PRODUCTION OF EXTRACELLULAR CARBOHYDRATES BY THE DIATOM, CYLINDROTHECA FUSIFORMIS REIMANN & LEWIN VAR. FUSIFORMIS

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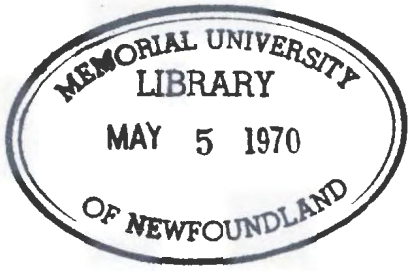
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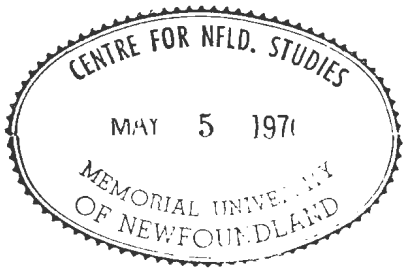
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PRODUCTION OF EXTRACELLULAR CARBOHYDRATES BY THE DIATOM, CYLINDROTHECA FUSIFORMIS REIMANN & LEWIN VAR. FUSIFORMIS

by

© Kwan-shong Young, B.Sc.

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ABSTRACT

The production of extracellular carbohydrate has been studied in the diatom, Cylindrotheca fusiformis Reimann & Lewin var. fusiformis, in bacteria-free culture. The concentration of extracellular carbohydrates was found to increase as the light intensity was decreased. An increase was also observed in connection with nitrogen-deficient cultures. Experiments indicated that extracellular carbohydrate production did not parallel the growth of the diatom. Higher levels of carbohydrate production were found during the early stages of growth and during the post-exponential phases. Carbohydrate production drop to a low level during the exponential phase of growth. The carbohydrates identified by thin layer chromatography were found to be the low molecular weight compounds: glucose, galactose, arabinose, xylose and galacturonic acid. It is suggested that the production of most of the extracellular carbohydrate may possibly be due to diffusion and to passive release from dead cells.

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The writer wishes to thank Dr. Robert R. L. Guillard of the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, who kindly provided the experimental diatom culture.

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INTRODUCTION

That natural waters may contain appreciable, though variable, quantities of dissolved organic materials has been known for a number of years. These substances have been studied by, among others, Vallentyne and Bidwell (1956), Vallentyne and Whittaker (1956), Duursma (1960), Williams (1961), Menzel (1964), and Ryther and Menzel (1965).

Jeffrey and Hood (1958) postulated that dissolved organic matter in natural waters originated from four main sources: 1) elaboration by living organisms, as extracellular metabolites and as excretory products, 2) decomposition of plants and animals, 3) leaching from soil, and 4) exchange or decomposition processes in sediments.

Algae, as important inhabitants of aquatic environments, excrete various kinds of metabolites into natural waters and into experimental culture media. Chemically, these substances have been identified as organic acids (Allen, 1956; Tolbert and Zill, 1956; Fogg and Watt, 1965), organic bases (Wangersky and Guillard, 1960), carbohydrates (Lewin, 1956; Jones, 1962; Moore and Tischer, 1964; Marker, 1965), nitrogenous compounds (Fogg and Boalch, 1958; Stewart, 1963; Hellebust, 1965), enzymes (Miller, 1959), volatile compounds (Armstrong and Boach, 1960), and vitamins (Nakamura and Gowans, 1964). Such substances, when liberated into the natural environment, are of importance, ecologically. They may serve as energy sources for bacteria, algae, zooplankton and for symbiotic relationships. Extracellular products of algae may also exert growth-promoting or inhibiting effects on other organisms. The ecological

effects of extracellular metabolites in natural waters have been reviewed by Lucas (1947, 1949, 1961), Saunders (1957), and Fogg (1962, 1966).

The present work continues from a study of diatom-bacteria interactions, by Moskovits (1961), in which he showed that the diatom, Nitzschia closterium (Ehr.) Wm. Smith appeared to be able to provide organic materials for bacterial growth. Since carbohydrates constitute an energy source readily utilizable by bacteria for growth, and since there is evidence for the production of extracellular carbohydrates by algae, it was considered of interest to investigate the nature and the quantities of extracellular carbohydrates produced by the marine diatom, Cylindrotheca fusiformis Reimann & Lewin var. fusiformis (formerly Nitzschia closterium (Ehr.) Wm. Smith), during the different phases of its growth and under different environmental conditions. Extracellular carbohydrates, here, refer to carbohydrates which can pass through a Morton bacterial filter (Morton, 1944) and which also give color reactions with anthrone reagent.

MATERIALS AND METHODS

The Experimental Diatom

1. The Organism and Its Source

The experimental organism used was the diatom, Cylindrotheca fusiformis Reimann & Lewin var. fusiformis[1], (clone MNC), obtained from Dr. R. R. L. Guillard of the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts[2]. The taxonomic status of this diatom was discussed by Reimann and Lewin (1964).

2. Culture Medium

Natural sea water has a complex and variable composition as regards organic and some inorganic compounds. This prevents obtaining consistent and reproducible results in experimental work. Another source of difficulty lies in the fact that additions of enrichment compounds to sea water, together with treatments such as autoclaving, bring about precipitate formation which also alters composition. Because of the need for a sea water whose composition had to be known and constant from time to time, and from which it was necessary to exclude all organic compounds except those deliberately added, it was decided to use synthetic sea water for all experimental work.

The synthetic sea water used was that employed by Moskovits (1961) in his experiments with Nitzschia closterium (Ehr.) Wm. Smith.

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[1]Originally designated as Nitzschia closterium (Ehr.) Wm. Smith.

[2]Originally isolated from the Sandy Hook, New Jersey, marine littoral by Dr. M. Maddux.

It was prepared in four separate parts: synthetic sea water base; buffer; inorganic enrichment; trace elements and chelator. All compounds used were analytical grade reagents, weighed on an analytical balance. Solutions were prepared with distilled water, completely dissolving one reagent before adding the next. In use the four components were added together, and the pH adjusted. The final solution is here referred to as "complete synthetic sea water".

a. Synthetic sea water base

The synthetic sea water base consisted of the major (conservative) elements as found in natural sea water. It was prepared according to the formula of Lyman and Fleming (1940), and was modified by Moskovits (1961) by omitting H_3BO_3, $SrCl_2$ and NaF. The composition of the synthetic sea water base is shown in Table I. The synthetic sea water base was prepared in 20-liter batches in a 25 liter polyethylene carboy. The solution thus prepared was a water-clear solution without any precipitate, whatsoever.

b. Buffer

The buffer used was tris(hydroxymethyl)aminomethane, $NH_2\text{-}C(CH_2OH)_3$, also referred to as TRIS. It buffers well between pH 7.5 and 8.5. Provasoli, McLaughlin, and Droop (1957) used TRIS routinely for culture work with marine algae. They found that it was not toxic for any of the organisms used (diatoms, dinoflagellates, blue-green algae, green algae). In present experiments, TRIS was used at a concentration of 0.1% (w/v).

TABLE I

Composition of synthetic sea water base

Salts	Amount*
NaCl	23.476 g
$MgCl_2.6H_2O$	10.629 g
Na_2SO_4(anhydrous)	3.917 g
$CaCl_2.6H_2O$	2.175 g
KCl	0.664 g
$NaHCO_3$	0.192 g
KBr	0.096 g

*Amounts are for 1 liter of distilled water.

c. Inorganic enrichment (nitrate, phosphate and silicate)

Nitrate was added as Ketchum and Redfield's (1938) solution A (KNO_3, 20.2 g; distilled water, 100 ml). Phosphate was added as Ketchum and Redfield's (1938) solution B ($Na_2HPO_4.12H_2O$, 4 g; distilled water, 100 ml). This was modified from the original fomrula which contained calcium, ferric and magnesium ions, already present in the sea water base and trace element solutions. Silicate was added as a stock solution of sodium silicate ($Na_2SiO_3.9H_2O$, 4.66 g; distilled water, 100 ml).

d. Chelator and trace elements

Droop (1961) pointed out that the primary purpose of a chelator in sea water culture media was to prevent the precipitation of divalent and trivalent ions, and particularly, of the ferric ion which would tend to form an insoluble ferric hydroxide. The chelator used here was the disodium salt of ethylenediamine tetraacetic acid (Na_2EDTA). This was incorporated into a trace element solution, the P1 metal mix of Provasoli, McLaughlin and Droop (1957) at a concentration of 0.1% (w/v). The composition of the P1 metal mix is given in Table II. In preparing this solution, the copper and cobalt sulphates were made up as separate stock solutions and requisite amounts of each were added to make the finished solution. In appearance, this solution was water-clear with a faint yellow color.

TABLE II

Composition of P1 Metal Mix

Salts	Amount*
Na_2EDTA	1.0 g
H_3BO_3	1.14 g
$MnSO_4.H_2O$	0.124 g
$FeCl_3.6H_2O$	0.049 g
$ZnSO_4.7H_2O$	0.0222 g
$CuSO_4.5H_2O$	0.00016 g
$CoSO_4.7H_2O$	0.00048 g

*Amounts are for 1 liter of distilled water.

e. Complete synthetic sea water

The complete synthetic sea water was prepared by mixing the above-described solutions and adjusting the pH in the following sequence:

1.	Synthetic sea water base	1 liter
2.	TRIS	1 g
3.	Sodium silicate stock solution	1 ml
4.	Ketchum and Redfield's solution A	2 ml
5.	Medium adjusted to pH 7 with 1 N HCl	
6.	Ketchum and Redfield's solution B	1 ml
7.	P1 metal mix	30 ml
8.	Medium adjusted to a final pH of 7.8 with 1 N KOH	

All pH adjustments were made with a Radiometer pH meter 22. The complete synthetic sea water[1] was a clear, colorless solution, which remained clear on autoclaving.

f. Growth factors

In the early aspects of the work, the writer obtained good growth of the experimental diatom, as a bacterized culture, in complete synthetic sea water. However, the bacteria-free culture grew very poorly in the same medium. Lewin and Lewin (1960) showed that some species of Nitzschia required either thiamin or cobalamin (vitamin B_{12}) for good growth. Provasoli (1963) indicated that there were strain

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[1]The salinity, as determined by titration with silver nitrate, ranged from 31.2 to 33.30 $^{0}/_{00}$, depending on the batch prepared (Moskovits, 1961).

differences for vitamin requirements for a number of species of diatoms including Nitzschia closterium in which some strains required B_{12} and thiamin, whereas others required thiamin only. Accordingly, it was decided to add these growth factors to the culture medium in the present work. The concentrations used were: thiamin hydrochloride,[1] 0.2 mg per liter; B_{12}[2], 1.0 µg per liter (Guillard, 1963).

Although there were no specific indications in the literature of requirements for biotin by marine diatoms, and particularly by species of Nitzschia (Provasoli, 1963; Droop, 1962; Provasoli, 1958), this growth factor, together with thiamin and B_{12}, appear to constitute the growth factors of greatest importance to algae (Provasoli, 1963). Biotin[3] was therefore also added at a concentration of 1.0 µg per liter (Guillard, 1963).

The vitamins were prepared for use as a single stock solution. Biotin was dissolved in glass distilled water and diluted to the proper concentration. It was autoclaved (15 minutes at 121°C) in a brown bottle which was then sealed with a sterile, puncturable rubber stopper. The required amounts of B_{12} and thiamin hydrochloride (obtained as sterile solutions) were then added to the biotin solution by the use of sterile syringes. The concentrations of vitamins in the stock solution were then: thiamin hydrochloride, 80 mg per liter; B_{12}, 0.4 mg per liter; biotin, 0.4

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[1]Parke, Davis and Company, Detroit, Michigan.

[2]British Drug Houses (Canada) Ltd., Montreal, P.Q.

[3]Nutritional Biochemicals Corporation, Cleveland, Ohio.

mg per liter. The stock solution was stored in the frozen state until used. In use, 1 ml of the stock solution was added to 400 ml of the sterile, complete synthetic sea water to give the desired, final concentration of growth factors. The experimental diatoms grew luxuriantly in the vitamin-enriched medium.

3. Temperature Control, Illumination and Agitation of Cultures

The culture experiments were carried out in an apparatus in which conditions of constant temperature, illumination and agitation were maintained (Fig. 1).

Constant temperature was achieved with the use of a water bath in which cooling was provided by a refrigeration system, thermostatically controlled. The water bath was a rectangular tank, 48¼ in. long, 21 1/8 in. high and 14 in. wide, constructed of welded ¼ in. angle iron. The framework was fitted on sides and bottom with a single thickness of ¼ in. plate glass bonded with a non-hardening silicon rubber cement. It was soon found necessary to modify this by adding another sheet of glass all around with airspace in between to eliminate the condensation of water vapor from the atmosphere on the glass surfaces. Since the illumination system was located underneath the tank, it would have been possible for condensate from the bottom of the tank to drip down onto the electrical contacts, causing a short circuit. Condensation on the sides of the tank tended to interfere with the observation of tank contents.

FIGURE 1

The experimental culture apparatus:

A. View showing constant temperature tank with coolant coils and with culture flasks in place. Control equipment and shaker are hidden behind far end of tank. Fluorescent tubes are immediately below the tank. Coolant tank, circulating pump and part of compressor may be seen on the floor. The black cloth on the frame above the tank was draped over sides of tank during experiments to block out daylight and room light.

B. View of the manner of attachment of the culture flasks to the control shaker rod.

C. View of wrist-action shaker. The shaker rests on the upper part of the control panel.

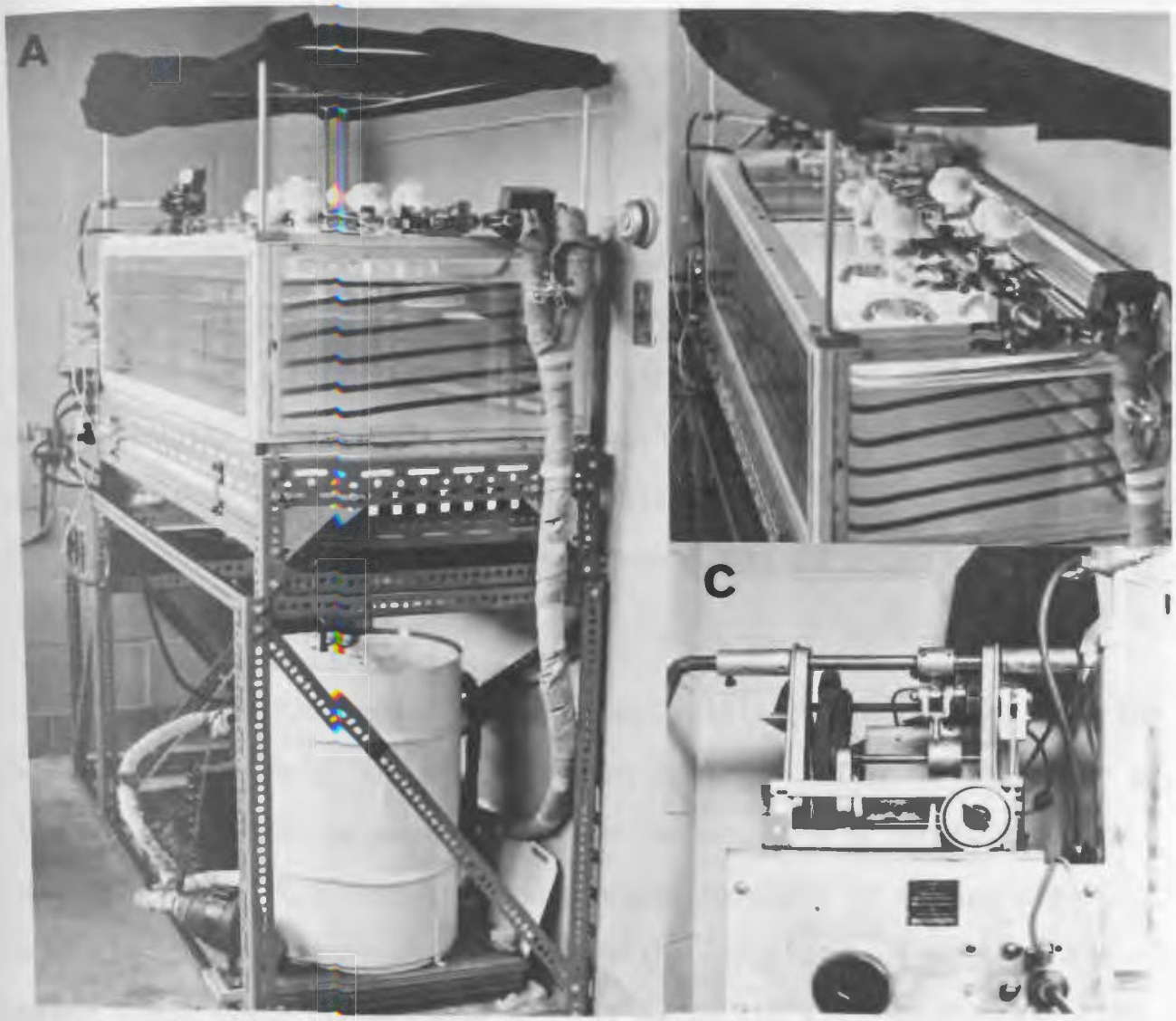

FIGURE 1.

a. Temperature control

Temperature control of the water bath was effected by a refrigeration system consisting of a twin cylinder, 580 rpm Tecumseh conventional condensing unit[1] (model 1400), driven by a ¼ hp, 110 V a-c motor. Copper coils from the refrigerating system were immersed in a reservoir containing a 1:1 mixture of ethylene glycol and water as the coolant[2]. The coolant reservoir consisted of one cylindrical metal can (containing the coolant) within another, the two cans being separated from one another by an insulating thickness of styrofoam. The coolant was maintained at 0°C through the use of an electronic thermostat.

An electric motor-driven pump circulated the coolant from the reservoir through ½ in. copper tubing bent to conform to the shape of the water bath and placed inside the latter. The water in the bath was maintained at a constant temperature by means of a separate electronic thermostat which actuated the circulating pump when the water temperature rose above or fell below the set temperature. The temperature of the bath was set at 15°C and was maintained within a range of ±0.2°C.

b. Illumination

Illumination was provided by a bank of eleven 30 W, cool-white, fluorescent tubes, placed beneath the bottom of the water bath. Except

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[1]Tecumseh Products Company, Tecumseh, Michigan.

[2]This mixture has a freezing point of -36°C (Merck Index, 1960, 7th edition, Merck and Company, Rahway, New Jersey).

at either end of the bath where there was a slight drop off in intensity and in which locations no experimental flasks were placed, the illumination in all parts of the bath was very uniform. A Luxtrol light control[1], (type WBD800) was used to vary light intensities for the various experiments. Although fluorescent light tubes give off much less heat than incandescent lamps of equivalent wattage, should the culture apparatus cooling system fail, the water of the bath could be sufficiently warmed to damage or kill the experimental diatoms. To eliminate the possibility of this event, an electronic thermostat was incorporated into the light circuit to shut off the illumination should the water bath temperature rise 0.75°C higher than the set temperature.

The illumination system was adjusted for the different light intensities used in the experiments, with a Brockway exposure meter[2], which read directly in foot candles. Light intensity measurements were made by placing the exposure meter with its photocell face down in a 2-liter beaker, and pushing the beaker down into the water bath to the level at which the bottoms of the experimental flasks would be located when these were placed into the bath. The maximum light intensity produced by the fluorescent tubes was found to be 760 ft-c.

A continuously operating aquarium filter was added to the water bath to maintain the clarity of the water.

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[1]Superior Electric Company, Bristol, Connecticut.

[2]Sekonic Electric Company, Tokyo, Japan.

c. Agitation

The shaking device, a Burrell[1] wrist-action shaker (model FF), was set up on a table immediately adjacent to one end of the water bath. An aluminum rod 3/4 in. in diameter and 4½ ft long was placed across the top of the water bath. One end of this rod was secured in the shaker sleeve. The remainder of the rod was supported by, and pivoted on, full pillow blocks at the mid-point and at the far end of the tank. Six pairs of screw finger-grip clamps were attached to the rod along its length at 6 in. intervals, enabling the rod to hold 12 1-liter Erlenmeyer flasks. The flask bottoms were immersed in the water of the bath to a depth of not less than 2 in. so that the 400 ml volume of culture medium in each flask was adequately covered.

The shaking action had to be such as to keep the cultures in gentle motion without splashing up onto the cotton plugs stoppering the flasks, thereby contributing to contamination of the cultures. Early in the experimental work, it was found that the shaking often became erratic and violent even at low oscillation speeds. To remedy this, a speed reduction pulley system was added to the shaker. An adjustable counter-balance was also added to compensate for fewer than the full complement of flasks.

4. Preparation of Bacteria-free Cultures

In order to study the production of extracellular carbohydrates by Cylindrotheca fusiformis Reimann and Lewin var. fusiformis, it was

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[1]Burrell Corporation, Pittsburgh, Pennsylvania.

necessary to prepare and use axenic cultures of the organism. Spencer (1952) pointed out that bacteria associated with diatoms were usually very tenaciously attached. This situation made the freeing of algal cells from bacteria by the process of single cell washing, as described by Pringsheim (1949), quite impractical.

Spencer (1952) isolated bacteria-free cultures of Nitzschia closterium var. minutissima by the use of penicillin and streptomycin. He found that this organism was not inhibited by treatment with 500 units per ml of penicillin, but was inhibited by 500 units per ml of streptomycin. Foter, Palmer and Maloney (1953) investigated the antialgal properties of a number of antibiotics for blue-green algae, green algae and diatoms. They found that both penicillin and chloromycetin inhibited the growth of blue-green algae, produced less inhibition of the growth of green algae, and did not inhibit diatom growth at all. Streptomycin inhibited the growth of all the organisms tested.

Although ZoBell (1946) estimated that approximately 95% of bacteria occurring in the sea were Gram-negative, it was believed in the present work, that there were opportunities for the air-borne, Gram-positive bacteria to contaminate the crude diatom cultures. Provasoli, Pintner and Packer (1951) used a combination of antibiotics (penicillin and chloromycetin) together with agar surface plating to isolate bacteria-free cultures of marine diatoms. Their method resulted in the elimination of both Gram-positive and Gram-negative bacteria which contaminated the cultures. Accordingly, it was decided to adopt the use

of Provasoli, Pintner and Packer's (1951) procedure. The concentrations of antibiotics used per ml of medium were: penicillin, 1,000 units; chloromycetin, 35 µg.

a. Preparation of antibiotic agar plates

Penicillin and chloromycetin were prepared as stock solutions. Penicillin stock solution was made by dissolving 1,000,000 units of penicillin G Sodium[1] in 32.25 ml of sterile, Pratt's (1947) phosphate stabilizer solution ($NaH_2PO_4 \cdot H_2O$, 0.298 g; Na_2HPO_4 (anhydrous), 0.205 g; NaCl, 0.818 g; glass distilled water, 1 liter). This gave a concentration of 31,000 units of penicillin per ml. In use, 1 ml of the penicillin stock solution was mixed with 30 ml of agar medium. The phosphate stabilizer solution was used to retard the destruction of the dissolved penicillin. It was kept in the frozen state when not in use.

The stock solution of chloromycetin was prepared by adding 30 ml of acetone to 1 gram of chloromycetin[2]. Complete solution was not effected. One ml of the solution was then transferred by sterile syringe to a carefully weighed, sterile serum bottle, which was stoppered with a sterile, puncturable, rubber stopper. The serum bottle was then additionally punctured with a cotton-plugged, sterile hypodermic needle, and placed in a vacuum desiccator to enable the acetone to

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[1]Glaxo-Allenburys (Canada) Ltd. The steri-vial contained 1,000,000 units.

[2]Obtained as Chloromycetin (intramuscular) from Parke, Davis and Company Ltd., Brockville, Ontario. The steri-vial contained 1 gram of the compound and was large enough to permit the addition of the indicated volume of acetone.

evaporate. Following complete evaporation of the acetone, the bottle was carefully weighed again in order to determine the amount of chloromycetin in the bottle. Then, the required volume of acetone was added to give a concentration of 0.00105 g per ml. In use, 1 ml of this working solution was mixed with 30 ml of agar medium to give a final concentration of 35 µg of chloromycetin per ml of agar medium. When agar medium at 45°C was mixed with the acetone solution in the petri dish, the acetone volatilized. No inhibition of diatom growth by acetone was therefore ever observed. Both stock and working solutions were stored at 0°C.

For surface plating of the diatoms the following medium was used: Bacto-peptone (Difco), 5 g; Oxoid agar no. 3, 15 g; complete synthetic sea water, 1 liter. This medium was tubed in 15 ml quantities for use. Peptone was used in order to reveal the presence of any bacteria or fungi associated with the diatoms during their purification.

The antibiotic agar plates were prepared by adding the penicillin and chloromycetin solutions to each petri dish. Thirty ml of agar medium were then poured in. The antibiotics and the medium were then mixed thoroughly by gently rotating the plate. The antibiotic agar plates were incubated at room temperature (25°C) for 24 hr as a check on any microbial, and particularly fungal, contamination prior to use.

b. Isolation of bacteria-free diatoms

The bacterized diatom culture was harvested by repeated

centrifugation and washing with sterile complete synthetic sea water. From the very dense suspension obtained, cells were transferred to an antibiotic agar plate and streaked as even as possible on one half of the plate. The plate was then incubated at 15°C, with illumination, for one week. Good growth was obtained.

To be certain that the diatoms were free from bacteria and fungi, a second transfer was made to a fresh antibiotic agar plate. This was done by scraping diatoms from the advancing edge of growth with a loop moistened with sterile synthetic sea water and streaking these on the second plate. After 10 to 15 days incubation of the second plate, the cells were again scraped off and inoculated into 400 ml of sterile, complete synthetic sea water with vitamins added. The culture was then incubated in the culture bath at 15°C with illumination.

c. Sterility testing

In order to be certain that the antibiotic-treated diatom cultures were, in actuality, free from bacteria and fungi, and remained so during the experiments, it was necessary to carry out sterility tests. Because the diatoms would be grown with constant shaking and also, because they would continuously contribute oxygen to the medium during photosynthesis, the resultant culture environments would be highly aerobic and would therefore tend to support aerobic rather than anaerobic contaminants. Accordingly, sterility tests for aerobic contaminants, only, were carried out.

The testing procedure was that given in the United States Pharmacopoeia (15th revision, 1955). The test medium was prepared by dissolving 2 grams of Bacto-yeast extract (Difco) and 6 grams of Bacto-peptone (Difco) in 1 liter of complete synthetic sea water and heating for 5 min at 121°C. On cooling, the medium was filtered through two thicknesses of Whatman no. 2 filter paper. The filtrate was dispensed in 50 ml portions in 125 ml Erlenmeyer flasks. These were cotton-plugged and autoclaved for 15 min at 121°C. The finished medium was a completely clear, yellowish-brown solution in which even the slightest turbidity produced by the growth of contaminants could be readily detected.

In use, 50 ml of the test medium was inoculated with 10 ml of the diatom culture being tested and incubated at room temperature for two weeks. Examinations for turbidity and surface fungal growth were made every third day. Antibiotic-treated cultures produced no turbidity or surface fungal growth while untreated cultures showed strong turbidities in 24 hr. Surface fungal growths were never observed.

Experimental Conditions

Moskovits (1961) pointed out that the eurythermality of the diatom Nitzschia closterium (Ehr.) Wm. Smith ranged from -1.7°C in the Bellingshausen Sea in the Antarctic to a maximum of 37.5°C in one of the Barataria Bay, Louisiana, salt marshes. It was difficult to decide on

an experimental temperature for an organism having such a wide temperature range. At high temperatures, while the organism would grow more rapidly, it would also exhaust available nutrients more rapidly. Growth factor requirements would also increase (Provasoli, 1958). At low temperatures, while nutrients and growth factors would not be exhausted as rapidly, the organism would grow more slowly and the experimental period would be prolonged. No literature was found to indicate that temperature affects the extracellular production of carbohydrates by algae. Since the culture apparatus was more readily maintained at 15°C than at higher or lower temperatures, it was decided to use that temperature for all the experiments.

Each of the first three experiments was carried out at a different light intensity. High (760 ft-c), intermediate (400 ft-c) and low (50 ft-c) light intensities were used. The nitrogen and phosphorus contents of the synthetic sea water were unaltered. The fourth experiment was carried out at the intermediate light intensity. In addition, the condition of nitrogen deficiency was created by reducing the nitrogen content of the medium from 5.6 µg per ml (normal medium) to 0.7 µg per ml.

Each experiment was terminated when the stationary phase of growth was reached.

General Experimental Procedure

1. Inoculation of Medium

Cultures used as inocula for the carbohydrate production

experiments were prepared by growing the bacteria-free diatom in 400 ml of complete synthetic sea water, with vitamins added, contained in cotton-plugged 1-liter Erlenmeyer flasks. These cultures were adapted to the temperature ($15^{o}C$ was employed throughout the experiments) and to the particular experimental light intensity for at least two weeks before the experiment, with at least two transfers to fresh medium being made during this period. The importance of pre-experimental adaptation has been stressed by Spencer (1954).

Cells were removed from the adapted cultures during the exponential phase of growth, and were processed as follows. Twenty ml aliquots of the culture were transferred to 35 ml sterile, screw-capped tubes, and centrifuged in an International high-speed,refrigerated ($5^{o}C$) centrifuge at 2,500 x g for 5 min. The supernatant was decanted, the cells were washed with sterile, synthetic sea water and recentrifuged. The very dense cell suspension obtained was diluted to the required concentration after making cell counts on it. The required volume of diluted cell suspension was then inoculated into 400 ml of sterile complete synthetic sea water with vitamins added. The starting cell concentrations for all experiments were maintained within the range of 2 to 5 x 10^{4} cells per ml.

For each experiment, ten flasks of media were inoculated and incubated in the culture bath. One of these was used for cell counts in order to follow the course of growth during the experiment. The other flasks were harvested at intervals for carbohydrate analysis. Each experiment was carried out in duplicate.

2. Cell Sampling and Counting

Following the start of an experiment, cell sampling and counting were carried out at intervals of three to four days. Although there was continuous agitation of the culture during the experiment, clumps of cells were always present in the bottom of the flask. In order to reduce sampling errors, the culture was shaken vigorously on a rotary shaker[1] for 10 min before sampling. Two 1-ml portions of the culture were then taken from the flask using a 1-ml large orifice pipette. The two aliquots were combined as one subsample in a 10-ml beaker. Four such subsamples were taken at each sampling period.

When agitation on the rotary shaker failed to completely disperse clumps of cells, ultrasonic treatment was resorted to. A MSE disintegrator[2] was used, operated for 5 min periods at 0.5 to 0.75 amp (low range). Within this range of ultrasonication, completely homogeneous cell suspensions were obtained and no destruction of cells occurred.

Cell counts were made on the suspensions thus prepared, using a Levy haemocytometer with double Neubauer rulings. Two counts were made on each subsample. A total of eight counts were therefore made at each sampling.

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[1]Yankee variable speed rotator; Clay-Adams, Inc., New York.

[2]Measuring and Scientific Equipment Ltd., London, England.

3. Sterility Testing

Immediately following the inoculation of diatoms, at the beginning of an experiment, sterility tests were carried out on each flask of culture. Since all but one of these flasks were not opened again until the cultures were harvested, no further tests were carried out. However, sterility tests were run on the flask from which samples for cell counts were removed. This was done in order to be certain that the growth curve, as determined by the growth in this one flask, was a valid representation of the growth occurring in all the other flasks.

4. Preparation of Cell-free Culture Medium

At intervals, during the course of an experiment, depending on the growth rate of the diatoms, two flasks of culture were harvested. The cells were separated from the culture medium by centrifugation and by filtration. Centrifugation was carried out in an International high-speed refrigerated ($5^{o}C$) centrifuge at 2,500 x g for 5 min. The supernatants were collected and their volumes measured. The cells were then washed with a small volume of complete synthetic sea water and recentrifuged. The original and the cell-wash supernatants were then pooled and filtered through a Morton bacterial filter (Morton, 1944) to make certain that no cells remained in the culture medium. The filtrate was frozen in preparation for the isolation of carbohydrates.

Isolation and Analysis of Carbohydrate

1. Isolation

Few direct determinations and analyses of dissolved carbohydrates in sea water have been carried out. Available analytical methods are not sufficiently sensitive for the small quantities of carbohydrates present in sea water. In addition, other organic compounds and inorganic salts in the sea water interfere with the reactions upon which the analyses are based. Lewis and Rakestraw (1955) compared the anthrone and the N-ethylcarbazole colorimetric determinations of total carbohydrate in sea water. They found that although there was no salt error with the N-ethylcarbazole method, the procedure was more laborious and exacting because of the light sensitivity and the rigid temperature requirements of the reagent. Zein-Eldin and May (1958) improved the N-ethylcarbazole method so as to make it more suitable for the determination of total carbohydrate in sea water. However, McLaughlin, Zahl, Nowak, Marchisotto, and Prager (1960) found the N-ethylcarbazole reaction to be most unsatisfactory. This was because non-carbohydrate materials, both organic and inorganic, gave rise to a variety of false color reactions. Antia and Lee (1963) tested the anthrone method with synthetic sea water whose composition was successively altered by adding or omitting certain components in order to determine the effect and correction of the salt effect. However, due to the presence of unknown compounds in natural sea water, it was still impossible to make direct measurements of carbohydrates. To obviate this difficulty, preliminary treatment, i.e., isolation of

carbohydrates from the sea water medium becomes necessary.

Jeffrey and Hood (1958) evaluated various methods for the isolation of organic compounds from sea water. Glucose, mannose, galactose and raffinose could be isolated with a recovery of 98 to 99% using an activated charcoal column and eluting with 5 to 15% ethanol. Hood (1967) suggested that polysaccharides could also be isolated using an activated charcoal column and eluting with 10 to 20% ethanol. It was therefore decided to adopt the use of these methods for the isolation of carbohydrates from the synthetic sea water culture media in which the diatoms were grown.

a. Preparation and use of activated charcoal columns

The charcoal used was Norite RKO[1] (50-200 mesh). It was prepared for use by soaking in glacial acetic acid for 1 to 2 hr at room temperature. The acid was then decanted and the charcoal was washed once with glass redistilled water. The charcoal was then packed in a glass column (2.5 x 25 cm) and washed repreatedly with glass redistilled water until the effluent was no longer acid as determined with Hydrion pH test paper. The column was then ready for use.

The cell-free culture medium was passed through the activated charcoal column. The column was then washed with glass redistilled water to remove the inorganic ions. Washing efficiency was determined by testing the effluents with 1 M $AgNO_3$. When the silver nitrate tests

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[1]American Norite Co., Inc., Jacksonville, Florida.

were negative, the column was eluted successively with 100 ml each of 5%. 10%, 15% and 20% ethanol. The alcoholic effluents were pooled and kept frozen prior to lyophilization.

b. Lyophilization of isolated carbohydrates

Although vacuum distillation could be used as a rapid method for concentrating the alcoholic effluents, it was considered that oligosaccharides and polysaccharides might be hydrolyzed in the concentrated sample. While this would not affect analysis for total carbohydrate, it would make it impossible to determine the original carbohydrates present by thin layer chromatography. For this reason, freeze-drying was resorted to[1]. The dried material was redissolved in 15 ml of glass redistilled water, and kept frozen until needed for analytical procedures.

2. Analysis of Total Carbohydrates

Although the carbohydrate isolation procedure eliminated the salt interference problem, the use of the N-ethylcarbazole method still posed problems because of the light sensitivity and the rigid temperature requirement of the reaction. It was therefore decided to adopt the use of the anthrone method for the determination of total carbohydrates in the experimental culture media. Scott and Melvin (1953), Lewis and Rakestraw (1955), Antia and Lee (1963) used various modification of this method, particularly with respect to the concentrations of anthrone and sulphuric acid used, reaction times and

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[1]Bellco freeze-drying apparatus no. 4110. Bellco Glass Inc., Vineland, New Jersey.

temperatures. After testing each of these modifications, the writer found that Antia and Lee's (1963) method gave the most reproducible results and therefore adopted it for the determination of total carbohydrates.

a. Preparation of glassware

All glassware used in the determination of total carbohydrate was soaked overnight in concentrated H_2SO_4, then successively rinsed in tapwater and glass redistilled water and finally oven-dried. Extreme care was exercised during glassware preparation in order to avoid contamination from cellulosic materials such as tissue paper fibers, cotton wool fluff, which are often found in the air of the laboratory environment.

b. Preparation of glucose standard solutions

A stock solution containing 500 mg of glucose per liter of glass redistilled water was prepared. A few drops of saturated $HgCl_2$ solution were added as preservative. In addition, the stock solution was kept frozen until used. For use, the stock solution was diluted to the required concentrations. The dilutions prepared were: 25, 12.5, 6.25, and 3.125 mg per liter. They were freshly prepared for each determination, not more than 12 hr before use. Until used, the dilutions were stored under refrigeration.

c. Preparation of anthrone reagent

Reagent grade anthrone was purified for use by recrystallization from hot benzene (Antia and Lee, 1963). The purified reagent was

stored in a desiccator. Precautions were taken to avoid contamination of the anthrone by cellulosic materials during its purification and storage. The reagent was freshly prepared just before use as a 0.1% (w/v) solution in concentrated H_2SO_4.

d. Analytical procedure

All determinations were carried out in duplicate. Five ml aliquots of the standard glucose solutions, "blanks" or unknown samples to be analyzed for total carbohydrate, were measured into 1.6 x 15 cm Pyrex test tubes which were then covered with aluminum foil caps to exclude air-borne contamination. Batches of 16 tubes were prepared in this way, each consisting of at least three glucose standards, one blank and the remainder unknowns, all in duplicate. Because of the considerable processing time involved, more unknowns could not be handled at any one time.

Before addition of anthrone reagent, each batch of tubes was cooled in an ice water bath for at least 30 min. To each tube, immersed in ice water and continuously shaken, 10 ml portions of anthrone reagent were added slowly, dropwise, from a burette. The rate at which the reagent was added was carried out very carefully so as to dissipate rapidly the heat produced by the reaction of sulphuric acid and water. When reagent addition was completed, each tube was shaken on a tube agitator for at least 30 sec to insure complete mixing of its contents. The tube was then returned to the ice water bath in a rack provided with handles so that an entire batch of tubes

could be immediately removed to another bath. The entire batch of tubes was cooled for an additional 2 min after the last tube had been added to the ice water bath. A batch of tubes was then successively transferred to a 20°C water bath for 5 min, then to a vigorously boiling water bath for a 6 min reaction period. The latter step was timed from the moment the bath temperature reached 98°C. At the end of the reaction period, the batch was plunged into the ice water bath for at least 3 min, after which it was replaced in the 20°C water bath for about 5 min. Then, with a minimum of delay, the tube contents were transferred successively to a 1 cm path absorption cell and the absorption read at 625 mμ on a Beckman DK-2 spectrophotometer. Distilled water was used as reference. Between successive measurements, the absorption cell was rinsed successively with distilled water, ethanol and ether. All sample readings were corrected with the blank reading. The results of samples from culture media (unknowns) were given in terms of mg per liter of glucose equivalent derived from a curve based on standard glucose solutions (Appendix II).

3. Thin-layer Chromatography (TLC) of Carbohydrates

Chromatographic techniques have become increasingly important tools for the separation of a wide variety of compounds of biological interest. Although paper chromatography has been used for many years for the separation of carbohydrates, it has a number of inherent disadvantages, the most important being a long elution time, which, together with temperature fluctuations during this period, produces erratic and irreproducible results.

Russel (1963) pointed out that thin layer chromatography (TLC) was one of the most rapid and sensitive means for the separation and identification of quite small amounts of compounds. The first attempts to separate sugar mixtures using TLC were by Stahl and Kaltenbach (1961) and by Pastuska (1961). Not only did this technique require little time for elution, but also smaller amounts of sugars (of the order of 0.05 μg) could be detected than was possible by paper chromatography. In view of these advantages, it was decided to use TLC for the qualitative analysis of carbohydrates in the present experiments.

Extensive studies of the use of TLC for carbohydrate analysis by various workers have involved the use of many different adsorbents, impregnating compounds and solvent systems. The proper selection of adsorbent for the separation of a given group of compounds is very important. The writer tested many adsorbents and adsorbent impregnating compounds including those employed in the methods of Stahl and Kaltenbach (1965), Waldi (1965a), Jacin and Mishkin (1965), Ovodov, Evtushenko, Vaskovsky, Ovodova and Solov'eva (1967), and Nemec, Kefurt and Jary (1967). The procedure using silica gel impregnated with 0.3 M disodium hydrogen phosphate or 0.3 M sodium dihydrogen phosphate as described by Ovodov, et al. (1967), gave the most satisfactory separation of hexoses, pentoses, sugar acids and oligosaccharides. The non-impregnated Silica gel G method described by Nemec, Kefurt and Jary (1967) was found useful for the separation of alcoholic sugars. Accordingly, these methods were followed for the qualitative analysis of carbohydrates in this study.

a. Preparation of TLC plates

TLC plates were prepared as described by Stahl (1965). Silica gel[1] containing a fluorescent material and a calcium sulphate binder was used as the adsorbent. In practice, a DESAGA[2] 112 cm long and 22 cm wide plastic aligning tray was set on a firm table. Five 20 x 20 cm clean, grease-free, glass plates were placed flush with one another on the alighing tray. Plate coating was done as follows. Thirty grams of silica gel were weighed out and placed in a 125 ml Erlenmeyer flask to whcih was added 60 ml of either glass distilled water, or 0.3 M sodium dihydrogen phosphate, or 0.3 M disodium hydrogen phosphate, depending on whether or not an impregnating compound was to be used. The flask was then stoppered, shaken vigorously for 15 to 25 sec, and swirled gently to get rid of air bubbles. The adsorbent slurry was then poured into the DESAGA adjustable TLC spreader which had been set to give an adsorbent layer 0.25 mm thick. The latter was moved from one end of the row of plates to the other at an even speed and without applying undue pressure to the spreader to give smooth, uniformly spread plates. Five to 6 sec were usually required to spread the plates. The whole operation, from the mixing of the adsorbent to the completion of the spreading needed to be completed within 5 min to avoid the setting of the calcium sulfate binder. The coated plates were dried and stored at room temperature before use.

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[1]SilicAR TLC-7GF; Mallinckrodt Chemical Works, St. Louis, Missouri.

[2]DESAGA, Heidelberg, Germany. (U.S. representative: C. A. Brinkmann, Westbury, Long Island, New York).

b. Carbohydrate standards

Carbohydrate standard solutions were prepared by dissolving 1 mg of the carbohydrate in 10 ml distilled water. The following compounds were used: glucose, galactose, fructose, mannose, rhamnose, fucose, arabinose, xylose, lyxose, ribose, sucrose, raffinose, galacturonic acid, mannuronic acid, sorbitol, mannitol, ribitol and erythritol. All the carbohydrates employed here were D-series except rhamnose and fucose. The solutions were kept frozen when not in use.

c. Solvents

The following solvents were freshly prepared before use:

(A) n-Butanol-acetone-water (4:5:1 v/v)

(B) n-Butanol-pyridine-water (8:4:3 v/v)

(C) Ethyl acetate-acetic acid-water (6:3:2 v/v)

(D) n-Butanol-ethanol-0.1 M phosphoric acid (1:10:5 v/v)

(E) Methyl ethyl ketone-acetic acid-methanol (6:2:2 v/v)

For the separation of hexoses and pentoses, solvents (A) and (B) were used for both the 0.3 M sodium dihydrogen phosphate and the 0.3 M disodium hydrogen phosphate impregnated plates, while solvent (C) was used for sodium dihydrogen phosphate impregnated plates only. For the separation of oligosaccharides and uronic acids, solvent (D) was used for both kinds of phosphate impregnated plates. Solvent (E) was used with non-impregnated plates for the separation of alcoholic sugars.

d. Detection reagents

Three detection reagents were used for the visualization of the carbohydrates. These were freshly prepared before use.

(A) Anisaldehyde-sulphuric acid reagent

This reagent was used by Stahl and Kaltenbach (1961) to detect as little as 0.05 µg of sugar. They found in addition, that it gave a variety of colors with different sugars. However, no color reactions were given by either uronic acids or alcoholic sugars.

The reagent consisting of a mixture of anisaldehyde, 5 ml; 95% ethanol, 90 ml; concentrated sulphuric acid, 5 ml; and glacial acetic acid, 1 ml, was sprayed evenly on the plate which was then heated in an oven at 90°C to 100°C for 5 to 10 min to develop the colors which appeared against a pink background.

(B) Benzidine-sodium metaperiodate reagent[1]

The reagent was prepared as two solutions. The first was a 0.1% aqueous solution of sodium metaperiodate. The second was prepared by dissolving 2.8 grams of benzidine in 80 ml of 96% ethanol. To this were then added 70 ml of water, 30 ml of acetone and 1.5 ml of I N HCl. In use, the plate was sprayed with the metaperiodate solution. While the plate was still damp, it was then sprayed with the benzidine solution. The carbohydrates appeared as white spots against a purple background.

(C) Concentrated sulphuric acid reagent

The plate was sprayed with concentrated sulphuric acid and then heated to 100-120°C for 3 to 10 min. The carbohydrates

[1]Described by Waldi (1965b) as spray reagent no. 18.

appeared as brown or black spots against a white background.

The detection reagents were applied with sprayers which used either compressed air or Freon as propellants.

e. Analytical procedure

Ascending TLC was the analytical procedure of choice, carried out in the following steps:

1. Sample application

Five ml of the previously freeze-dried sample was again freeze-dried and dissolved in 3 drops of glass redistilled water in a small conical tube. Aliquots of the sample solutions and the standard sugar solutions were applied on a line parallel to and at least 2 cm from one edge of the coated plate. Margins of at least 2 cm were also maintained on each side. A plastic template which bridged the TLC plate served as a hand rest and as an indicator of the staring line. Samples and standards were usually spotted at the starting line at intervals of 15 mm using micropipettes. Spot size was kept as small as possible and was usually less than 3 mm in diameter. Each spot was derived from 15 µl of sample or 1 µl of a standard sugar solution. Glucose was spotted on every plate in order to determine the Rg-value. The coating of the plate was scored 5 mm from each side to eliminate edge effects (Brown and Benjamin, 1964). The plate was then ready for development.

2. Development

Both rectangular and circular covered glass tanks were used as developing chambers. Solvent was added to the tank at least 15 min before the plates were added, with solvent-soaked filter paper placed within the tank wall to saturate the atmosphere. The plates were developed at room temperature (23-25°C) for a distance of 13 to 15 cm from the starting line to the solvent front. The plates were then removed from the tank, placed flat on a table to inhibit further development, and finally dried at room temperature.

3. Visualization

When the plates were completely dry, they were first examined under UV light[1] and then treated with detection reagents.

4. Determination of Unknowns

Because the colors developed on the chromatograms are very unstable with time, the results were recorded immediately upon visualization. Also, when a series of thin layer chromatograms is investigated, use of a reference substance rather than the use of the solvent front to obtain Rf-values[2], has been recommended by Gänshirt (1965).

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[1] Short wave UVS-11 Mineralite ultra-violet lamp; Ultra-violet Products, Inc., San Gabriel, California.

[2] $Rf = \frac{\text{Distance of center of spot from starting point}}{\text{Distance of solvent front from starting point}}$

Since glucose was used as the reference compound, in the present analytical work, Rg-values were used instead of Rf-values, where:

$$Rg = \frac{\text{Distance of sample spot from starting point}}{\text{Distance of glucose spot from starting point}}$$

The "unknown" spots from the experimental culture media were identified by the correspondence of their Rg-values with those of known carbohydrates in at least two different solvent systems or two different salt-impregnated adsorbents. Color reactions with detection reagents were also used to identify the unknowns.

RESULTS

Growth Curves

Replicate growth curves of the diatom for each of the four experimental conditions are shown in Fig. 2, 3, 4 and 5. Cell concentration data for the start, for the end of the exponential phase of growth and for the end of the experiment, for each experiment, are summarized in Table III. For each experiment, good agreement was obtained between the growth curves of the replicates. Differences were particularly evident when cell concentrations became high after the exponential phase of growth and may have been due to cell sampling or cell counting errors, or both.

In the experiments involving adequate nutrition under variations in light intensity (Experiments 1, 2, and 3), although all the three experiments reached a somewhat similar cell concentration at the end of the experiments (245.875 to 259.375 x 10^4 cells per ml), better growth was found in the high and intermediate light intensity experiments (Experiments 1 and 2). It required, for both of these experiments, six days to reach the end of the exponential phase of growth with cell concentrations ranging from 215.625 to 223.75 x 10^4 cells per ml, and 13 to 15 days to reach the stationary phase of growth. In the low light intensity experiment (Experiment 3), it required 12 days to reach the end of the exponential phase of growth with cell concentrations ranging from 178.75 to 188.5 x 10^4 cells per ml, and 21 days to reach the stationary phase of growth. In the nitrogen

FIGURE 2

Growth of and total carbohydrate production by the experimental diatom under high light intensity (760 ft-c) and in normal synthetic medium (Experiment 1).

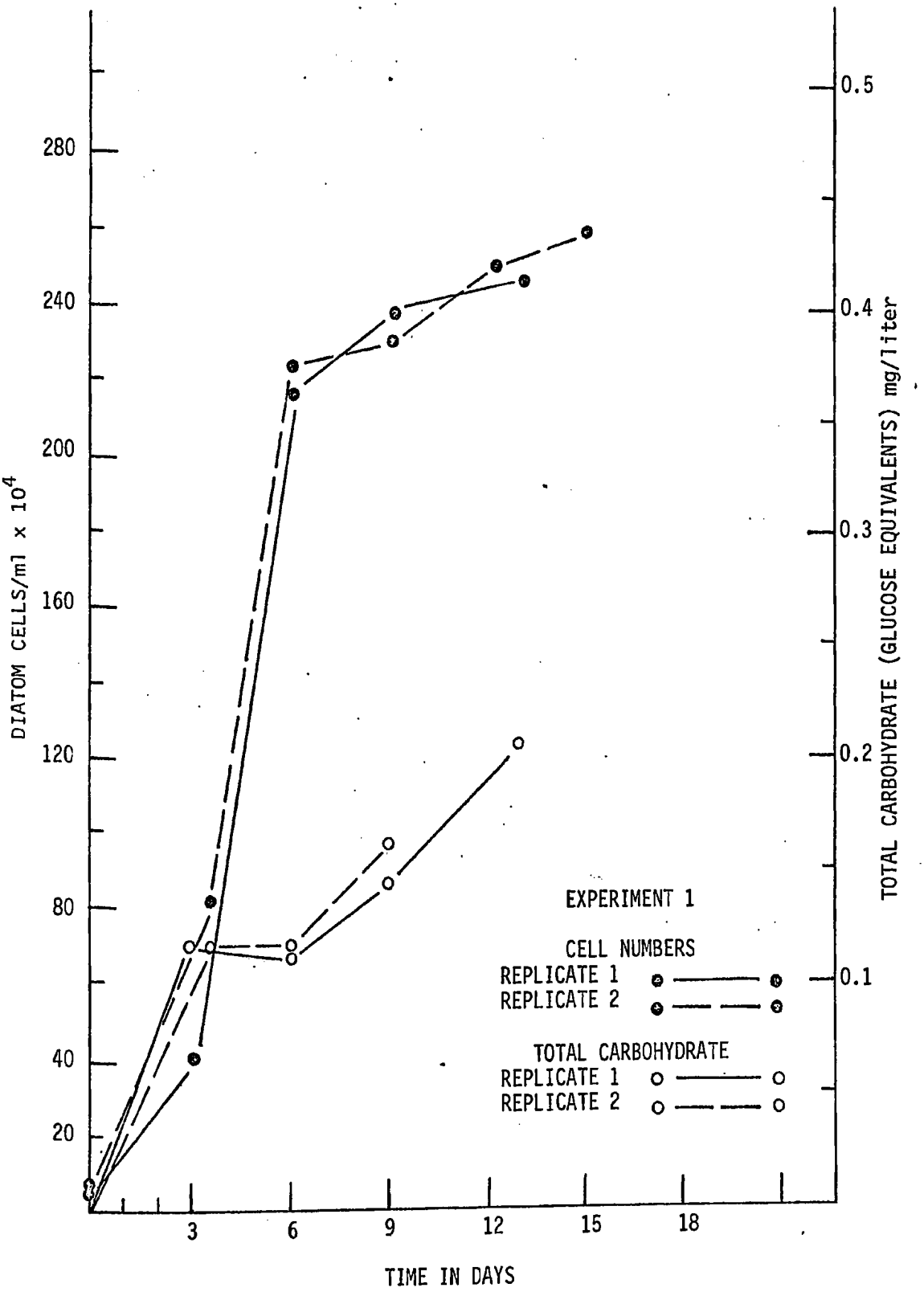
DIATOM CELLS/ml x 10^4
280
240
200
160
120
80
40
20
0.5
0.4
0.3
0.2
0.1
TOTAL CARBOHYDRATE (GLUCOSE EQUIVALENTS) mg/liter
3
6
9
12
15
18
TIME IN DAYS
EXPERIMENT 1
CELL NUMBERS
REPLICATE 1
REPLICATE 2
TOTAL CARBOHYDRATE
REPLICATE 1
REPLICATE 2

FIGURE 3

Growth of and total carbohydrate production by the experimental diatom under intermediate light intensity (400 ft-c) and in normal synthetic medium (Experiment 2).

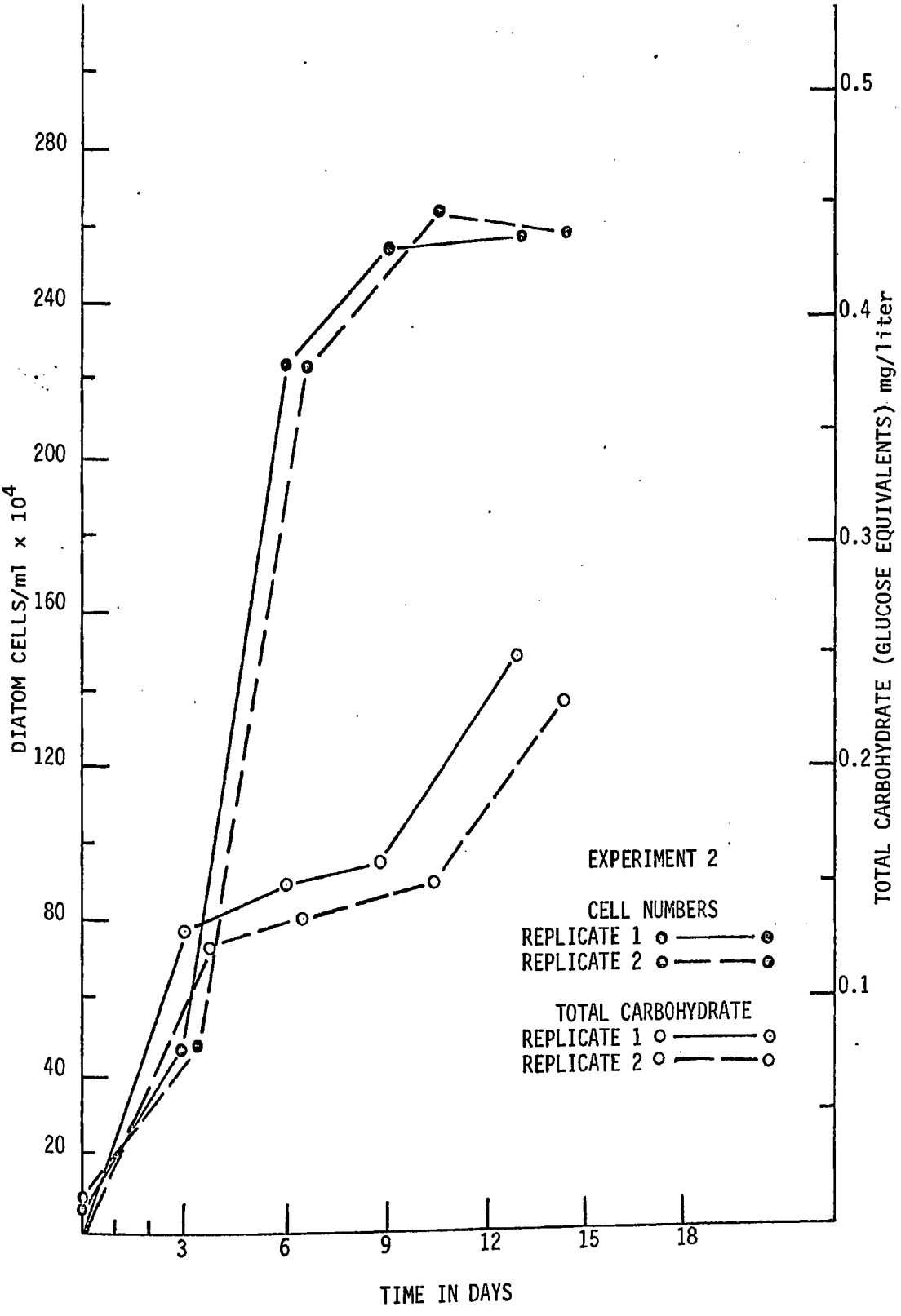
DIATOM CELLS/ml x 10^4
280
240
200
160
120
80
40
20
0.5
0.4
0.3
0.2
0.1
TOTAL CARBOHYDRATE (GLUCOSE EQUIVALENTS) mg/liter
3
6
9
12
15
18
TIME IN DAYS
EXPERIMENT 2
CELL NUMBERS
REPLICATE 1
REPLICATE 2
TOTAL CARBOHYDRATE
REPLICATE 1
REPLICATE 2

FIGURE 4

Growth of and total carbohydrate production by the experimental diatom under low light intensity (50 ft-c) and in normal synthetic medium (Experiment 3).

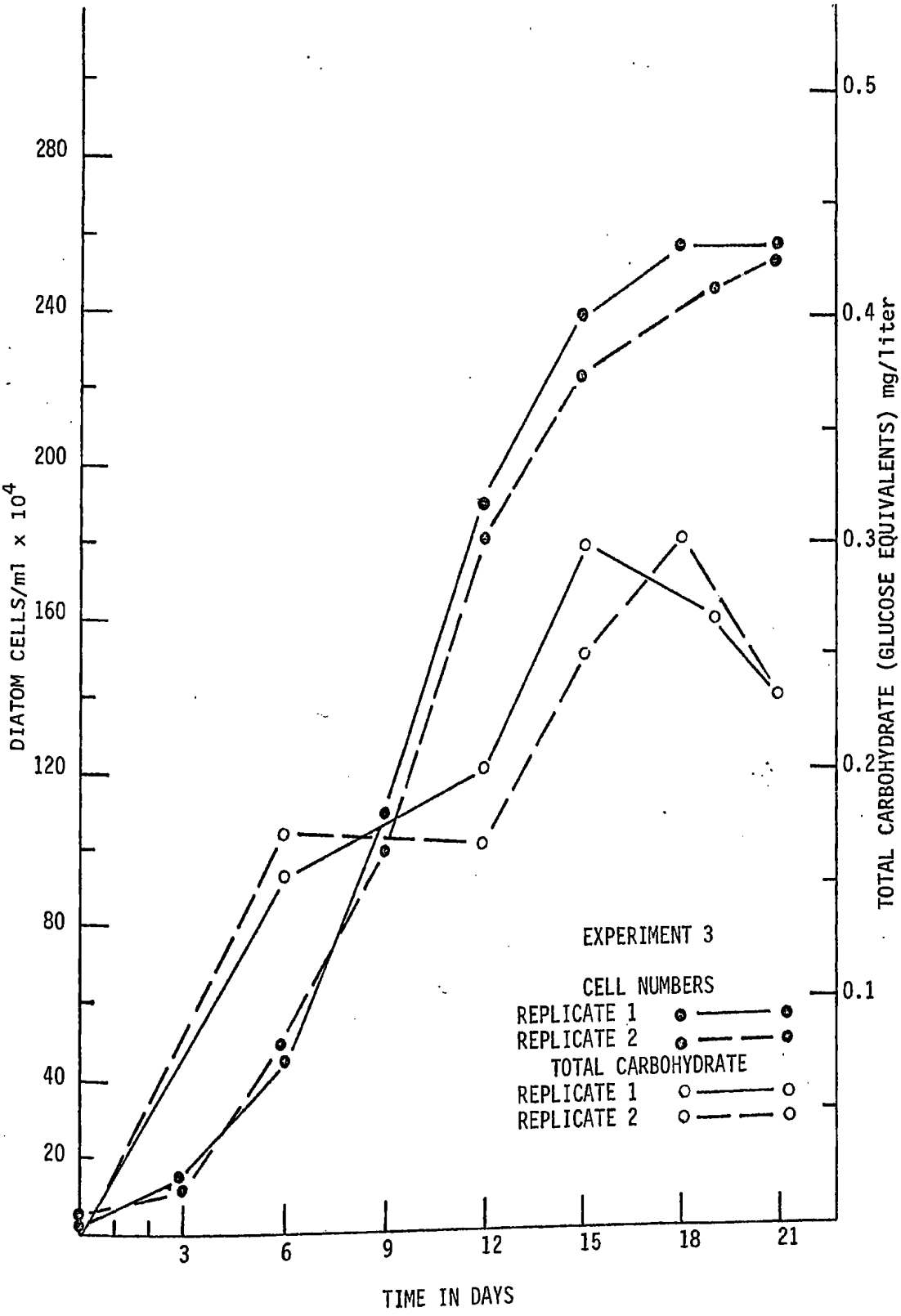
DIATOM CELLS/ml x 10^4
280
240
200
160
120
80
40
20
0.5
0.4
0.3
0.2
0.1
TOTAL CARBOHYDRATE (GLUCOSE EQUIVALENTS) mg/liter
3
6
9
12
15
18
21
TIME IN DAYS
EXPERIMENT 3
CELL NUMBERS
REPLICATE 1
REPLICATE 2
TOTAL CARBOHYDRATE
REPLICATE 1
REPLICATE 2

FIGURE 5

Growth of and total carbohydrate production by the experimental diatom under intermediate light intensity (400 ft-c) and in nitrogen-deficient synthetic medium (Experiment 4).

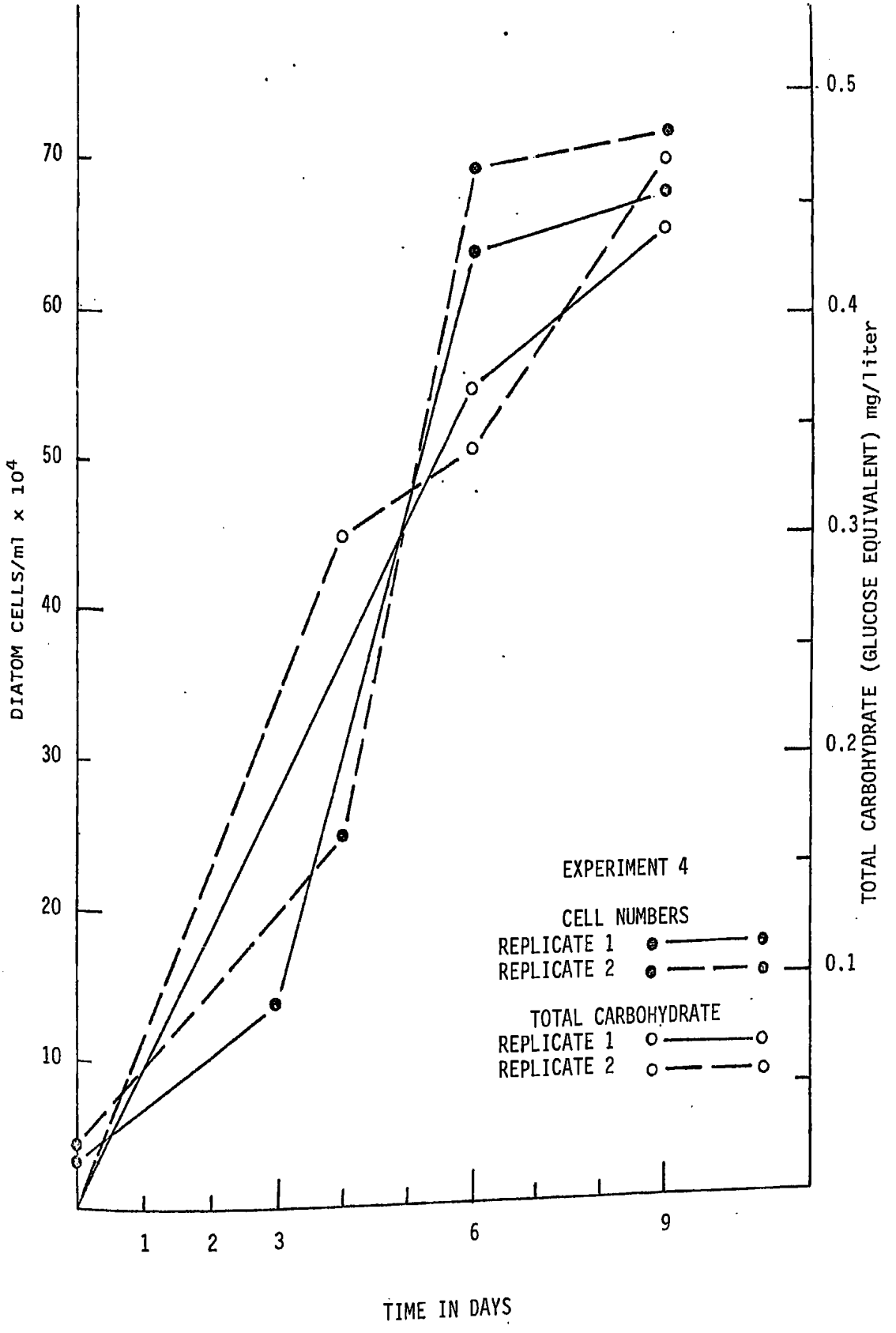
DIATOM CELLS/ml x 10^4
70
60
50
40
30
20
10
0.5
0.4
0.3
0.2
0.1
TOTAL CARBOHYDRATE (GLUCOSE EQUIVALENT) mg/liter
EXPERIMENT 4
CELL NUMBERS
REPLICATE 1
REPLICATE 2
TOTAL CARBOHYDRATE
REPLICATE 1
REPLICATE 2
1
2
3
6
9
TIME IN DAYS

TABLE III

Summarized experimental data for cell and carbohydrate concentrations from all experiments

Experiment	Replicate	Start		End of Exponential Phase of Growth		End	
		Cell concn*	Carbohydrate concn**	Cell concn	Carbohydrate concn	Cell concn	Carbohydrate concn
1	1	3.75	0.117 (3 days)	215.625 (6 days)	0.111	245.875 (13 days)	0.207
	2	4.875	0.117 (3 days)	221.625 (6 days)	0.113	259 (15 days)	ndc
2	1	4	0.130 (3 days)	223.75 (6 days)	0.148	258.375 (13 days)	0.250
	2	5	0.125 (3 days)	222.875 (6 days)	0.135	259.375 (14 days)	0.230
3	1	3.75	0.169 (6 days)	188.5 (12 days)	0.199	259.375 (21 days)	0.234
	2	4	0.173 (6 days)	178.750 (12 days)	0.169	257 (21 days)	0.234
4	1	3.5	nd	63.75 (6 days)	0.365	68.125 (9 days)	0.436
	2	4	0.300 (3 days)	69.375 (6 days)	0.339	71.875 (9 days)	0.469

*Cells x 10^4 cells per ml.
**mg (as glucose equivalents) per liter of culture medium.
ndc: not determined because of contamination.
nd: not determined.

deficiency experiment (Experiment 4) which was carried out at the intermediate light intensity, growth was poor as compared to that obtained in the intermediate light intensity experiment with adequate nutrition (Experiment 2). Although both of the experiments required six days to reach the end of the exponential phase of growth, the nitrogen deficiency experiment only showed 63.75 to 69.375 x 10^4 cells per ml, while the intermediate light intensity experiment showed 222.875 to 223.75 x 10^4 cells per ml. At the termination of the experiments (13 to 14 days for Experiment 2; 9 days for Experiment 4), the nitrogen deficiency experiment had a cell concentration ranging from 68.125 to 71.875 x 10^4 cells per ml as compared to 258.375 to 259.375 x 10^4 cells per ml for the intermediate light intensity experiment.

Cell Condition, Color and Size

In the high, intermediate and low light intensity experiments, the majority of the cells were actively motile[1] from the beginning of the experiment through the exponential phase of growth. A few non-motile cells (chromatophores intact), but very few dead cells (chromatophores absent) were also observed during the same period. As the stationary phase of growth was approached, more non-motile cells and more dead cells were observed. In the nitrogen deficiency experiment, from start through exponential phase of growth, most of the cells were found to be non-motile. Some feebly motile cells[2] and a few dead cells were also

[1]Cells showed continuous rapid movement over more or less extended distances.

[2]Cells showed intermittent slow movement over short distances only.

observed. Increasing numbers of dead cells were observed as the stationary phase of growth was approached.

The chromatophores (two) of the cells from the high, intermediate and low light intensity experiments and from the nitrogen deficiency experiment were greenish-yellow, golden yellow, yellowish-brown and pale greenish-yellow in color, respectively. In gross appearance, the experimental cultures showed a yellow, a brownish-yellow, a deep brown and a pale yellow color, respectively.

Measurements of specimens of the diatom in the living state and taken at random, from both[1] the intermediate light intensity experiment (10 specimens) and the nitrogen deficiency experiment (8 specimens), gave an over-all cell length ranging from 25 to 35 μ. The width, measured at the widest part of spindle-shaped body, had a range of 3 to 5 μ. Measurements of specimens of the diatom from the low light intensity experiment (20 specimens) gave a range of 25 to 58 μ in length and 3 to 7 μ in width.

Total Carbohydrate Production

Replicate curves for total quantities of carbohydrate (as glucose equivalents) produced by the diatom under each of the experimental conditions are shown in Fig. 2, 3, 4 and 5. These data for the start, for the end of the exponential phase of growth and for the end of the experiment, for each experiment, are summarized in Table III. For

[1]The cells of the high light intensity experiment were not measured.

each experiment, the carbohydrate production curves of the replicates matched each other very well except in the nitrogen deficiency experiment. For the latter experiment, carbohydrate production at three days incubation was not determined in the first replication.

Each of the experiments showed a somewhat similar pattern of carbohydrate production in that there was little or no increase in carbohydrate production during the exponential phase of growth. A definite increase was found as the stationary phase of growth was approached. In the low light intensity experiment, additionally, a decrease in carbohydrate production was found as the stationary phase of growth was approached.

In the light intensity variation experiments, slightly higher carbohydrate production was found in the low light intensity experiment than in the intermediate or high light intensity experiments at comparable phases of growth. Progressively less carbohydrate was produced in the latter two experiments, in the order indicated. In addition, the low light intensity experiment, showed a decrease in carbohydrate production, at the end of the experiment, to a concentration of 0.234 mg per liter, which was very much the same concentration obtained (0.230 to 0.250 mg per liter) at the end of the intermediate light intensity experiment. Although diatom growth was considerably poorer in the nitrogen deficiency experiment than in the high, intermediate and low light intensity experiments, it showed the highest carbohydrate production of all the experiments.

Qualitative Analysis of Carbohydrates

The chromatographic results which represent the best separations obtained are shown in Table IV. The rest of the chromatographic data are shown in Appendix I. All the samples obtained from the experimental cultures showed the same pattern of separation in any of the systems (impregnating compounds and solvents) employed. No qualitative differences in carbohydrate content were found either in the samples collected at different stages of growth in any one experiment or under the different experimental conditions established. The identified carbohydrates were glucose, galactose, xylose, arabinose and galacturonic acid.

From all the experimental samples collected, three unidentified (U) compounds were also found. These had Rg-values of 0 (U_1), 0.1 (U_2), and 1.64 (U_3) in disodium hydrogen phosphate-impregnated and solvent (D)-developed plates. U_1 showed a deep blue color with anisaldehyde-sulphuric acid reagent, while U_2 and U_3 failed to give any color reaction with the same reagent. The U_2 spot was larger in size than the spots of any of the other compounds encountered, whether identified or not. This may mean that it was present in a larger quantity than were any of the other compounds. Using ultraviolet visualization, the best separation obtained with sodium dihydrogen phosphate-impregnated and solvent (D)-developed plates showed five spots having Rg-values of 0, 0.45, 0.60, 0.85 and 1.32. These absorbed ultraviolet and therefore showed no fluorescence. Such compounds remained unidentified because none of the carbohydrate standards used showed any ultraviolet absorption.

TABLE IV

Representative TLC data* obtained with carbohydrate standards and unknowns

Standards		Unknowns Identified		Unknowns Unidentified		Colors with anisaldehyde-H_2SO_4 reagent
Compounds	Rg-value	Compound	Rg-value	Compound	Rg-value	
Galacturonic acid	0.21	Galacturonic acid	0.25			-
Raffinose	0.38					Dark blue
Mannuronic acid	0.62					-
Galactose	0.85	Galactose	0.85			Dark green
Glucose	1.00					Light blue
Arabinose	1.00					Yellowish-gree
Sucrose	1.05					Dark blue
Ribose	1.13					Dark blue
Xylose	1.32	Xylose	1.30			Grey
Fructose	1.50					Dark blue
Lyxose	1.87					Brown
Mannose	1.90					Brown
Rhamnose	2.65					Light green
		Glucose, arabinose sucrose**	1.00			Blue
				U_1	0	Dark blue
				U_2	0.10	-
				U_3	1.64	-

*These results represent the best separations obtained utilizing the following system:
Solvent: n-butanol-ethanol-0.1 M phosphoric acid (1:10:5 v/v)
Impregnating compound: 0.3 M disodium hydrogen phosphate
Detection reagents: 1) anisaldehyde-sulphuric acid reagent
2) benzidine-sodium metaperiodate reagent

**This unknown, since it has an Rg-value identical with, or similar to, the Rg-values for glucose, arabinose and sucrose, may actually present any one or a combination of these compounds. These compounds were separated and identified with the other systems (Appendix I) employed, although not separable or identifiable with the present system.

DISCUSSION

The results from the high, intermediate and low light intensity experiments and from the nitrogen deficiency experiments showed, in general, somewhat similar patterns of carbohydrate production. Assuming that carbohydrate production is expressed as quantities produced per cell, a higher level of carbohydrate production was found in the early stages than in any of the other phases of growth, for each of the experiments.

Nalewajko, Chowdhuri and Fogg (1963, cited in Watt, 1966), using the green alga, Chlorella pyrenoidosa, found that the percentage extracellular release[1] in the culture medium increased with dilution of the population density. Fogg, Nalewajko and Watt (1965), examining water from a lake containing Oscillatoria agardhii, Fragilaria crotonensis and Tabellaria flocculosa var. asterionelloides as predominant species, found that when the water samples were diluted with membrane-filtered water from the same source, the percentage extracellular release increased upon dilution of the suspension. They suggested that there was a tendency to equilibrium between the extra- and intracellular concentrations of the metabolites concerned. A similar result was obtained by Watt (1966) upon the dilution of a natural phytoplankton population. Here, too, the percentage extracellular released showed a

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[1] Samples were incubated with inorganic-C^{14}, then membrane-filtered. The organic matter in the filtrates were determined and expressed as percentage of the total C^{14} fixed (Watt, 1966).

marked increase when the dilution was in excess of 90%.

Fogg (1966) postulated two types of possible kinetic relationships in the release of extracellular products of algae. Type I involved liberation of low molecular weight metabolic intermediates, such as glycollate, into the surrounding medium. Type II was concerned with the liberation of high molecular weight compounds, such as polysaccharides or polypeptides. Fogg (1966) suggested that, in the Type I situation, the extra- and intracellular concentration gradients of an intermediate must be maintained in an equilibrium during the active growth of the cells. In order to maintain such an equilibrium the quantity of the intermediate liberated would be a function of the volume of a well-stirred culture. It also follows that at very low algal population densities, the quantities of extracellular products liberated per cell per given volume of culture medium must be greater than those liberated in cultures of higher population density.

In the writer's experiments, usually 10 ml of washed diatom inocula were transferred to 400 ml of fresh media to obtain the required starting cell populations. Therefore, the resultant dilutions were greater than 95% for each of the experiments. If the extracellular production of carbohydrates by the experimental diatom is in accord with the hypothesis of equilibrium, then increased carbohydrate production in the early stages of the experiments may have been the

result of dilution. Furthermore, the carbohydrates identified by TLC were low molecular weight compounds, presumably able to pass through the cytoplasmic membranes of the cells.[1]

Another possible explanation for the relatively high concentration of carbohydrate found in the early stages of the nitrogen deficiency experiments, where more dead cells were observed than in the other experiments, may be the release of carbohydrates from the dead cells. Marker (1965), also observed a relatively high level of extracellular production of carbohydrates during the "lag" phase of growth of flagellates Isochrysis galbana and Prymnesium parvum. He suggested that the breaking up of the inoculum cells and the subsequent release of considerable quantities of organic matter into the culture medium might be the reason for high carbohydrate production at that phase of growth.

In each of the experiments, extracellular carbohydrate production did not increase with rapid cell multiplication during the exponential phase of growth, but increased only with the onset of the stationary phase. Assuming that the equilibrium between the extra- and intracellular carbohydrates of the diatom is reached before the exponential phase of growth, then according to Fogg's (1966) Type I hypothesis, there will be no further increase in carbohydrate

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[1] Stadelmann (1962), reviewing the permeability of algae, concluded that some diatoms had surprisingly high rates for permeability to sugars, such as glucose and sucrose.

concentration in the culture medium during the active growth of the cells. However, this hypothesis only applies to the low molecular weight compounds. According to Fogg's (1966) Type II hypothesis, if the extracellular products were high molecular weight compounds, then the amounts liberated would be proportional to cell growth. The results of other workers (Fogg, 1952; Allen, 1956; Jones, 1962), support this hypothesis. On the other hand, Muscatine (1965), using the isolated symbiotic algae from the hydra, Chlorohydra viridissima, showed that the quantities of the extracellular products increased with time, although these products were not high molecular weight compounds, but rather low molecular weight compounds, primarily as maltose, with small amounts of glucose, glycolic acid, an unidentified oligosaccharide of glucose, and alanine.

The diatom used in the present study was thought to have mucilaginous capsular material (J. C. Lewin, 1958). Whether or not this capsular material was carbohydrate in nature and was released into the surrounding medium was not demonstrated in the present work. Some diatoms, such as the fresh water Navicula pelliculosa (J. C. Lewin, 1955) and the marine littoral diatom, Amphipleura rutilans (Trentepohl) Cleve (R. A. Lewin, 1958), secrete extracellular mucilaginous capsules, which do not dissolve in the surrounding medium.

Guillard and Wangersky (1958) found that extracellular carbohydrate production did not parallel the increase in cell numbers

during the exponential phase of growth in any of the marine and brackish water flagellate cultures used.[1] As the cultures entered the stationary phase of growth, the extracellular carbohydrate production began to increase. These authors refer in their discussion to the experiments of J. C. Lewin (1955) demonstrating that the diatom, Navicula pelliculosa, accumulated extracellular carbohydrates in the light under conditions of nitrogen, phosphorus or silicon deficiency. Marker (1965) similarly observed that high concentrations of extracellular carbohydrates were found only during the stationary phase of growth of his flagellate cultures. He suggested that most of the extracellular carbohydrate found resulted from cell autolysis.

If mineral deficiencies and cell autolysis are the only factors responsible for the accumulation and therefore for the high concentration of carbohydrates observed, then extracellular carbohydrate production will be high during the stationary phase of growth where the nutrients are almost exhausted, and cells begin to die. On the other hand, during the exponential phase of growth, with adequate nutrients, extracellular carbohydrate production will not increase greatly during this period, although as Guillard and Wangersky (1958) pointed out, some cells die even under the most favorable circumstances, liberating minute quantities of organic material into the medium.

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[1]These belonged to the genera: Isochrysis, Monochrysis, Prymnesium, Dunaliella, Pyraminomonas, Rhodomonas, Chlamydomonas.

The pattern of carbohydrate production observed in the present work and in the studies of Guillard and Wangersky (1958) and Marker (1965) is apparently not a universal one. Jones (1962), using the red alga, Porphyridium cruentum, found that the extracellular production of soluble polysaccharide was concomitant with cell growth. Muscatine (1965) found that the excretion of extracellular material, mostly maltose, by the symbiotic algae increased with time. A similar pattern was reported by Fogg (1952) in the liberation of carbohydrate material by the blue-green alga, Anabaena cylindrica.

In the experiments involving light intensity variation with adequate nutrition, slight increases in carbohydrate production were found as the light intensity was decreased. Similar results were found in the flagellate culture experiments of Marker (1965), where a decrease in light intensity reduced the level of intracellular carbohydrate and increased the concentration of extracellular carbohydrate. Watt (1966) found that in a natural phytoplankton population, the percentage extracellular release increased as the light intensity decreased. The highest values were obtained in complete darkness. The lowest values were found within the range of optimum light intensities. However, at saturation light intensities, a high percentage extracellular release was obtained. In the present experiments, both the high and intermediate light intensities[1] resulted

[1]According to Ryther (1956), diatoms required 1000 to 2000 ft-c for light saturation. Humphrey and Subba Rao (1967) found that the diatom, Cylindrotheca closterium (Ehr.) Reimann & Lewin, required light saturation of 1000 ft-c or more. Maddux and Jones (1964), using a continuous culture

in growth rates which appeared to be identical and which were greater than that found at the low light intensity. Because intracellular carbohydrate was not measured in order to obtain intracellular-extracellular carbohydrate ratios, it was not possible to determine, on the basis of extracellular carbohydrate production, the optimum light intensity.

The increased carbohydrate production observed in the low light intensity experiments agrees with the results of Marker (1965), except for the additional decrease of carbohydrate production observed in the stationary phase of growth. However, a similar decrease was observed by Guillard and Wangersky (1958) in their experiments with Isochrysis galbana. No explanation was given for this phenomenon. It is known that some species of algae are able to grow heterotrophically. Bristol-Roach (1928) measured the growth rates of a species of Scenedesmus in the light in a mineral medium, in the dark with added glucose, and in the light with added glucose. She found that under low light intensity, best growth occurred in the light with added glucose cultures, somewhat less growth occurred in the light in the purely mineral medium, while the least growth occurred in the dark with added glucose. This appeared to indicate that under low light intensity, the

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method to grow the diatom, Nitzschia closterium (Ehr.) Wm. Smith (now Cylindrotheca fusiformis, Reimann & Lewin var. fusiformis) in a light-and-dark cycle, found that the maximum growth rate was obtained by increasing light intensity from 1883 to 3229 lux (175 to 300 ft-c). The experimental conditions and methods these workers used were so different from the present work that the writer was not able to use their information.

growth obtained was a function of both heterotrophic and autotrophic modes of nutrition. The decrease in carbohydrate production observed in the writer's low light intensity experiments is difficult to explain unless the possibility exists that there is an uptake of carbohydrate by the cells in order to maintain growth under no-so-favorable conditions.

In the nitrogen deficiency experiments, carbohydrate production was higher than in any of the other experiments. Microscopic examination showed that most of the cells were not in a healthy condition. In fact, more dead cells were observed here than in any of the other experiments. Thus, the higher carbohydrate production observed may have been due to the death and autolysis of the cells. Marker (1965) obtained a similar result from nitrogen-deficient flagellate cultures.

In addition to the possibility that higher carbohydrate production is due to cell autolysis, it may be also attributable to an alteration in metabolic pathways. J. C. Lewin (1955) reported that the diatom, Navicula pelliculosa produced a gelatinous capsule around the cell in culture media deficient in certain necessary elements. These capsules were found to be composed solely of glucuronic acid. As a result of nitrogen deficiency the chief photosynthetic products of cells may change from protein to carbohydrate and then to lipid (Bongers, 1956; Van Oorschot, 1955; both cited in Syrett, 1962). Considerable fat accumulation occurred from four to six days after the onset of nitrogen deficiency in Chlorella pyrenoidosa (Fogg and Collyer, 1953). In other

species of Chlorella, such as Chlorella vulgaris and Chlorella strain "A", fat accumulation did not begin until much later, and carbohydrate was a major photosynthetic product in cells deprived of nitrogen (Van Oorschot, 1955). In the latter case, large amounts of low molecular weight carbohydrates, if accumulated in the cells, could diffuse into the medium according to Fogg's (1966) Type I hypothesis.

Carbohydrate production in the present series of experiments was quite low as compared with the results obtained by other workers. The identified carbohydrates: galactose, glucose, arabinose, xylose and galacturonic acid, were simple, low molecular weight compounds.

Fogg (1952) found as much as 7 mg of carbohydrate material per liter of filtrate from Anabaena cylindrica cultures. This material was later identified by Bishop, Adams and Hughes (1954) as a homogeneous, complex polysaccharide consisting of glucose, xylose, glucuronic acid, galactose, rhamnose and ribose.

R. A. Lewin (1956), using 18 species of green algae[1], found that the amounts of extracellular polysaccharide produced ranged from 3 to 113 mg per liter. In all the species but one, the polysaccharides consisted primarily of galactose and arabinose. In this exceptional species, Chlamydomonas ulvaënsis, glucose and xylose predominated. Minor components of the polysaccharides were fucose, rhamnose, mannose,

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[1]These belonged to the genera: Chlamydomonas, Chlorosarcina and Gloeocystis.

uronic acids, and several unidentified compounds.

The highest values for extracellular carbohydrates obtained by Guillard and Wangersky (1958) for a number of species of algae, including flagellates, dinoflagellates[1], diatoms[2], greens[3] and a blue-green[4], ranged from 1.5 mg per liter for the diatom, Cyclotella sp., to 123 mg per liter for the flagellate, Prymnesium parvum.

Marker (1965), using the flagellates, Prymnesium parvum and Isochrysis galbana confirmed Guillard and Wangersky's (1958) results, and found even higher concentrations of carbohydrate (over 200 mg per liter) in salinity-effect experiments with Isochrysis galbana during a 30-day incubation period. He also found that the polysaccharides released from the flagellates consisted mainly of glucose, galactose and arabinose, with small quantities of xylose and ribose.

Moore and Tischer (1964), using eight species of green and blue-green algae[5], found that the amounts of extracellular polysaccharides

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[1]These belonged to the genera: Amphidinium, Gymnodinium.

[2]These belonged to the genera: Cyclotella, Nitzschia, Melosira, Actinocyclus.

[3]These belonged to the genera: Chlorella, Chlorococcum.

[4]This belonged to the genus Synechococcus.

[5]These belonged to the genera: Chlamydomonas, Chlorella, Palmella, Oocystis, Nostoc and Anabaena.

produced ranged from 174 to 557 mg per liter. The polysaccharides of each of the species, except Anabaena flos-aquae appeared to be composed of four monosaccharides: a hexose, a pentose, a methylpentose and a uronic acid. Anabaena flos-aquae lacked the methylpentose component. Hydrolysates of the polysaccharides contained glucose, galactose, arabinose, xylose, ribose, fucose, rhamnose, and glucuronic acid.

Muscatine (1965) found that as much as 85.5% of total C^{14} fixed by the symbiotic algae from Chlorohydra viridissima was released into the medium mainly in the form of maltose, but with small amounts of glucose, glycolic acid, an unidentified oligosaccharide of glucose, and alanine.

Hellebust (1965), investigating organic compounds excreted by a number of species of phytoplankton[1], detected mannitol, arabinose and glucose on chromatograms from the neutral fraction of electrodialyzed culture medium of some of the species.

It can thus be seen that the quantities of extracellular carbohydrate produced by algae vary considerably. These variations may well be due to the species of algae used, the culture conditions employed, the analytical methods employed, and the manner in which the results are expressed. These factors will differ from worker to worker.

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[1]These belonged to the following groups: Chrysophyta (including Chrysophyceae and Bacillariophyceae), Pyrrophyta, Chlorophyta and Cyanophyta.

In the literature, high values for extracellular carbohydrate production were usually obtained when dissolved polysaccharides were liberated. Low values, however, were obtained in the writer's experiments. Although in the writer's experiments, dissolved polysaccharides were not isolated, as by co-precipitation, the possibility still existed that such materials may have been present. If this was the case, then the polysaccharides were present in soluble form in only very small amounts, thus giving low values for total carbohydrate production. Continuing this line of reasoning, then spots U_1 and U_2 on the chromatograms may have been the polysaccharides because of their low Rg-values. There is also the possibility that since dissolved polysaccharides from the experimental diatoms were not at all isolated by the procedures employed, only small quantities of simple, low molecular weight carbohydrates were isolated.

It is of interest to consider the possible origin of the extracellular carbohydrates reported by other workers. Bishop, Adams and Hughes (1954), investigating an extracellular polysaccharide from the blue-green alga, Anabaena cylindrica, found that this material had the same chemical composition as the cellular material. Jones (1962), using the red alga, Porphyridium cruentum, found that this organism excreted an acidic polysaccharide-protein complex which appeared to be similar to the cell material. Marker (1965) found that the extracellular carbohydrates from flagellate cultures appeared to have the same composition as the intracellular carbohydrates.

Parson, Stephens and Strickland (1961), examining the members of a number of groups of marine phytoplankton[1], found that these had very similar intracellular compositions when grown under similar physical and chemical conditions, regardless of the size of the organisms, or the groups to which they belonged. The intracellular carbohydrates of all the diatoms[2] examined included glucose, galactose, mannose, ribose, xylose, rhamnose, fucose and hexouronic acids. According to Fogg's (1966) Type I hypothesis, these low molecular weight carbohydrates should diffuse into the surrounding medium. The possible significance of this is that some of these compounds may then, through the mediation of extracellular enzymes, undergo polymerization to become extracellular polysaccharides. Although extracellular algal enzymes have been identified (Miller, 1959), no data has been found on any such enzyme involved in the formation of extracellular polysaccharides.

Finally, it is worth discussing briefly, some of the data available on the occurrence of carbohydrates or carbohydrate-like materials[3] in natural waters. Vallentyne and Bidwell (1956) found that the total free sugars in a lake mud ranged from 24 to 72.5 mg per kg dry weight of ethanol-insoluble sediment. These were glucose, galactose,

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[1]These belonged to the following groups: Chlorophyta, Chrysophyta (including Chrysophyceae and Bacillariophyceae), Pyrrophyta and Cyanophyta.

[2]These belonged to the genera: Chaetoceros, Skeletonema, Coscinodiscus, Phaeodactylum.

[3]Those substances giving reactions with the reagents, such as anthrone or N-ethyl carbazole, commonly used in the determination of carbohydrates both in natural water and in cultures (Collier, 1953; Zein-Eldin and May, 1958).

arabinose, xylose, and ribose. Vallentyne and Whittaker (1956) found only trace amounts of sucrose and glucose in filtered lake water. Wangersky (1952) found a substance giving some indications of being a rhamnoside in concentrations up to 100 mg per liter in the Gulf of Mexico. Lewis and Rakestraw (1955), examining water samples from The Scripps Institute of Oceanography pier and from the kelp beds off La Jolla, California, found carbohydrates in concentrations ranging from 0.16 to 0.45 mg per liter. Water samples from coastal lagoons of the San Diego area gave concentrations of from 0.3 mg per liter (Encinas) to 7.9 mg per liter (Del Mar). Collier (1958) reported that the carbohydrate concentrations over the continental shelf of the Gulf of Mexico ranged from 0.0 to 3 mg per liter. During a "red tide" period, concentrations varied from 0.0 to 19.4 mg per liter. Collier (1958) indicated that the highest concentrations of carbohydrates were found in the rivers, tidal streams, and marshes bordering the estuaries. Concentrations generally decreased seaward. He also suggested that carbohydrates and other organic materials released by plankton blooms might serve as substrates for marine bacteria, which in turn could supply the plankton with growth factors such as B_{12}. Wangersky (1959), analyzing the dissolved carbohydrates in Long Island Sound through an entire plankton cycle, found that there was no carbohydrate in the water during the spring diatom bloom, but found between 0.5 to 1.5 mg per liter toward the end of a July dinoflagellate bloom. Walsh (1965a), investigating dissolved carbohydrates in Cape Cod waters, showed that the highest values ranging from 1.16 to 3.17 mg per liter were found

in the highly productive estuaries. Other waters had values ranging from 0.4 to 1.0 mg per liter. He suggested that the phytoplankton played an important role in the regulation of carbohydrate concentrations in natural waters. Walsh (1965b), investigating the diurnal fluctuations of dissolved carbohydrate in Oyster Pond, Cape Cod, found that this was most marked at the two meter depth, where it was highest at noon (3.54 mg per liter), and lowest in the morning (1.42 to 1.57 mg per liter). He suggested that carbohydrate was produced by algae in the day time, during photosynthesis, and used by them at night as a readily available source of energy for respiration. Walsh (1966) also found, in examining two Cape Cod ponds, that the greater carbohydrate concentrations were found in the pond having the higher productivity. Donnelly and Burklew (1966), observing carbohydrate distribution during a "red tide" in Apalachee Bay, Florida, reported values of 0.1 to 5.3 mg per liter, depending on area and depth. Walsh and Douglass (1966), investigating the relationship between the vertical distribution of dissolved carbohydrate and oxygen saturation in the Sargasso Sea off Bermuda, found that the highest value (0.75 mg per liter) was obtained at the 75-meter depth where the oxygen saturation was over 120%. They suggested that the overproduction of phytoplankton was a source of dissolved carbohydrate in the Sargasso Sea.

Although the carbohydrate concentration of natural waters varies in time, and from one body of water to another, it should be remembered that different analytical procedures used in determination

can give varying results. Although the concentration of carbohydrates in the natural waters may be low, its rate of turn-over may be sufficiently high to be of considerable ecological importance (Fogg, 1962).

REFERENCES

ALLEN, M. B. 1956. Excretion of organic compounds by Chlamydomonas. Arch. Mikrobiol. 24:163-168.

ANTIA, N. J. and C. Y. LEE. 1963. Studies on the determination and differential analysis of dissolved carbohydrate in seawater. Manuscript Report Series, 168, Fish. Res. Bd. Can. 117 p.

ARMSTRONG, F. A. J. and G. T. BOALCH. 1960. Volatile organic matter in algal culture media and seawater. Nature. 185:761-762.

BISHOP, C. T., G. A. ADAMS, and E. O. HUGHES. 1954. A polysaccharide from the blue-green alga, Anabaena cylindrica. Can. J. Chem. 32:999-1004.

BONGERS, L. H. J. 1956. Aspects of nitrogen assimilation by cultures of green algae (Chlorella vulgaris, strain A and Scenedesmus). Mededel. Landbouwhogeschool Wageningen. 56:1-52.

BRISTOL-ROACH, B. M. 1928. On the influence of light intensity and glucose on the growth of a soil alga. Ann. Botany. 42:317-345.

BROWN, T. L. and J. BENJAMIN. 1964. Useful thin-layer chromatographic techniques. Anal. Chem. 36:446-447.

COLLIER, A. 1953. The significance of organic compounds in sea water. Trans. 18th N. Am. Wildl. Conf. 463-472.

_____ 1958. Some biochemical aspects of red tide and related oceanographic problems. Limnol. Oceanog. 3:33-39.

DONNELLY, P. V. and M. A. BURKLEW. 1966. Carbohydrate distribution in the northwest coastal waters of Florida. Florida Board Conserv., St. Petersburg, Marine Lab. Prof. Papers Ser. no. 8:39-42.

DROOP, M. 1961. Some chemical considerations in design of synthetic culture media for marine algae. Botan. Marina. 2:231-246.

DROOP, M. R. 1962. Organic micronutrients, p. 141-159. In R. A. Lewin (ed.), Physiology and biochemistry of algae. Academic Press, Inc., New York.

DUURSMA, E. K. 1960. Dissolved organic carbon, nitrogen, and phosphorus in the sea. Neth. J. Sea Res. 1:1-148.

FOGG, G. E. 1952. The production of extracellular nitrogenous substances by a blue-green alga. Proc. Roy. Soc., London. 139B: 372-379.

FOGG. G. E. 1962. Extracellular products, p. 475-489. In R. A. Lewin (ed.), Physiology and biochemistry of algae. Academic Press, Inc., New York.

_____ 1966. The extracellular products of algae, p. 195-212. In H. Barnes (ed.), Oceanography and marine biology, Vol. 4. George Allen and Unwin, Ltd., London.

FOGG, G. E. and G. T. BOALCH. 1958. Extracellular products in pure cultures of a brown alga. Nature. 181:789-790.

FOGG, G. E. and D. M. COLLYER. 1953. The accumulation of lipides by algae, p. 177-181. In J. S. Burlew (ed.), Algal culture from laboratory to pilot plant. Carnegie Institution of Washington Publ. 600. Washington, D.C.

FOGG, G. E., C. NALEWAJKO, and W. D. WATT. 1965. Extracellular products of phytoplankton photosynthesis. Proc. Roy. Soc., London. 162B:517-534.

FOGG, G. E. and W. D. WATT. 1965. The kinetics of release of extracellular products of photosynthesis by phytoplankton. Mem. Ist. Ital. Idrobiol. 18 (Suppl.): 165-174.

FOTER, M. J., C. M. PALMER, and T. E. MALONEY. 1953. Antibiotics and Chemotherapy. 3:505-508.

GÄNSHIRT, H. 1965. Documentation of thin-layer chromatograms, p. 40-44. In E. Stahl (ed.), Thin-layer chromatography, a laboratory handbook. Academic Press, Inc., New York.

GUILLARD, R. R. L. 1963. Organic sources of nitrogen for marine centric diatoms, p. 93-104. In C. H. Oppenheimer (ed.), Symposium on marine microbiology. C. C. Thomas, Springfield, Illinois.

GUILLARD, R. R. L. and P. J. WANGERSKY. 1958. The production of extracellular carbohydrates by some marine flagellates. Limnol. Oceanog. 3:449-454.

HELLEBUST, J. A. 1965. Excretion of some organic compounds by marine phytoplankton. Limnol. Oceanog. 10:192-206.

HOOD, D. W. 1967. Personal communication.

HUMPHREY, G. F. and D. V. SUBBA RAO. 1967. Photosynthetic rate of the marine diatom, Cylindrotheca closterium. Australian J. Marine Freshwater Res. 18:123-127.

JACIN, H. and A. R. MISHKIN. 1965. Separation of carbohydrates on borate-impregnated silica gel G plates. J. Chromatog. 18: 170-173.

JEFFREY, L. M. and D. W. HOOD. 1958. Organic matter in sea water: an evaluation of various methods for isolation. J. Marine Res. 17:243-271.

JONES, R. F. 1962. Extracellular mucilage of the red alga, Porphyridium cruentum. J. Cell. Comp. Physiol. 60:61-64.

KETCHUM, B. M. and A. C. REDFIELD. 1938. A method for maintaining a continuous supply of marine diatoms by culture. Biol. Bull. 75:165-169.

LEWIN, J. C. 1955. The capsule of the diatom, Navicula pelliculosa. J. Gen. Microbiol. 13:162-169.

_____ 1958. The taxonomic position of Phaeodactylum tricornutum. J. Gen. Microbiol. 18:427-432.

LEWIN, J. C. and R. A. LEWIN. 1960. Auxotrophy and heterotrophy in marine littoral diatoms. Can. J. Microbiol. 6:127-134.

LEWIN, R. A. 1956. Extracellular polysaccharides of green algae. Can. J. Microbiol. 2:665-672.

_____ 1958. The mucilage tubes of Amphiplura rutilans. Limnol. Oceanog. 3:111-113.

LEWIS, G. J., Jr., and N. W. RAKESTRAW. 1955. Carbohydrate in sea water. J. Marine Res. 14:253-258.

LUCAS, C. E. 1947. The ecological effects of external metabolites. Biol. Rev. 22:270-295.

_____ 1949. External metabolites and ecological adaptations. Symp. Soc. Exptl. Biol. 3:336-356.

_____ 1961. On the significance of external metabolites. Symp. Soc. Exptl. Biol. 15:190-206.

LYMAN, J. and R. H. FLEMING. 1940. Composition of sea water. J. Marine Res. 3:134-146.

MADDUX, W. S. and R. F. JONES. 1964. Some interactions of temperature, light intensity and nutrient concentration during the continuous culture of Nitzschia closterium and Tetraselmis sp. Limnol. Oceanog. 9:79-86.

MARKER, A. F. H. 1965. Extracellular carbohydrate liberation in the flagellates Isochrysis galbana and Prymnesium parvum. J. Marine Biol. Assoc. U.K. 45:755-772.

McLAUGHLIN, J. J. A., P. A. ZAHL, A. NOWAK, J. MARCHISOTTO, and J. PRAGER. 1960. Mass cultivation of some phytoplanktons. Ann. N.Y. Acad. Sci. 90:856-865.

MENZEL, D. W. 1964. The distribution of dissolved organic carbon in the Western Indian Ocean. Deep-Sea Res. 11:757-765.

MILLER, J. D. A. 1959. An extracellular enzyme produced by Monodus. Brit. Phycol. Bull. 7:22

MOORE, B. G. and R. G. TISCHER. 1964. Extracellular polysaccharides of algae: effects on life-support systems. Science. 145:586-587.

MORTON, H. E. 1944. A new style assembly for fritted filters. J. Bacteriol. 47:379-380.

MOSKOVITS. G. 1961. Studies on diatom-bacteria interactions. Ph.D. Thesis. A. and M. College of Texas. 341 pp.

MUSCATINE, L. 1965. Symbiosis of hydra and algae, III. Extracellular products of algae. Comp. Biochem. Physiol. 16:77-92.

NAKAMURA, K. and C. S. GOWANS. 1964. Nicotinic acid-excreting mutants in Chlamydomonas. Nature. 202:826-827.

NALEWAJKO, C., N. CHOWDHURI and G. E. FOGG. 1963. Excretion of glycolic acid and the growth of a planktonic Chlorella, p. 171-183. In Studies on microalgae and photosynthetic bacteria. Japanese Society of Plant Physiologists. Univ. Tokyo Press, Tokyo.

NEMEC, J., K. KEFURT and J. JARY. 1967. Thin-layer chromatography of aldonic acids, lactones, aldoses, and alditols. J. Chromatog. 26:116-120.

OVODOV, Y. S., E. V. EVTUSHENKO, V. E. VASKOVSKY, R. G. OVODOVA, and T. F. SOLOV'EVA. 1967. Thin-layer chromatography of carbohydrates. J. Chromatog. 26:111-115.

PARSON, J. R., K. STEPHENS, and J. D. H. STRICKLAND. 1961. On the chemical composition of eleven species of marine phytoplankton. J. Fish. Res. Bd. Can. 18:1001-1016.

PASTUSKA, G. 1961. Untersuchungen über die qualitativ und quantitativ Bestimmung der Zucker mit Hilfe der Kieselgelschicht-Chromatographie, II Mitteilung. Z. Anal. Chem. 179:427-429.

Pharmacopoeia of the United States of America. 1955. 15th revision. U.S. Pharmacopoeial Convention, Inc., Easton, Pennsylvania.

PRATT, R. 1947. Stabilization of penicillin in aqueous solutions of low concentrations of phosphates. Nature. 159:233-235.

PRINGSHEIM, E. G. 1949. Pure culture of algae. Cambridge Univ. Press, London.

PROVASOLI, L. 1958. Nutrition and ecology of protozoa and algae. Ann. Rev. Microbiol. 12:279-308.

_____ 1963. Organic regulation of phytoplankton fertility, p. 165-219. In M. N. Hill (ed.), The Sea, Vol. 2. Interscience Publishers, New York.

PROVASOLI, L., J. J. A. McLAUGHLIN and M. R. DROOP. 1957. The development of artificial media for marine algae. Arch. Microbiol. 25:392-428.

PROVASOLI, L., I. J. PINTNER and L. PACKER. 1951. Use of antibiotics in obtaining pure cultures of algae and protozoa. Proc. Soc. Protozool. 2:6.

REIMANN, B. E. F. and J. C. LEWIN. 1964. The diatom genus Cylindrotheca Rabenhorst, with a reconsideration of Nitzschia closterium. J. Roy. Microscop. Soc. 83:283-296.

RYTHER, J. H. 1956. Photosynthesis in the ocean as a function of light intensity. Limnol. Oceanog. 1:61-70.

RYTHER, J. H. and D. W. MENZEL. 1965. On the production, composition and distribution of organic matter in the western Arabian Sea. Deep-Sea Res. 12:199-209.

RUSSEL, J. H. 1963. Thin-layer chromatography. Rev. Pure Appl. Chem. 13(1):15-29.

SAUNDERS, G. W. 1957. Interrelations of dissolved organic matter and phytoplankton. Botan. Rev. 23:389-409.

SCOTT, J. A. Jr., and E. H. MELVIN. 1953. Determination of dextran with anthrone. Anal. Chem. 25:1956-1661.

SPENCER, C. P. 1952. On the use of antibiotics for isolating bacteria-free cultures of marine phytoplankton organisms. J. Marine Biol. Assoc. U.K. 31:97-106.

_____ 1954. Studies on the culture of a marine diatom. J. Marine Biol. Assoc. U.K. 33:265-290.

STADELMANN, E. J. 1962. Permeability, p. 493-528. In R. A. Lewin (ed.), Physiology and biochemistry of algae. Academic Press, Inc., New York.

STAHL, E. 1965. Instruments used in thin-layer chromatography and their operation, p. 5-29. In E. Stahl (ed.), Thin-layer chromatography, a laboratory handbook. Academic Press, Inc., New York.

STAHL, E. and U. KALTENBACH. 1961. Dünnschicht-Chromatographie. VI. Mitteilung. Spurenanalyse von Zuckergemischen auf Kieselgur G-Schichten. J. Chromatog. 5:351-355.

_____ and _____ 1965. Sugars and derivatives, p. 461-469. In E. Stahl (ed.), Thin-layer chromatography, a laboratory handbook. Academic Press, Inc., New York.

STEWART, W. D. P. 1963. Liberation of extracellular nitrogen by two nitrogen-fixing blue-green algae. Nature. 200:1020-1021.

SYRETT, P. J. 1962. Nitrogen assimilation, p. 171-188. In R. A. Lewin (ed.), Physiology and biochemistry of algae. Academic Press, Inc., New York.

TOLBERT, N. E. and L. P. ZILL. 1956. Excretion of glycolic acid by algae during photosynthesis. J. Biol. Chem. 222:895-906.

VALLENTYNE, J. R. and R. G. S. BIDWELL. 1956. The relation between free sugars and sedimentary chlorophyll in lake mud. Ecology. 37:495-500.

VALLENTYNE, J. R. and J. R. WHITTAKER. 1956. On the presence of free sugars in filtered lake water. Science. 124:1026-1027.

VAN OORSCHOT, J. L. P. 1955. Conversion of light energy in algal cultures. Mededel. Landbouwhogeschool Wageningen. 55:225-276.

WALDI, D. 1965a. Dünnschicht-Chromatographie einiger Zucker und Zuckeralkohole. J. Chromatog. 18:417-418.

_____ 1965b. Spray reagents for thin-layer chromatography, p. 483-502. In E. Stahl (ed.), Thin-layer chromatography, a laboratory handbook. Academic Press, Inc., New York.

WALSH, G. E. 1965a. Studies on dissolved carbohydrate in Cape Cod waters. I. General survey. Limnol. Oceanog. 10:570-576.

_____ 1965b. Studies on dissolved carbohydrate in Cape Cod waters. II. Diurnal fluctuation in Oyster Pond. Limnol. Oceanog. 10:577-582.

WALSH, G. E. 1966. Studies on dissolved carbohydrate in Cape Cod waters. III. Seasonal variation in Oyster Pond and Wequaquet Lake, Mass. Limnol. Oceanog. 11:249-256.

WALSH, G. E. and J. DOUGLASS. 1966. Vertical distribution of dissolved carbohydrates in the Sargasso Sea off Bermuda. Limnol. Oceanog. 11:406-408.

WANGERSKY, P. J. 1952. Isolation of ascorbic acid and rhamnosides from sea water. Science. 115:685.

_____ 1959. Dissolved carbohydrates in Long Island Sound (1956-1958). Bull. Bingham Oceanog. Collection. 17:87-94.

WANGERSKY, P. J. and R. R. L. GUILLARD. 1960. Low molecular weight organic base from the dinoflagellate, Amphidinium carteri. Nature. 185:689-690.

WATT, W. D. 1966. Release of dissolved organic material from the cells of phytoplankton populations. Proc. Roy. Soc., London. 164B. 521-551.

WILLIAMS, P. M. 1961. Organic acids found in Pacific Ocean waters. Nature. 189:219-220.

ZEIN-ELDIN, Z. P. and B. Z. MAY. 1958. Improved N-ethyl carbazole determination of carbohydrates with emphasis on sea water samples. Anal. Chem. 30:1935-1941.

ZOBELL, C. E. 1946. Marine microbiology. Chronica Botanica Co., Waltham, Mass.

APPENDIX I

TABLE V

TLC data obtained with carbohydrate standards and unknowns using a number of systems

STANDARDS	Chromatographic Systems*						
	1	2	3	4	5	6	7
Glucose	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Rhamnose	7.73	2.86	3.24	3.70	5.00	3.12	0.92
Xylose	3.45	2.10	2.16	2.12	2.93	1.81	1.25
Ribose	3.00	1.74	2.12	2.00	3.14	1.58	0.85
Fructose	1.64	1.00	1.45	1.17	2.00	1.22	
Fucose	3.18	2.08	1.90	1.85	3.21	2.00	
Arabinose	1.36	1.37	1.31	2.12	2.07	1.53	1.00
Galactose	0.64	0.80	0.65	0.75	1.29	0.88	0.84
Lyxose	4.27	1.50	1.61	1.50	3.00	-	0.80
Mannose	1.64	1.37	1.30	1.44	1.86	1.27	1.30
Sucrose	0	0	0	0.19	1.07		
Raffinose	0	0	0	0	0.43		
Galacturonic acid					0.27		
Mannuronic acid					0.80		
Ribitol							0.78
Mannitol							0.60
Erythritol							6.79
Sorbitol							0.55
Identified Compounds							
Galactose	0.59		0.63	0.60	1.27		
Glucose	1.00		1.05	1.00			
Xylose	3.57	2.10	2.16			1.81	
Arabinose		1.40			2.05	1.52	
Galacturonic acid					0.27		
Tentatively Identified Compounds							
Sucrose, Raffinose	0	0	0	0.10			
Arabinose, Fructose	1.46		1.30				
Xylose, Lyxose					2.94		

TABLE V (continued)

Tentatively Identified Compounds	1	2	3	4	5	6	7
Galactose, Glucose Fructose		0.88					
Xylose, Arabinose				2.10			
Glucose, Sucrose					1.00		
Glucose, Galactose						1.00	
Unidentified Compounds							
**					0	0	
**					0.13		
**					3.50		

*System 1: Solvent: n-butanol-acetone-water (4:5:1 v/v)
Impregnating Compound: 0.3 M disodium hydrogen phosphate
Detection Reagent: anisaldehyde-sulphuric acid reagent

System 2: Solvent: n-butanol-pyridine-water (8:4:3 v/v)
Impregnating Compound: 0.3 M disodium hydrogen phosphate
Detection Reagent: anisaldehyde-sulphuric acid reagent

System 3: Solvent: n-butanol-acetone-water (4:5:1 v/v)
Impregnating Compound: 0.3 M sodium dihydrogen phosphate
Detection Reagent: anisaldehyde-sulphuric acid reagent

System 4: Solvent: n-butanol-pyridine-water (8:4:3 v/v)
Impregnating Compound: 0.3 M sodium dihydrogen phosphate
Detection Reagent: anisaldehyde-sulphuric acid reagent

System 5: Solvent: n-butanol-ethanol-0.1 M phosphoric acid (1:10:5 v/v)
Impregnating Compound: 0.3 M sodium dihydrogen phosphate
Detection Reagent: 1) anisaldehyde-sulphuric acid reagent
2) benzidine-sodium metaperiodate reagent

TABLE V (continued)

System 6: Solvent: ethyl acetate: acetic acid: water (6:3:2 v/v)
Impregnating Compound: 0.3M sodium dihydrogen phosphate
Detection reagent: anisaldehyde-sulphuric acid reagent

System 7: Solvent: methyl ethyl ketone-acetic acid-methanol (6:2:2 v/v)
Impregnating Compound: none
Detection reagent: 1) anisaldehyde-sulphuric acid reagent
2) concentrated sulphuric acid reagent

**These unidentified compounds may possibly be U_1, U_2 and U_3. The first spot (Rg-value, 0) showed a dark blue color with anisaldehyde-sulphuric acid reagent as found in U_1. The second spot (Rg-value, 0.13) was larger in size than any other spots employed and therefore resembled the behavior of U_2. The third spot (Rg-value, 3.50) had the highest value of the unidentified compounds for the particular solvent system employed and therefore resembled the behavior of U_3.

FIGURE 6

A standard glucose curve for the determination of total carbohydrate by the anthrone procedure.

APPENDIX II