred that Trentepohlia formed less carotenoid pigment in a soil-water medium, than it did when part of a lichen thallus. Considering Ahmadjian's suggestion, this would seem to indicate that one function of the soil-water extract is to provide a source of organic nitrogen or of preformed organic carbon, but it is also possible that soil-water extract functions as a buffer or a source of some inorganic nutrient such as iron.

Lipid Content

A determination of the lipid content of Trentepohlia (I.U. 1227) revealed that in healthy cultures (harvested during the exponential growth phase) lipids comprise 17 percent of the dry weight. These data compare well with those reported by Moore (1975), who found that lipids in Ulothrix zonata, Cladophora glomerata, and Spirogyra sp. composed 11.9-16.1 percent of the dry weight. In Scenedesmus dimorphous and Cosmarium laeve lipids made up 22.5-25.9 percent of the dry weight. In Trentepohlia (I.U. 1227) approximately 90 percent of the lipid fraction is triglycerides, with smaller amounts of fatty acids, diglycerides, sterols, glycolipids, and phospholipids.

Pigmented oil globules were obvious from microscopic examination of the cells of Trentepohlia (I.U. 1227). The oil globules in this genus have previously been reported by Howland (1929). She noted that these globules were orange-red in color, probably from carotenoids. This was also the case with Trentepohlia (I.U. 1227). The oil globules appeared to increase in size and number as the culture aged, but it should be noted that this increase could be an apparent one because the globules become more obvious as the green pigmentation disappears from the chloroplasts. It seems reasonable to assume that these globules of triglyceride are food reserves which accumulate under certain

environmental conditions favorable for the formation of triglycerides, specifically nitrogen starvation. However, Jacobs and Ahmadjian (1969) found that Trebouxia phycobionts accumulated lipid containing globules and suggested that the lipids represent a type of storage product that is more suitable to the lichen symbiosis than starch. In arid climates, lichens have an adequate water balance for metabolism for only a few hours a day, and lipids represent a storage product which could give the greatest amount of energy in the shortest amount of time.

Fatty Acids

Parker et al. (1967) found that in each of 11 species of blue-green algae the fatty acid composition was unique and reproducible. However, Moore (1975) reported that in five species of green algae the seasonal variation of fatty acids within taxa was greater than the differences between taxa. The fatty acid composition of Trentepohlia (I.U. 1227) does not vary greatly with environmental conditions (Table 9). The most common fatty acid in Trentepohlia (I.U. 1227) was oleic ($C_{18:1}$) followed in decreasing order by $C_{16:3}$, hexadecenoic ($C_{16:1}$), palmitic ($C_{16:0}$), and myristic ($C_{14:0}$). The most significant change in the fatty acid composition was observed when sugar was omitted from the medium. The percentage of oleic acid was significantly less and the percentage of $C_{16:3}$ fatty acid increased

significantly when sugar was omitted from the medium. The $C_{14:1}$ fatty acid was observed only in those cultures that did not contain sugar.

Varying the light regime or the source of nitrogen did not appear to have an appreciable effect on the content of fatty acids. Even in cultures grown in the dark the composition appeared to be similar to that of the cultures grown under constant illumination or a 12 hour photoperiod. The percentage of oleic acid is a little lower in the dark grown cultures, but replicate experiments would be needed to determine if the difference is significant.

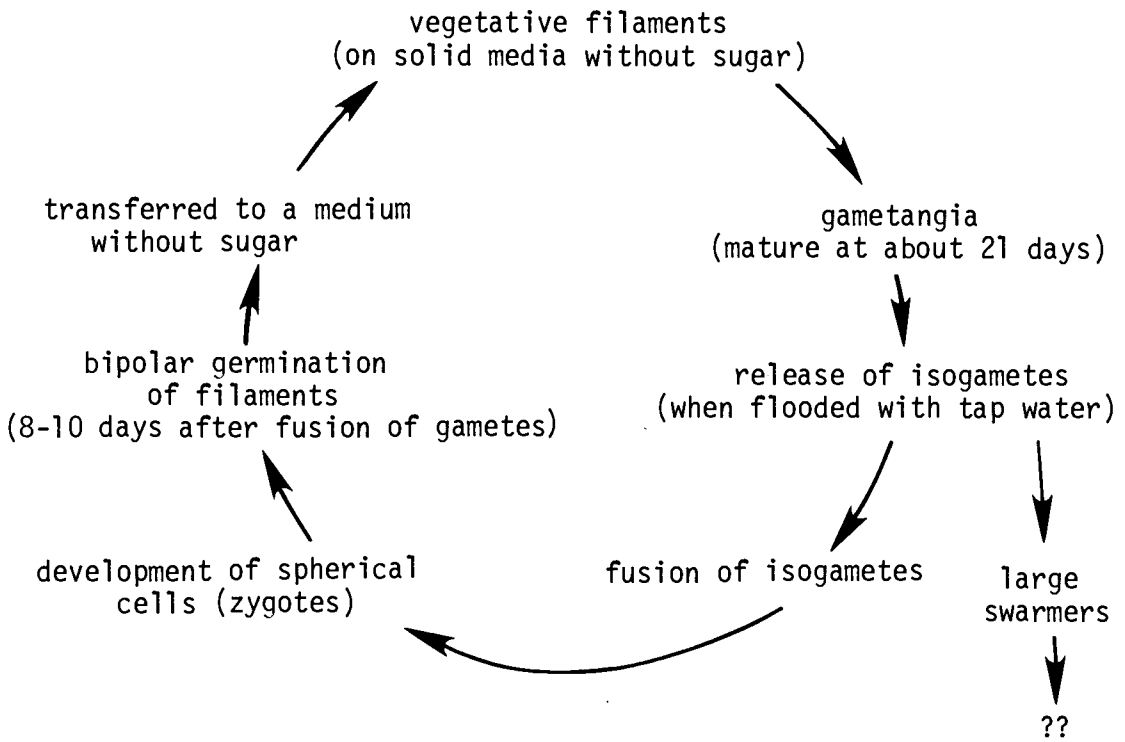
Moore (1975) reported $C_{18:1}$ and $C_{16:1}$ as the predominant fatty acids in five species of green freshwater algae, and significant amounts of $C_{16:0}$, $C_{14:0}$, $C_{18:2}$, $C_{18:3}$, and $C_{16:1}$ were reported. In Trentepohlia (I.U. 1227) $C_{18:1}$ was also the predominant fatty acid, and significant amounts of $C_{14:0}$, $C_{16:1}$, and $C_{16:0}$ were detected, but only small amounts of $C_{18:2(3?)}$. The $C_{16:3}$ fatty acid which was 25.1-26.6 percent of the total when dextrose was present was not reported by Moore.

Demort et al. (1972) found 28 fatty acids in 10 species of estuarine phytoplankton: $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ were common to all species. The chlorophyta were distinguished by large amounts of the C_{18} acids and high percentages of $C_{20:5}$. Only a trace of $C_{18:3}$ was observed in Trentepohlia (I.U. 1227) and no

$C_{20:5}$ was detected. Moore (1975) also reported significant amounts of $C_{18:3}$ but only a trace of $C_{20:5}$ in algae collected in the spring. The data of Demort et al. and Moore suggest that fatty acid composition may be altered by the habitat as well as the season. It would be interesting to compare the fatty acid composition of several Chlorophyta from diverse habitats such as lichen phycobionts, aerial algae, freshwater phytoplankton, and marine phytoplankton.

Morphology and Sexual Reproduction

Asexual reproduction was not observed but, from observations of the development of Trentepohlia (I.U. 1227) in culture it is possible to construct a reasonably definitive sexual life cycle:



Parthenogenetic development of isogametes into new filaments has been reported for this genus by Printz (1939), but Smith (1950) indicates that reports of such development should be accepted with caution, unless the zoospores can be distinguished morphologically from the isogametes. Parthenogenetic development in Trentepohlia (I.U. 1227) cannot be ruled out, but in light of the observations of this species and the above proposed life cycle, it does not appear probable. All observed germinations of new filaments were initiated bipolarly from the zygote, and all filaments developing in this manner were capable of producing isogametes. Only one report of the direct observation of the fusion of isogametes has been found in the literature, and it is likely that sporangia have often been confused with gametangia. By altering the photoperiod Uyenco (1965) induced the liberation of zoospores from sporangia 4-7 days after the transfer of the alga from one culture medium to a fresh one. He was unsure whether the ellipsoidal reproductive structure were gametangial or sporangial in nature, but reported observing, "biflagellated zoospores and isogametes (in various stages of fusion)."

The development of sporangia or gametangia under conditions of stress would seem to have an adaptive advantage. However, in a subaerial alga such as Trentepohlia, which has been reported to survive extreme desiccation in the

vegetative phase and which requires moisture for motile isogametes or zoospores, reproduction seems to occur when environmental conditions are near optimum (Howland, 1929). Howland also reported that inadequate moisture inhibited the development of sporangia (gametangia ?) and that temperature and moisture were critical for the release of zoospores (isogametes ?). Ahmadjian (1967b) has noted that green algae do not usually produce zoospores or gametes when the alga is part of a lichen thallus, but Trentepohlia is an exception, and sporangia have often been observed in this genus when it is part of a lichen thallus.

The production of gametangia only in the absence of a source of organic carbon in this species remains unexplained. If indeed organic carbon is dervied from the mycobiont then sexual reproduction may be a means of dispersal when the symbiotic relationship is stressed. Direct observation of the lichen in nature may help to explain this relationship.

Howland (1929) reported variation in the position, size, and shape of the sporangia (gametangia ?) in Trentepohlia. In Trentepohlia aurea she noted four types of sporangia. In the present study of Trentepohlia (I.U. 1227) there were numerous variations in gametangial types. In fact each gametangium seemed to be unique. This may be a result of stress induced by an artifical environment in

the culture tube or flask. Direct observation in the natural environment would be necessary to determine if this variation in gametangia actually occurs. It has been shown that filaments isolated from a lichen thallus and grown in culture are morphologically distinct from the filaments when they are part of the lichen thallus (Uyenco, 1965). Since the mycobiont seems to influence the morphology of the phycobiont, the question of whether the morphological characteristics of Trentepohlia (I.U. 1227) in a lichenized condition or in culture are taxonomically definitive or "typical" would appear to be moot. Even though the shape of the gametangium is variable, the position is always the same. They are borne laterally on a single stalk cell, never in a terminal or intercalary position on the axis.

No explanation of the large swarmers is apparent. They were not observed functioning as isogametes, and their ultimate fate is unknown. It is possible by flooding the organism with tap water that some of the gametangia released isogametes prematurely (before gametogenesis was complete) and these larger swarmers had failed to undergo as many divisions as the smaller, presumably normal swarmers.

SUMMARY

1. Cultures of Trentepohlia (I.U. 1227) were maintained on a defined axenic medium for 19 months.
2. Trentepohlia (I.U. 1227) grew well on both organic and inorganic sources of nitrogen. Comparable growth was achieved with NH_4Cl (0.4-0.7 g/l) and 10 g/l proteose peptone. Cultures with media containing urea did not support as much growth as those containing proteose peptone or NH_4Cl when soil-water extract was not included in the medium.
3. Trentepohlia (I.U. 1227) is a facultative heterotroph. Dextrose, ribose, and both steroisomers of arabinose enhanced growth in the dark and in the light, but optimum growth was obtained with a sugar source in the light. Six other sugars tested failed to promote growth.
4. The data indicate that proteose peptone acts only as a source of nitrogen, and does not provide organic carbon to Trentepohlia (I.U. 1227).
5. The ratio of chlorophyll a to b was affected by light regime and the source of nitrogen, but the total amount of chlorophyll as a percentage of dry weight remained constant under all imposed conditions.
6. The carotenoids in this organism were determined to be alpha and beta carotene, neurosporine, violaxanthin,

and lutein.

7. Observable quantities of carotenoids and fat globules, which are presumably a food reserve, are probably present in significant amounts only when the organism is subjected to nitrogen starvation.
8. The lipids made up approximately 17 percent of the dry weight of Trentepohlia (I.U. 1227). Triglycerides composed about 90 percent of the total lipid fraction, and fatty acids, sterols, diglycerides, glycolipids, and phospholipids made up the remainder.
9. Of the 14 fatty acids isolated from this organism, oleic acid was the most abundant under all imposed cultural conditions; significant amounts of $C_{14:0}$, $C_{16:1}$, and $C_{16:0}$ were also noted.
10. The fatty acid composition did not vary greatly with the nitrogen source, but the percentage of oleic acid $C_{18:1}$ was greatest in the cultures in a 12 hour photoperiod, less in those under constant illumination, and least in those cultures in darkness.
11. A significant difference in the fatty acid composition was observed when sugar was deleted from the medium. This reduced the amount of oleic acid by 20-35 percent and the percentage of $C_{16:3}$ nearly doubled. The $C_{14:1}$ fatty acid was observed only in cultures without sugar.

12. The development of gametangia was triggered by growing the alga on solid media without a source of organic carbon.
13. The isogametes could be released by flooding the medium with sterilized tap water, after a minimum of 21 days following the transfer of the alga to a medium without sugar.
14. The fusion of the biflagellated isogametes and the subsequent development of the zygote with bipolar development of new filaments were observed.
15. The filaments developing from zygotes were observed to develop gametangia, and the subsequent release of isogametes, formation of a zygote and development of filaments were noted.
16. The size and shape of the gametangia were highly variable but they were always borne laterally on a single stalked cell.
17. Large swarmers composed seven percent of the swarmers released. They were not observed to function as gametes, and their ultimate fate and function are not known.

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