Plant Physiol. (1974) 54, 129–132

Effects of Cation Levels of the Nutrient Medium on the Biochemistry of *Chlorella*

I. CONCENTRATION SERIES[1]

Received for publication February 5, 1973 and in revised form April 5, 1974

JEAN J. MACCARTHY AND GLENN W. PATTERSON
Department of Botany, University of Maryland, College Park, Maryland 20742

ABSTRACT

The effects of variations in nutrient cation levels on the growth and biochemistry of *Chlorella* were investigated. This study involved concentration-series experiments in which the levels of Mg^{2+}, K^+, and Ca^{2+} varied from deficiency to toxicity levels for growth. The nutrient sufficiency concentrations of Mg^{2+} and K^+ were 0.08 and 0.10 meq/l, respectively. Deficiencies of Mg^{2+} or K^+ reduced the growth rate, as well as cellular total nitrogen and unsaturated fatty acid levels. K^+ deficiency increased total lipid levels, while total fatty acids were unaffected. Increasing Mg^{2+} or K^+ concentrations in the nutrient media were accompanied by corresponding increases in growth rate and certain biochemical fractions. Calcium was without effect except at a toxicity level. Cellular sufficiency concentrations for Mg^{2+} and K^+ were 0.3 and 1.2% of the dry weight, respectively.

Algae have been recognized as a potential source of food (8) and as possible bioregenerative life support systems for space vehicles (6). The mineral nutrition of these organisms is of considerable interest. Although some work has been done in this field, more extensive studies are necessary to resolve algal nturient requirements and the effect of nutrition on algal growth and metabolism.

A mineral nutrition experiment was initiated to determine the precise cation requirements of *Chlorella sorokiniana* and the effect of cation concentration on the biochemistry of *Chlorella*. The first part of this study involved concentration-series experiments. There were three separate concentration series, *i.e.*, a Mg^{2+} series, a K^+ series, and a Ca^{2+} series. The response of *Chlorella* to a wide range of cation concentrations was determined in each series, and cells grown in each culture treatment were analyzed for total nitrogen, elemental accumulation, and lipid content. The concentration-series experiments were also an essential preliminary investigation and provided pertinent data on sufficiency and deficiency values of Mg^{2+} and K^+ for the second part of this study, a factorial experiment (4).

MATERIALS AND METHODS

Experimental Design. Concentration-series experiments were set up in duplicate for Ca^{2+}, Mg^{2+}, and K^+. In each series experiment the level of the cation being tested was varied from deficiency through the optimum levels for growth and on up to a toxicity level (Table I). The levels of all other elements in the nutrient medium remained constant and sufficient, with the exceptions of Na^+ and Cl^-, which were permitted to vary as required in the experiment.

Algal Culture Conditions. A green alga, *Chlorella sorokiniana* Shihira et Krauss (strain 7-11-05) was used. It was transferred from agar slants into sterile medium containing glucose and was precultured to an absorbance of 0.3 to 0.5 before being transferred into 350 ml of the sterile culture media described in Table I. This medium was contained in sterilized culture tubes measuring 4.5 by 43 cm with conical tips, and each culture tube was equipped with a bubbling tube and an outlet tube held in place with a silicone stopper. A carbon source was provided by a 1% CO_2 in air mixture that was bubbled through the medium and also served to keep the cells in suspension. Inoculated tubes were placed at random in positions in a glass water bath maintained at 38.0 ± 0.5 C. Illumination was provided at an intensity of 1600 ft-c by two banks of six 40-w Westinghouse cool-white lamps. The pH of each culture was adjusted to 6.5 with dilute NaOH.

Growth Rates The growth rates were calculated in number of doublings per day using the following formula:

$$(79.7/\Delta t)\log_{10}(N/N_o) = K$$

Where N = final absorbance, N_0 = initial absorbance, Δt = growth period in hours, and K = growth rate in number of doublings per day. Absorbance measurements were taken with a Coleman junior spectrophotometer set at 550 nm and using optically matched Pyrex glass tubes (1.7 × 14.9 cm). The initial absorbance of each culture each day (before growth) was between 0.0007 and 0.005 (calculated by dilution of a more dense suspension). The growth period was 23.5 hr, since this represented a time during which cells were growing logarithmically. Cultures were taken through 15 consecutive serial transfers over a period of 15 days, and all transfers were made aseptically in a transfer chamber. Routine checks for bacterial contamination were made using an enriched organic medium.

Harvesting Procedures. Cells for N and elemental analyses were harvested (while growing logarithmically) by centrifugation at 600*g* for 20 min. The sedimented cells were resuspended in distilled water and immediately centrifuged for an additional 20 min to remove remaining salts of the medium. This was followed by overnight drying on a freeze-dryer. Prior to elemental and N analyses, the cells were oven-dried at 60 C. Scott (7) has shown that *C. pyrenoidosa* cells can be washed up to two times in distilled water without losing a significant amount of their mineral content.

Cells for lipid analysis were also harvested (while growing

[1] Scientific Article No. 1866, Contribution No. 4776 of the Maryland Agricultural Experimental Station.

Table I. *Composition of Concentration Series Media*

Trace elements in each series: Co, Cu, Mn, Zn (as Na_2 EDTA chelates), 1 mg/l each; Mo (as MoO_3), 1 mg/l; Fe (as Na EDTA chelate), 5 mg/l.

Mg^{2+}	K^+	Ca^{2+}	Na^+	NO_3^-	SO_4^{2-}	$H_2PO_4^-$	Cl^-
meq/l							
A. Magnesium series							
0.01	0.33	0.10	5.89	5.0	1.0	0.33	0
0.05	0.33	0.10	5.85	5.0	1.0	0.33	0
0.10	0.33	0.10	5.8	5.0	1.0	0.33	0
1.0	0.33	0.10	4.9	5.0	1.0	0.33	0
10.0	0.33	0.10	0	5.0	1.0	0.33	4.1
100.0	0.33	0.10	0	5.0	1.0	0.33	94.1
200.0	0.33	0.10	0	5.0	1.0	0.33	194.1
500.0	0.33	0.10	0	5.0	1.0	0.33	494.1
1000.0	0.33	0.10	0	5.0	1.0	0.33	994.1
B. Potassium series							
1.0	0.003	0.10	5.23	5.0	1.0	0.33	0
1.0	0.016	0.10	5.22	5.0	1.0	0.33	0
1.0	0.033	0.10	5.20	5.0	1.0	0.33	0
1.0	0.16	0.10	5.07	5.0	1.0	0.33	0
1.0	0.33	0.10	4.9	5.0	1.0	0.33	0
1.0	9.33	0.10	0	5.0	1.0	0.33	4.1
1.0	99.33	0.10	0	5.0	1.0	0.33	94.10
1.0	499.33	0.10	0	5.0	1.0	0.33	494.1
C. Calcium series							
1.0	0.33	0.001	5.0	5.0	1.0	0.33	0
1.0	0.33	0.10	4.9	5.0	1.0	0.33	0
1.0	0.33	1.0	4.0	5.0	1.0	0.33	0
1.0	0.33	10.0	0	5.0	1.0	0.33	5.0
1.0	0.33	100.0	0	5.0	1.0	0.33	95.0

logarithmically) by centrifuging at 600*g* for 20 min. The wet, sedimented cells were immediately extracted for lipids.

Lipid Extraction. Sedimented cells were immediately resuspended in 2 to 5 ml of methanol and were extracted overnight with chloroform-methanol (2:1, v/v) in a Soxhlet apparatus equipped with a Whatman double thickness extraction thimble. The extract was evaporated to dryness, redissolved in chloroform, filtered into a weighed beaker, and evaporated to dryness, redissolved in chloroform, filtered into a weighed beaker, and evaporated once again to dryness. The dry extract was weighed, and this weight was considered to be the total lipid. The total lipid extract was saponified with 4% KOH solution in aqueous ethanol (80% v/v) for 45 min at 70 C. Saponified lipid in ethanol was brought up to 250 ml with water, transferred to a liquid-liquid extraction apparatus and extracted for 60 to 90 min with added anhydrous diethyl ether, then acidified with 6 N HCl, and extracted overnight. The resulting ether extract was evaporated to dryness, and the fatty acids in the extract were methylated by heating with 5 ml of BCl_3-methanol reagent, (Applied Science Labs) for 5 min. Fatty acid methyl esters were extracted by washing three times with *n*-hexane. The hexane extract was evaporated to dryness. The esters were immediately redissolved in *n*-hexane, transferred into glass vials, and sealed under nitrogen.

Fatty Acid Identification and Estimation. TLC and GLC were used in the separation and identification of fatty acid methyl esters.

TLC was used to separate esterified fatty acids into bands corresponding to their degrees of unsaturation. Thin layer plates of 12% $AgNO_3$ on Silica Gel G were spread at a thickness of 0.75 mm on glass plates. Plates were activated at 90 C for 90 min, and allowed to cool before applying fatty acid methyl esters as a narrow band. Plates were developed in 20% (v/v) anhydrous diethyl ether in *n*-hexane, air dried, and then sprayed with 0.2% dichlorofluorescein in ethanol. Separation of the esters into bands could then be observed using an UV lamp. The esters, eluted from these bands with anhydrous diethyl ether, were redissolved in *n*-hexane and chromatographed by GLC for identification by comparison with standards of known fatty acid methyl esters.

GLC was also used in the quantitative analyses. Glowall Chromalab Model A-110 and Model A-310 gas chromatographs were used. These were equipped with Honeywell recorders with disc integrators, argon ionization detectors, and columns of 1.8 m × 3.4 mm (i.d.) packed with Gas Chrom P (Applied Science Labs.) coated with 15% diethylene glycol succinate. The columns were maintained at a temperature of 160 C. Argon carrier gas pressure was 15 p.s.i. Fatty acid samples were injected into the column with a 10-μl syringe. Quantitation was achieved with a methyl stearate standard and by using the disc integrator to measure the peak areas on the chromatograms. Standards of known fatty acid methyl esters were also used for qualitative purposes.

Total Nitrogen Analysis. Total N determinations were made on samples of dried cells (not in excess of 100 mg) using the method of Ranker (5) and the Kemmerer-Hallett distillation unit (2). Salicylic acid was used in conjunction with concentrated H_2SO_4 during the digestion process. A sodium sulfate-copper sulfate-selenium mixture was used as the catalyst.

Table II. *Effect of Cation Levels on Growth and Biochemical Composition of Chlorella sorokiniana*

Level	Growth Rate	Total-Nitrogen	Total Lipid	Total Fatty Acids	Saturated Fatty Acids	Unsaturated Fatty Acids	Unsaturated Fatty Acids-Saturated Fatty Acids
meq/l	*doublings/day*	*% dry wt.*					*ratio*
Mg^{2+}							
0.01	4.7	4.9	22.5	4.3	2.8	1.2	1.41
0.05	8.5	7.1	18.7	3.8	1.7	2.1	1.2
0.10	8.7	8.9	22.5	7.9	2.2	5.7	2.6
1.0	8.6	9.0	19.4	6.4	1.8	4.4	2.5
10.0	8.6	8.9	19.2	5.6	1.6	4.0	2.5
100.0	8.6	8.4	16.9	4.3	1.5	2.7	1.7
200.0	6.9	7.2	14.8	3.1	1.3	1.8	1.4
500.0	4.6[1]						
K^+							
0.003	4.8	6.9	26.3	6.1	4.8	2.8	0.58
0.016	6.4	7.3	19.9	6.5	2.8	4.0	1.4
0.033	7.4	8.4	19.7	7.3	2.8	4.3	1.7
0.166	9.1	9.8	20.9	6.9	2.1	3.9	1.9
0.333	9.2	9.7	20.2	7.9	2.3	4.7	2.0
9.33	8.9	9.8	19.9	7.8	1.8	4.7	2.6
99.33	8.9	9.6	17.0	5.6	1.7	3.8	2.2
499.33	1.5[1]						
Ca^{2+}							
0.001	9.2	9.2	22.9	9.6	2.8	6.6	2.3
0.101	9.2	9.8	20.8	9.1	2.9	6.1	2.1
1.00	9.2	9.7	20.2	9.2	2.8	6.3	2.3
10.0	9.2	9.0	19.8	7.9	2.4	5.6	2.3
100.0	8.3	5.6	13.1	3.8	1.9	2.5	1.3

[1] One replicate only. All other samples in duplicate except growth rate data which are averages of 15 determinations.

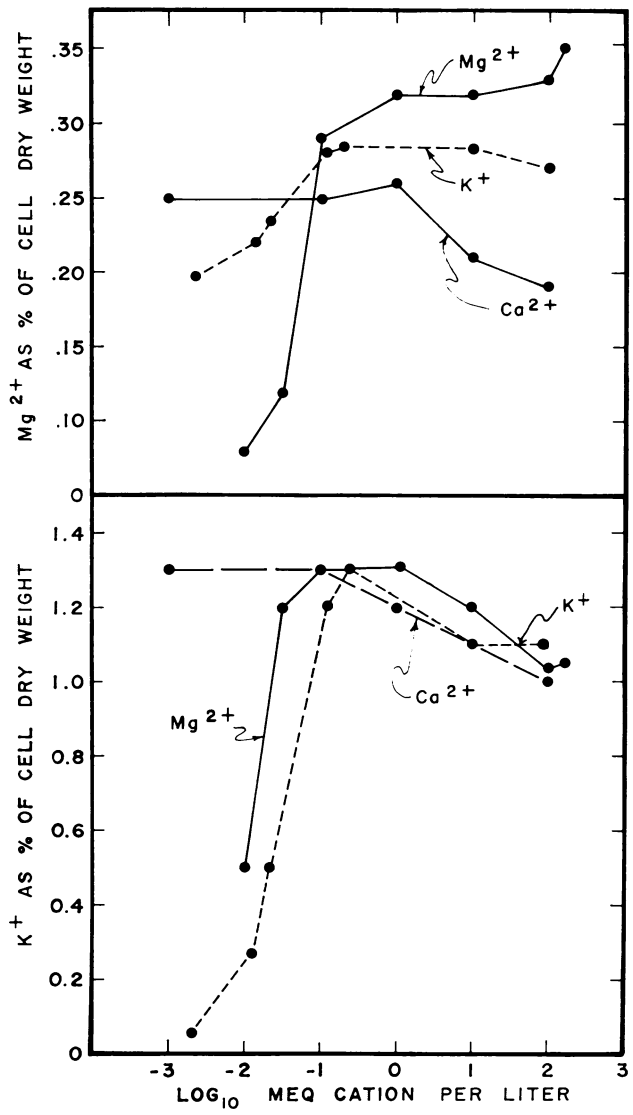

FIG. 1. Effect of nutrient concentrations of Mg^{2+}, K^+, and Ca^{2+} on levels of Mg^{2+} and K^+ as per cent dry weight of *C. sorokiniana*.

Elemental Analysis. A sample of dried ground cells (not in excess of 100 mg) was ashed in an aluminum pan overnight at 450 C in a muffle furnace. This ashed material was dissolved in 5 ml of 0.6 N HCl, filtered, and made up to 100 ml with distilled water. Mg^{2+}, Ca^{2+}, K^+, and Na^+ were determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 303 using an air-acetylene flame.

RESULTS AND DISCUSSION

Mg^{2+} Series. At the lowest level of Mg^{2+}, the growth rate was 4.7 doublings per day (Table II). Elevating the Mg^{2+} level increased the growth rate to a maximum of about 8.6 doublings per day. This growth rate was maintained over a wide range of Mg^{2+} concentrations, to a toxic level of 200 meq/l. A plot of these data indicates that for the growth of *C. sorokiniana* the critical concentration of Mg^{2+} is 0.08 meq/l (3). (Gauch has suggested that sufficiency value may be a more appropriate term than the formerly used "critical concentration" [1]). In this paper, the sufficiency value is defined as the lowest concentration of an element giving maximal or near maximal growth.

Cellular levels of Mg^{2+}, K^+ (Fig. 1), total N, total fatty acids, and unsaturated fatty acids (Table II) were reduced under conditions of Mg^{2+} deficiency. As the nutrient medium Mg^{2+} concentration was raised, the level of each of these fractions increased and remained high over a wide range of Mg^{2+} concentrations. At Mg^{2+} toxicity the level of each fraction (with the exception of Mg^{2+} levels in cells) dropped again. The unsaturated fatty acid-saturated fatty acid ratio was likewise affected. Saturated fatty acid levels were reduced by increasing Mg^{2+} concentration, whereas total lipid levels were unaltered. However, total lipid was reduced at Mg^{2+} toxicity.

The cellular sufficiency level of Mg^{2+} was determined as 0.29% of the dry weight (Fig. 1).

K^+ Series. The growth rate was 4.8 doublings per day at the lowest K^+ concentration. Increasing nutrient medium K^+ levels were accompanied by increasing growth rates until the nutrient solution K^+ sufficiency level was reached (0.10 meq/l) and no further increases in growth rate were experienced (3). At K^+ toxicity (499 meq/l), growth rate declined sharply (Table II).

Cellular levels of Mg^{2+}, K^+ (Fig. 1), total N, and unsaturated fatty acids (Table II) were low when K^+ was deficient. Increasing nutrient medium K^+ concentrations were reflected by increases in each of these fractions. The unsaturated fatty acid-saturated fatty acid ratio (low when K^+ was deficient) also followed this trend. The highest levels of total lipid and saturated

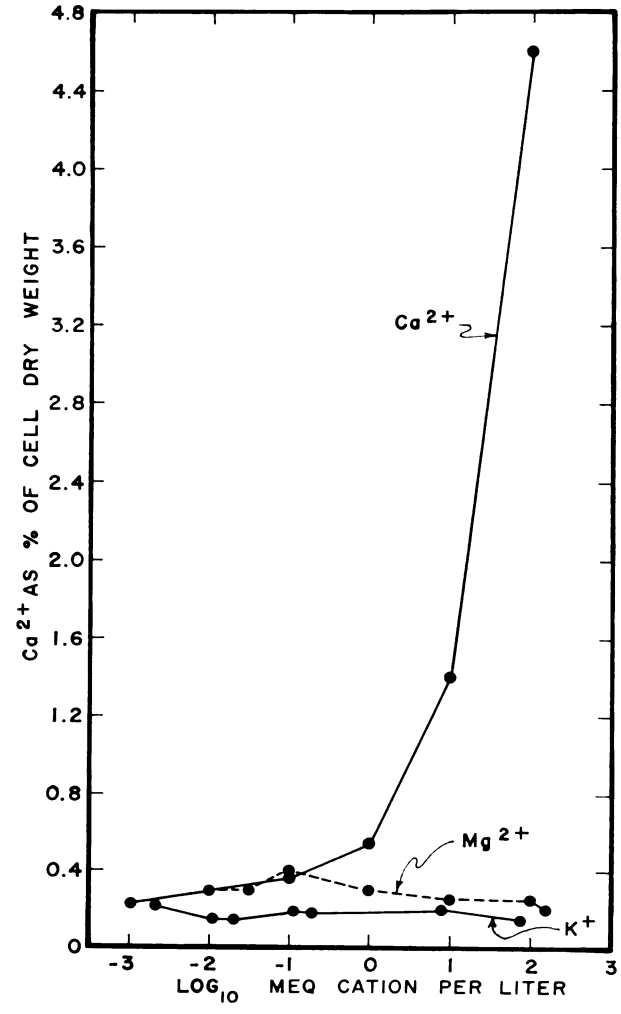

FIG. 2. Effect of nutrient concentrations of Mg^{2+}, K^+, and Ca^{2+} on the level of Ca^{2+} as per cent dry weight of *C. sorokiniana*.

fatty acids occurred when K^+ was deficient. The cellular sufficiency concentration of K^+ was 1.2% of the dry weight (Fig. 1).

Ca^{2+} Series. Ca^{2+} had no effect except at toxicity (100 meq/l). Here, there was an increased assimilation of Ca^{2+} (Fig. 2), accompanied by a reduction in growth rate and other cellular fractions (Table II).

Data from the Mg^{2+} and K^+ series experiments indicate that when either of these elements is deficient in the culture medium, normal metabolic routes are altered. This is shown by reductions in total N and unsaturated fatty acids. The unusually high levels of total lipid and saturated fatty acids produced under K^+ deficiency conditions reinforces this conclusion. Maximal cellular levels of total N, total fatty acids, and unsaturated fatty acids were attained by cells grown in the range of Mg^{2+} or K^+ concentrations giving optimal growth.

Acknowledgment—The authors wish to thank Professor H. G. Gauch for his interest and helpful suggestions during the research and writing of the manuscript.

LITERATURE CITED

1. GAUCH, H. G. 1972. Inorganic Plant Nutrition. Dowden, Hutchinson and Ross, Stroudsburg, Pa. p. 431.
2. KEMMERER, G. AND L. T. HALLETT. 1927. Improved micro-kjeldahl ammonia distillation apparatus. Ind. Eng. Chem. 19: 1295–1296.
3. MACCARTHY, J. J. 1972. The effect of nutrient medium cation levels on the biochemistry of *Chlorella*. Ph.D. dissertation. University of Maryland, College Park.
4. MACCARTHY, J. J. AND G. W. PATTERSON. 1974. The effect of cation levels of the nutrient medium on the biochemistry of *Chlorella*. II. The factorial experiment. Plant Physiol 54: 133–135.
5. RANKER, E. R. 1927. A modification of the salicylic-thiosulfate methods suitable for the determination of total nitrogen in plants, plant solutions, and soil extracts. J. Assn. Off. Agric. Chem. 10: 230–251.
6. RICHARDSON, B., D. M. ORCUTT, L. ROSNER, AND R. L. MILLER. 1969. Some characteristics of *Oocystis polymorpha*, a thermotolerant green alga suitable for mass culture. Sam-Tr-69-3, USAF School of Aerospace Medicine, Aerospace Medical Division (AFSC), Brooks Air Force Base, Texas.
7. Scott, G. T. 1944. Cation exchanges in *Chlorella pyrenoidosa*. J. Cell. Comp. Physiol. 23: 47–58.
8. SPOEHR, H. A. 1951. *Chlorella* as a source of food. Proc. Amer. Phil. Soc. 95: 62–67.