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GLUCOSE TRANSPORT AND METABOLISM IN *GYMNODINIUM BREVE*

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Key Word Index—*Gymnodinium breve*; dinoflagellate; Florida red tide; glucose transport and metabolism.

Abstract—Florida's red tide organism, *Gymnodinium breve*, utilized exogenous glucose in the light for the synthesis of cellular components. Glucose was not taken up in the dark. Kinetic parameters for glucose uptake include a K_{FD} of 11 μM and a V_{max} of 1×10^{-10} mol of glucose taken up/mg cellular protein/hr. Glucose uptake was competitively inhibited by phloridzin ($K_i = 40\,\mu M$), mannose ($K_i = 120\,\mu M$), and 2-deoxy-D-glucose ($K_i = 190\,\mu M$) and non-competitively inhibited by galactose ($K_i = 125\,\mu M$). Kinetics and inhibition of glucose uptake are consistent with a facilitated diffusion transport system.

INTRODUCTION

For purposes of laboratory cultivation of marine dinoflagellates, comparatively nutrient-deficient media are employed and artificial lighting is provided. Such conditions select for photoautotrophs but often create the mistaken impression that such organisms are obligate photoautotrophs.

Organic nutrients, primarily from land run-off, have been implicated in Florida's red tide outbreaks although attempts to stimulate blooms in the laboratory using organic enrichment of media have not been totally successful. Although Ryther [1] found that high concentrations of organic nutrients could extend the duration of red tides, he believed high levels were not necessary for their development. This view was substantiated by Aldrich [2] who showed no stimulation of *Gymnodinium breve* growth in the light or dark on a number of organic carbon sources. Conversely, Doig [3] *et al.* have demonstrated that seasonal pulses of nutrient enrichment, i.e. land run-off, are more significant in red tide outbreaks than previously suspected and that a continuous flow of nutrients in the form of treated sewage, may be important in sustaining blooms.

The heterotrophic capabilities and response of *G. breve* to carbohydrate carbon sources have been examined and are reported in this paper.

RESULTS

The isolation of whole cells using 6% sucrose NH-15 solution in Hopkins vaccine tubes is a method designed to minimize cellular destruction, as laboratory cultures of *G. breve* are exceedingly fragile. By centrifuging through 6% sucrose, the following objectives are accomplished: (1) following exposure to a radioactive substrate the cells are effectively washed free of external label during centrifugation (zero time samples are at background levels). (2) the denser sucrose solution tends to buoy the cells against the centrifugal force and thereby prevent lysis. Six per cent sucrose is used because this concentration does not cause shrinkage of the cells due to osmotic shock (1.095 Osm/l. for NH-15, 1.27 Osm/l. for 6% sucrose NH-15), but is dense enough to prevent lysis at 70–140 *g*. In addition, any bacterial contaminants are not concentrated up to 100 *g*. Unfortunately, low speed centrifugation is not sufficiently gentle to retain 100% viability of the sample.

G. breve cultures are generally contaminated with Gram-negative motile rods, as described by Wilson [8]. Owing to the aforementioned fragility of cultures, removal of bacterial contaminants by conventional physical methods is not possible, nor is the administration of antibiotics successful. Although several antibiotics, notably chloramphenicol, kanamycin and tetracycline do inhibit bacterial growth in cultures, they also have an irreversible detrimental effect on *G. breve* including loss of chloroplast integrity and cell lysis. Under no conditions of antibiotic administration did *G. breve* survive 2 generations (longer than *ca* 7 days). We have noted, however, that when cultures of *G. breve* were subjected to lighting intensities of greater than 4300 lx, the dinoflagellate population ceased swimming and settled out, while the bacterial population remained disperse. The separatory funnel technique allows the recovery of viable, concentrated *G. breve* cells while, at the same time reducing bacterial contamination to below microscopically detectable limits (< 6000 cells/ml). For uptake experiments, micro-pore (0.2 μm) filtered NH-15 from the original culture provides the most suitable medium for *G. breve* resuspension. NH-15 (see Table 1) based nutrient agar plates inoculated with experimental cultures obtained in this fashion, show no bacterial growth after 7 days and only 1–4 colonies/cm^2 after 10 days. Plated unsettled cultures show numerous colonies after 2–3 days.

In the solvent fractionation of cells labeled with glucose-[^{14}C], 30% of the glucose taken up was metabolized to chloroform-soluble material. Non-dialyzable macromolecular water-soluble material accounts for a further 15% and the remaining 55% of the radioactivity is recovered at the denatured interface between the aqueous and organic phases.

Following hydrolysis and chromatography of the macromolecular water phase, four aniline–diphenylamine reactive spots are observed. Three are identified

Table 1. Composition of NH-15 medium

Compound	Concn (ppm)	Stock solutions: Vitamins 8	Concn (ppm)	Metals T	Concn (ppm)	Sulfides	Concn (ppm)
NaCl	24000	Inositol	1000	$FeCl_3 \cdot 6H_2O$	280	NH_4Cl	200
$MgSO_4 \cdot 7H_2O$	6000	Thymine	800	H_3BO_3	300	$NaHCO_3$	200
$MgCl_2 \cdot 6H_2O$	4500	Choline chloride	500	Na_2SiO_3	500	$Na_2S \cdot 9H_2O$	150
$CaCl_2$	700	Orotic acid	260	TiO_2	83	KH_2PO_4	100
KCl	600	Thiamine	200	$ZrOCl_2$	40	$MgCl_2 \cdot 6H_2O$	40
Tris buffer (pH 8.1–8.2)	400	Nicotinic acid	100	$MnCl_2 \cdot 4H_2O$	37		
K_2HPO_4	10	Pyridoxine	40	$BaCl_2$	15		
KNO_3	10	Pyrodoxamine diHCl	20	NH_4VO_3	12		
EDTA	10	PABA	10	K_2CrO_4	11		
Thiamine	10	Putrescine	8	H_2SeO_3	10		
Adenine sulfate	1	Riboflavin	5				
Vitamin B_{12}	0.001	Folic acid	2·5				
Biotin	0·0005	Biotin	0·5				
Vitamins 8	0·1 ml of mix	Vitamin B_{12}	0·05				
Sulfides	5 ml of mix						
Metals T	5 ml of mix						

as galactose, xylose, and arabinose. The fourth spot migrates as a disaccharide should migrate and compares most closely with cellobiose. It remains unidentified as the colour reaction is not that of cellobiose. Surprisingly, glucose or cellobiose are not detectable under either of the hydrolysis conditions.

A quantitative amino acid analysis of the hydrolysed interface is shown in Table 2.

Glucose uptake

Glucose uptake follows first order kinetics at all concentrations examined. Uptake in the dark proceeds at a decreasing rate for 2 hr when it ceases altogether (Fig. 1). At 2 μM substrate concentration, the uptake of glucose is more rapid than that of either mannose or galactose. Uptake is also first order for these sugars. 3-*O*-Me-D-glucose and 2-deoxy-D-glucose, both non-metabolizable glucose analogs, are not accumulated (Fig. 2). The K_{FD} (a constant of facilitated diffusion analogous to the Michaelis constant K_m) for glucose transport is 11.4(±)2 μM and the V_{max} is $1(\pm)0.1 \times 10^{-10}$ mol of glucose taken up/mg cell protein/hr.

Phloridzin, galactose, mannose, and 2-deoxy-D-glucose inhibit the uptake of glucose. The results are presented as double reciprocal plots in Figs. 3–6. Phloridzin, mannose, and 2-deoxy-D-glucose act as competitive inhibitors of glucose uptake, while galactose acts as a

Table 2. Labeled amino acid analysis of denatured debris at the interface arising from the solvent fractionation

Amino acid	nmol*	nmol carbon	CPM	Sp. act.†
Asp	1590	6360	23700	3.73
Thr	1090	4340	9380	2.16
Ser	1170	3510	4280	1.21
Glu	1870	9370	19900	2.12
Pro	944	4720	3430	0.73
Gly	1910	3820	1390	0.36
Ala	1770	5300	10500	1.98
Val	1100	5480	6240	1.14
Met	404	2020	4480	2.22
Ile	768	4610	6470	1.40
Leu	1530	9200	14900	1.62
Tyr	520	4680	8530	1.82
Phe	803	7230	11800	1.63
Lys	1030	6160	4850	0.79
His	547	3280	884	0.27
Arg	793	4760	4150	0·87

Lyophilized interface arising from the solvent fractionation was hydrolysed for 18 hr at 105° in 6N HCl containing 0.1% PhOH. Amino acid ratios were determined quantitatively on an amino acid analyser.* The sp. act. of each amino acid was determined by summation of radioactivity observed in analysis of collected fractions and division by the nmol of carbon present in in each fraction from the analyser†.

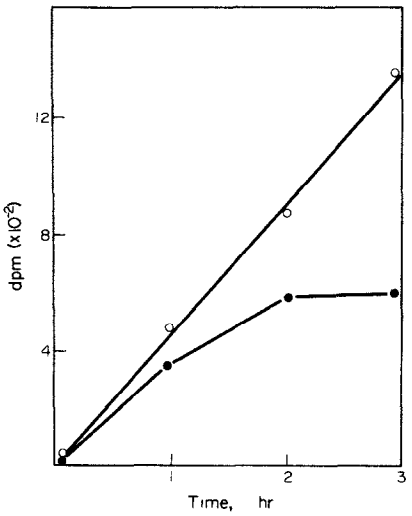

Fig. 1. Comparison of uptake of glucose in the light and dark. Identical cultures of *G. breve* were supplemented with 8 μM glucose-[^{14}C] (100 mCi/mmol) and were examined for uptake in the light (○) and in the dark (●). Culture illumination was 4000 lx, temp. 24°. Dark incubations were performed by wrapping the culture tubes in aluminum foil. Each point is the result of whole cell samples assayed for radioactivity by liquid scintillation techniques.

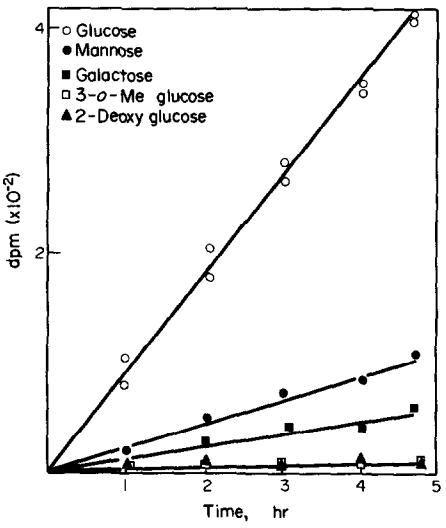

Fig. 2. Comparative rates of uptake for glucose, galactose, mannose, 2-deoxy-D-glucose and 3-*O*-Me-D-glucose. Substrate concentration in each case was 2 μM. Each point is the result of a whole cell assay of radioactivity. All sp. act. of substrates were 50–51 mCi/mmol. The relative rates of uptake for glucose–mannose–galactose were 10:2:1. 2-Deoxy-D-glucose and 3-*O*-Me-D-glucose were not concentrated by *G. breve*.

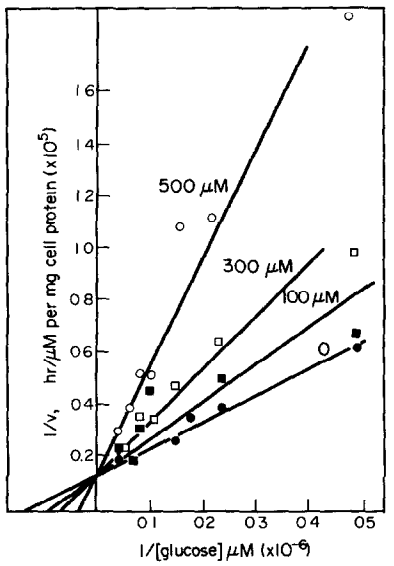

Fig. 4. Competitive inhibition of glucose uptake by mannose. Glucose-[^{14}C] concn: 2–40 μM; mannose concn: (●) 0 μM, (■) 100 μM, (□) 300 μM, (○) 500 μM. Cultures were incubated 2 hr under standard culture conditions and whole cells were assayed for radioactivity every 30 min. Glucose uptake was linear at each mannose concentration. Double reciprocal plots of glucose uptake at each mannose concentration intersect at the $1/v$ axis, indicative of competitive inhibition.

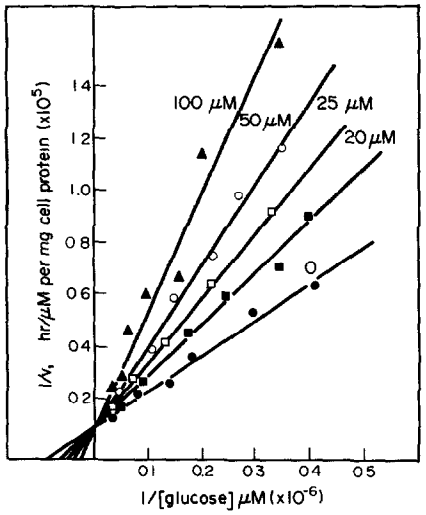

Fig. 3. Competitive inhibition of glucose uptake by phloridzin. Glucose-[^{14}C] concn: 2–40 μM; phloridzin concn: (●) 0 μM, (■) 20 μM, (□) 25 μM, (○) 50 μM, (▲) 100 μM. Cultures were incubated 2 hr under standard culture conditions and whole cells were assayed for radioactivity every 30 min. Glucose uptake was linear at each phloridzin concentration. Double reciprocal plots of glucose uptake at each phloridzin concentration intersect at the $1/v$ axis, indicative of competitive inhibition.

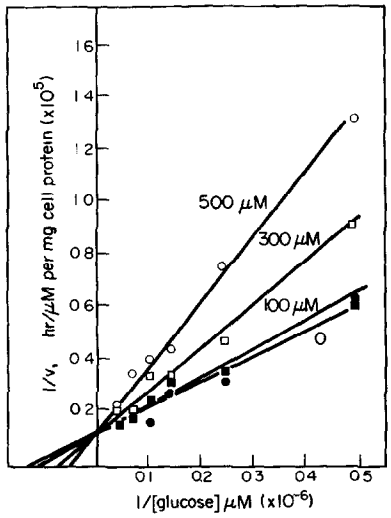

Fig. 5. Competitive inhibition of glucose uptake by 2-deoxy-D-glucose. Glucose-[^{14}C] concn: 2–40 μM; 2-deoxy-D-glucose concn: (●) 0 μM, (■) 100 μM, (□) 300 μM, (○) μM. Cultures were incubated 2 hr under standard culture conditions and whole cells were assayed for radioactivity every 30 min. Glucose uptake was linear at each 2-deoxy-D-glucose concentration. Double reciprocal plots of glucose uptake at each 2-DOG concentration intersect at the $1/v$ axis, indicative of competitive inhibition.

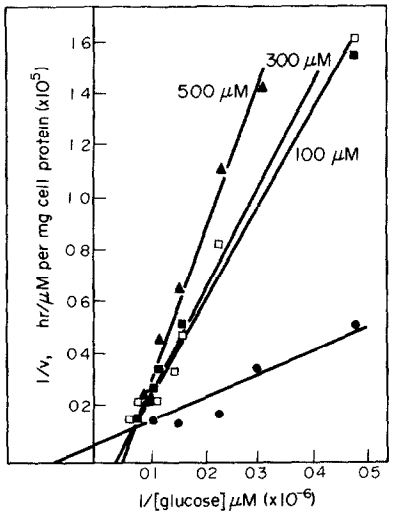

Figure 6. Non-competitive inhibition of glucose uptake by galactose. Glucose-[^{14}C] concn: 2–40 μM; galactose concn: (●) 0 μM, (■) 100 μM, (□) 300 μM, (▲) 500 μM. Cultures were incubated 2 hr under standard culture conditions, and whole cells were assayed for radioactivity every 30 min. Glucose uptake was linear at each galactose concentration. The double reciprocal plot of glucose uptake rate vs inhibitor concentration at various glucose concentrations indicate that galactose inhibition is non-competitive in nature.

non-linear, non-competitive inhibitor [9]. The inhibitor constants K_i are determined graphically by plotting the slope of the lines against their respective inhibitor concentrations.

DISCUSSION

Following separatory funnel 'settling', bacterial numbers are reduced to less than 6000 cells/ml and cultures obtained in this fashion show linear rates of glucose-[^{14}C] uptake. If *G. breve* cultures containing bacterial at normal concentrations (3×10^6 cells/ml) are utilized for transport and metabolism studies, the rate of ^{14}C uptake by the cells continues to rise exponentially throughout the incubation. This increase was found to be due to the uptake of ^{14}C bacterial metabolites. Although this is potentially an interesting interspecies metabolic relationship, in order to examine the response of *G. breve* to exogenous carbohydrates, it is necessary to eliminate bacterial contributions. It is for this reason the separatory funnel technique is used. If the separatory funnel technique is repeated a second time to further reduce bacterial levels, uptake and metabolism again are linear and correspond directly to cell number and not bacterial concentration (which are now present at a calculated level of 12/ml).

Further, if *G. breve* cells are removed from settled cultures by low speed centrifugation, and the supernatants are incubated in the presence of glucose-[^{14}C], pellets then obtained by high speed centrifugation (to centrifuge any bacteria which may still be present) show no accumulation of ^{14}C. Uptake and metabolism ceases at the time *G. breve* cells are removed.

G. breve can utilize glucose-[^{14}C] for the synthesis of cellular components in the light. The glucose taken up is rapidly metabolized as no pools of free glucose are detected. The only labeled carbohydrates found in polysaccharides are galactose, xylose and arabinose. Glucose apparently is not an important component of either structural or storage polysaccharides. These findings cast doubt on previous suggestions that *G. breve* is enclosed in a simple cellulose cell envelope. In addition, the storage granules present in *G. breve* which fail to stain with iodine [10], if they are indeed polysaccharide in nature, are believed to be composed of galactose, xylose, and/or arabinose.

Over half the label derived from the metabolism of glucose-[^{14}C] is found in amino acids released by acid hydrolysis. This is not surprising as reports have shown ATP synthesized via cyclic photophosphorylation is used predominantly for protein synthesis in algal and chloroplast preparations in the light [11]. All of the 16 amino acids detected were labeled with ^{14}C but the sp. act. was not uniform. The pattern of radioactivity (Table 2) is not what would be expected if $^{14}CO_2$ were being supplied as the labeled carbon source. If $^{14}CO_2$ were being produced and were then used to synthesize amino acids following fixation through photosynthesis, the highest sp. act. should be expected in glycine, serine, alanine and aspartic acid [12, 13].

Generally, during photosynthesis in the presence of $^{14}CO_2$, glycine is labeled more rapidly than any other amino acid [12]. In the case of glucose-[^{14}C] utilization by *G. breve*, however, the sp. act. of glycine is one of the lowest detected. In the absence of labeled bicarbonate, and in the presence of glucose-[^{14}C], label from glucose probably is diluted by competition with autotrophic pathways for the synthesis of glycine from CO_2. The high sp. act. in aspartate is not surprising as one would expect glycolysis and the tricarboxylic acid cycle to be responsible for the major fraction of its carbon skeleton. It thus seems that *G. breve* is able to utilize glucose carbon for the synthesis of cellular components without total prior degradation to CO_2.

Glucose is not taken up by *G. breve* in the dark. This is in agreement with the findings of Aldrich [2] that *G. breve* is incapable of growth on glucose in the dark. If other exogenous substrates are transported in the manner of glucose (only in the light) it may help explain why *G. breve* is an obligate phototroph. An organism capable of heterotrophic growth would continue to utilize glucose in the dark, deriving ATP via oxidative phosphorylation. In fact, many photosynthetic organisms capable of an heterotrophic existence develop light-labile active transport systems in the dark [14]. The transport system for glucose in *G. breve* appears to be neither active nor inducible.

The evidence compiled is consistent with the existence of a facilitated diffusion system for the transport of glucose. The uptake of glucose is concentration dependent and is saturated at high glucose concentration. Uptake can be competitively inhibited by several compounds including phloridzin, mannose and 2-deoxy-D-glucose. Mannose, a sugar which can be metabolized, may inhibit uptake by competition for a reaction step other than transport, such as phosphorylation by hexokinase. Galactose, which certainly should be a substrate for a hexokinase reaction inhibits non-competitively.

Phloridzin and its aglycone phloretin have been reported to act as competitive inhibitors of glucose uptake in a number of transport systems. Phloridzin does not

translocate across cell membranes, but rather, binds through the monosaccharide portion of the molecule to specific receptors associated with the transport system. In addition, there are phenolic receptor sites which bind other portions of the phloridzin molecule, and hence it becomes an excellent competitive inhibitor of glucose transport because it essentially immobilizes a portion of the available transport sites [15]. In *G. breve*, phloridzin is a more efficient competitive inhibitor of glucose transport than either mannose or 2-deoxy-D-glucose.

2-Deoxy-D-glucose is not metabolized, nor is it concentrated by *G. breve*. Yet, it is capable of competitive inhibition; inhibition taking place at the transport level. Because 2-deoxy-D-glucose apparently binds to the glucose transport system but is not accumulated, transport of 2-deoxy-D-glucose and of glucose is not active.

Among those tested, phloridzin, mannose and 2-deoxy-D-glucose are the only compounds which showed competitive inhibition patterns. The glucose transport system in *G. breve* shows a high degree of specificity. It appears that only monosaccharides with a high stability in the C1 conformation have any great affinity for the transport receptors (Fig. 7). As determined by LeFevre [15], the relative stability of sugars in the C1 conformation is 2-deoxy-D-glucose > D-glucose > D-mannose > D-galactose > D-xylose... It is evident from LeFevre's findings that the stability of the C1 conformation of galactose is *much* less than that of mannose. This finding may explain the reason galactose does not inhibit glucose uptake competitively—the affinity of galactose must be excedingly low owing to the decreased fraction of galactose in the C1 conformation.

3-*O*-Me-D-glucose is not concentrated by *G. breve*, nor does it inhibit glucose uptake. The 3-hydroxyl group in glucose is in a predominantly equatorial configuration. The modification of the 3-hydroxyl to a 3-methoxy should not decrease its stability in the C1 conformation,

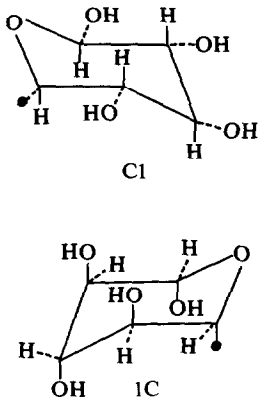

Fig. 7. C1 and 1C chair configurations of D-glucose. Note that in the C1 conformation, all OH groups are in equatorial positions as well as the C-6 carbon. In the 1C conformation, all OHs and C-6 are in axial positions. According to Reeves [16], C1 is the stereochemically favoured conformation, as the substituent groups are least sterically hindered.

as the bulky 3-*O*-Me group is in a sterically uncrowded equatorial position. Thus, an explanation for the lack of inhibition of glucose uptake is that the 3-hydroxyl group must be intact for attachment to the transport molecule.

Evidence indicates the process of transport is not directly linked to energy consumption as inhibitors of oxidative phosphorylation and electron transport (2,4-dinitrophenol and *o*-phenanthroline) fail to have any effect on glucose uptake. Because no free glucose is detected intracellularly in *G. breve* upon exposure to glucose-[^{14}C], it can be postulated that all glucose transported is immediately metabolized, suggesting that transport is the rate-limiting step in glucose utilization. Apparently, light is necessary for the synthesis of ATP needed for metabolic processes, as glucose uptake is inhibited in the dark. As *G. breve* is incapable of utilizing exogenous glucose in the absence of light, it may best be classified as a photomixotrophic organism [3].

The process of glucose uptake by *G. breve* can best be characterized as a passive carrier-mediated system. Intracellular metabolism keeps the internal glucose concentration near zero. This situation allows a continuous carrier-mediated flow of glucose into the cells.

CONCLUSIONS

(1) *G. breve* can utilize carbohydrates for the synthesis of cellular components. (2) Glucose is utilized in the light principally for the synthesis of amino acids without total oxidation to CO_2. The predominant monosaccharides obtained after acid hydrolysis (galactose, xylose, arabinose) were also synthesized from glucose, as were lipid-soluble compounds. (3) The kinetics of glucose uptake and inhibition are consistent with a facilitated diffusion transport system. Glucose uptake is much greater in the light than it is in the dark. The K_{FD} for glucose uptake is about 11 μM and the V_{max} is about 1×10^{-10} mol of glucose taken up/mg cellular protein/hr. (4) The receptors of the carrier molecules have a high affinity for sugars in the C1 conformation. Phloridzin ($K_i = 40$ μM), mannose ($K_i = 120$ μM), and 2-deoxy-D-glucose ($K_i = 190$ μM) inhibits glucose uptake competively. Galactose ($K_i = 125$ μM) inhibits glucose uptake in a non-competitive manner. 3-*O*-Me-D-glucose, *o*-phenanthroline, and 2,4-dinitrophenol have no effect on glucose transport. (5) Even though *G. breve* possesses a specific system for glucose uptake, it does not provide for a significant ancillary source of carbon, as the maximum rate of uptake is low. It does not preclude the possibility that glucose, in addition to other nutrients, both organic and inorganic, can stimulate (or sustain) Florida red tide blooms.

EXPERIMENTAL

Unialgal cultures of *G. breve** were grown in the laboratory, 2.5 l. in 3 l. cotton-plugged conical flasks. The cultures were maintained in the completely defined artificial seawater medium NH-15 of Gates and Wilson as modified in ref. [4]. The cultures were kept unshaken at 24° in a transparent Plexiglas 60 × 240 cm constant temp. bath and were illuminated by 6 dual banks of 40 W Sylvania GRO-LUX Wide Spectrum† fluorescent lamps at an incident illumination of 4000 lx. Cultures grown under these conditions reach a maximum cell density of 2–4 × 10^7 cells/l. in 17 days, from an inoculum of 10^6 cells/l. *G. breve* cells were counted using either a Coulter Counter Model B (10–60 μm) or direct observation of 0.1 ml of culture medium under low

* Unialgal cultures courtesy of E. A. Joyce, K. A. Steidinger, Fla. Dept. Nat. Resource. Mar. Res. Lab., St. Petersburg, Fla.

† Sylvania Bulletin 0-285, Danvers, Massachusetts.

power magnification. Bacterial counts were obtained microscopically in a Petroff–Hausser counting chamber.

Preparation of cultures. Cultures in early log-phase of growth were used for all studies. A 2 l. sterile separatory funnel was filled with a 2 l. culture of *G. breve* containing 5×10^3 cells/ml and 3×10^6 bacteria/ml, and was then illuminated at 4300 lx intensity for 20 min. *G. breve* cells, presumably light-saturated, collected above the stopcock (bacteria remained disperse) and were drained in a minimum vol. (*ca* 2 ml) into a sterile 140 ml Kimax screw-capped tube containing 100 ml micropore (0.2 μm) filtered original culture medium. When lighting intensities were reduced to below 4000 lx, cells commenced swimming. Appropriate dilutions were made to yield a final *G. breve* concn of 1–2 × 10^4 cells/ml and a final bacterial concn calculated to be not greater than 6×10^3 cells/ml. Bacterial contamination in cultures so obtained was assessed using Difco Bacto-nutrient agar, a medium recommended for use in determining the agar plate count of bacteria in the bacteriological examination of water. This medium contains (in g/l.): beef extract, 3; peptone, 5; agar, 15. NH-15 medium (see Table 1), the culture medium and aq. basis for the nutrient agar, contains organic chelators, trace metals, numerous vitamins, several sources of nitrogen and sources of phosphorus and sulfur. Before and after each expt, cultures were examined under high power magnification to determine if the cells had retained normal size, shape and motility. Cultures less than 90% motile were discarded.

Isolation of whole cells. Hopkins vaccine tubes were filled to 1 ml with 6% sucrose dissolved in NH-15 medium. Aliquots (10 ml) of the experimental culture were carefully layered on top of the sucrose soln and the tubes were centrifuged at 70 *g* for 4 min. The culture supernatant and the sucrose solns were removed with a pipet, leaving 10 μl of sucrose soln containing the whole cell pellet. The pellet was removed with the aid of a micro-syringe. The latter was rinsed with 2 × 0.1 ml vols of dist. H_2O following removal and expulsion of the pellet.

Determination of radioactivity. All radioactive pellets and their appropriate syringe washes were suspended in 10 ml Aquasol (New England Nuclear, Inc.) liquid scintillation fluid in Beckman Poly Q vials. Radioactivity was assessed using a liquid scintillation counter; ^{14}C efficiency was 60%.

Developed TLC plates were assayed for radioactivity in two ways. First, 5 × 20 cm plates were scanned for radioactivity using a gas flow counter. Total deflection was set at 10 000 DPM. Secondly, 1 mm–1 cm fractions were scraped sequentially from origin to solvent front and were suspended each in 10 ml Aquasol for counting. In all cases, appropriate controls such as unlabeled cells, liquids, and plates were assayed for background radiation. The substrates in all cases were uniformly labeled with ^{14}C (sp. act. 50–200 mCi/mmol) with the exception of 2-deoxy-D-glucose (1-^{14}C, 50 mCi/mmol).

Metabolism of glucose. Following exposure of 100 ml to 20 μM glucose-[^{14}C] (51 mCi/mmol) for 6 hr at standard culture conditions, the labeled cells (1×10^6) were extracted with $CHCl_3$ according to the method of ref. [5]. The organic phase was flash-evaporated and an aliquot was assayed for radioactivity. The H_2O layer was concentrated to 10 ml by flash-evaporation, transferred to dialysis tubing (MW exclusion 16000–18000) and then exhaustively dialysed against dist. H_2O.

The retained material following dialysis was hydrolysed in 0.5 N HCl for 3 hr at 100° or for 18 hr in 3 N H_2SO_4 at 100°. The hydrolysates were reduced in vol. and were applied to 5 × 20 cm Si gel TLC plates. The plates were developed in one direction ×3 according to the method of ref. [6] to optimize monosaccharide separation. Sugars were identified by spraying with aniline–diphenylamine and comparing color reaction and migration of standards. Radioactivity of the fractions was assayed as outlined earlier.

The denatured debris at the interface from the solvent separation was filtered on Gelman type A-E glass fiber filters, washed with 5% TCA and MeOH and lyophilized. Protein was determined according to ref. [7]. Portions were hydrolysed in 6N HCl/0.1% PhOH for 18 hr at 105°. Amino acid analysis was performed on an amino acid analyser to obtain a quantitative amino acid profile. Fractions of 0.4 ml were also collected and assayed for radioactivity.

Uptake of carbohydrates. Labeled substrates were added to the cultures aseptically in a minimal vol. Experimental incubations (100 ml in 140 ml Kimax screw-capped tubes) did not exceed 6 hr and were carried out under standard culture conditions.

Whole cells samples (10 ml) were prepared every 30 mins. The rate of glucose-[^{14}C] uptake with respect to increasing substrate concn was examined from 2 to 40 μM. Comparison of glucose uptake in the light and in the dark was investigated at 8 μM substrate concn. Dark incubations were performed by wrapping the culture vessel in Al foil. The relative rates of uptake of glucose, galactose, mannose, 2-deoxy-D-glucose, and 3-*O*-Me-D-glucose were examined at 2 μM substrate concn.

Inhibitors of glucose uptake. Glucose-[^{14}C] (100 mCi/mmol) and unlabeled inhibitors were thoroughly mixed in 5 ml NH-15. The mixture was added to cultures as in the previous expts. The cultures were incubated for 2 hr and 10 ml samples were removed every 30 min. Glucose-[^{14}C] conc ranged from 2 to 40 μM in all cases. Unlabeled inhibitors were used at concentrations as follows: galactose 0–0.5 mM, mannose 0–0.5 mM, phloridzin 0–0.1 mM, 2-deoxy-D-glucose 0–0.5 mM, 3-*O*-D-glucose 0–0.5 mM, 2,4 dinitrophenol 0–1.0 mM, *o*-phenanthroline 0–0.5 mM.

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