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Heterotrophic Nutrition of the Marine Pennate Diatom *Nitzschia angularis* var. *affinis**

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Abstract

The nutritional pattern for heterotrophic growth of *Nitzschia angularis* var. *affinis* (Grun.) Perag. is more complex than for other diatom species studied previously. This species grew slowly in the dark in the presence of single amino acids, either glutamate or alanine; other amino acids when supplied singly were not used as substrates. Carbon from glutamate was converted to cell carbon with an efficiency of 43%. Glutamine was inhibitory both in the light and in the dark, and aspartate inhibited heterotrophic growth on glutamate. Glucose and tryptone supplied singly did not support heterotrophic growth, but when combined, together they allowed for rapid growth of *N. angularis* (generation time of 16 h). Glucose in combination with glutamate, alanine, aspartate, or asparagine (but not with any other amino acids) also supported growth in the dark, at a rate considerably more rapid than with glutamate alone. In the presence of excess glucose and limiting concentrations of glutamate, approximately 50% of the cell carbon for heterotrophic growth came from glucose, while in combination with tryptone about 25% of the cell carbon came from glucose. Amino acids were taken up by cells grown either photoautrophically or in the dark in the presence or absence of organic substrates; uptake rates were somewhat higher for dark-grown than for light-grown cells. Glucose was taken up only by dark-grown cells; induction of a glucose uptake system in the dark required the presence of glutamate but not of glucose. The rates of uptake of glutamate and glucose by cells incubated in the dark with glutamate were sufficiently high to account for the observed rates of growth on these substrates in the dark. The uptake systems of *N. angularis* have relatively high affinities for glucose (K_s = 0.03 mM) and glutamate (K_s = 0.02 mM).

Introduction

Diatom species constitute the predominant marine microalgal flora in most coastal habitats of the temperate region. For a complete understanding of the ecological relationship among the species in a community, dynamics of the food web, etc., it is necessary to learn as much as possible about the physiological and metabolic behavior of individual species comprising the community. With this in mind, we have been interested for some time in heterotrophy as a possible alternate mode of nutrition among the diatoms, including: a determination of species capable of utilizing organic substrates for dark growth, which carbon sources may function as substrates, how the carbon sources are taken up and metabolized, rates of growth in the dark, and the efficiency with which the various substrates are utilized.

In previous studies, we concentrated on the heterotrophic nutrition of the marine centric diatom *Cyclotella cryptica* Reimann, Lewin and Guillard, which utilizes glucose as a substrate for dark growth (Lewin, 1963; Hellebust, 1970, 1971a, b), and on the marine pennate diatom *Cylindrotheca fusiformis* Reimann and Lewin, which utilizes lactate, succinate, fumarate, and malate (Lewin and Hellebust, 1970; Hellebust and Lewin, 1972). Both species exhibited a lag phase when incubated in the dark in the presence of a suitable substrate, with cell division commencing only after about 2 days. This

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lag phase represented the time needed for development of transport systems specific for uptake of the substrate. A surprising finding was that only darkness itself (or dim light) was required for induction of the particular transport system (a transport system for glucose uptake in *Cyclotella cryptica* and a transport system for lactate uptake in *Cylindrotheca fusiformis*); the presence of the substrate was not necessary. Furthermore, both of these transport systems were inactivated by light. Thus, light conditions controlled the transport system for glucose and for lactate in these two species. A similar light control of galactose uptake in another clone of *Cyclotella cryptica* and of glucose uptake by an unidentified species of *Coscinodiscus* has since been demostrated by White (1974).

In order to learn as much as possible concerning modes of heterotrophic nutrition found among species of diatoms, we are currently experimenting with three pennate species that possess entirely different nutritional patterns from the species studied earlier. These three species are all capable of utilizing glutamate as a substrate for dark growth. One of the species, *Navicula pavillardi* Hustedt, could be grown in the dark in the presence of glutamate, but glucose was not utilized as a substrate (Lewin and Hellebust, 1975). Growth of *N. pavillardi* on glutamate in the dark took place without an initial lag phase; growth on tryptone commenced after a 2-day lag phase, which could be abolished by the simultaneous presence of glucose.

The metabolism of a second species, *Nitzschia angularis* var. *affinis* Grun. Perag., is the subject of the present paper. *N. angularis* in many ways resembles *Navicula pavillardi* in its nutritional responses, but there are also significant differences. *N. pavillardi* is a fairly large-celled species with a consequent slow growth rate, which caused us some experimental difficulties. Since the growth rate of *Nitzschia angularis* is much faster, both in the light and in the dark, rates of uptake of radioactively labeled substrates could be more readily measured with this latter species.

Materials and Methods

Organism

The strain of *Nitzschia angularis* was found originally growing on *Chondrus crispus* in Herring Cove, Nova Scotia, Canada. It was isolated into bacteria-free culture by streaking material on an agar surface and was designated as Strain No. 35-M (Lewin and Lewin, 1960).

Culture Medium

The cells were grown in sea water enriched with inorganic nutrients and vitamins (thiamine, biotin, vitamin B_{12}). The culture medium employed was essentially medium "f" as specified by Guillard and Ryther (1962), except that it was found necessary to add an increased amount of sodium metasilicate (Si concentration = 10 ppm) to obtain the maximum cell yield from the cultures.

Dark-Growth Experiments

For those experiments in which substrates were tested for heterotrophic growth, 5 ml of culture medium were put into each test tube, which was then covered with a glass cap. Organic compounds were either added to the culture medium before autoclaving, or they were filter-sterilized and added after autoclaving, depending on the stability of the particular compound being tested. All culture media were adjusted to pH 8.

Inoculum was taken from log-phase cultures growing in the light in the enriched sea-water medium described above and was homogenized in a sterilized Potter Tissue-Homogenizer to disperse clumps. A uniform cell suspension was then inoculated into each tube to give an initial cell concentration of 2 to 4 x 10^3 cells/ml. The cultures were grown at 20°C. For determination of growth rates in the dark, cells were generally counted every other day at the beginning of an experiment and at longer intervals later on. The contents of duplicate tubes were homogenized and counted independently using a hemacytometer. Averaged cell numbers were plotted on semi-log graph paper and the doubling time of the populations was read from the graphs.

Uptake of ^{14}C-(Uniformly)-Labeled Substances

Axenic cultures and experimental conditions were used. Detailed descriptions of methods have been presented earlier (Hellebust and Guillard, 1967; Lewin and Hellebust, 1975). All of the results of the uptake experiments are expressed as averages of duplicate determinations.

Cell-Carbon Determinations

Carbon contents of cells incubated in the dark with glutamate as a substrate

Table 1. Organic compounds tested as possible substrates supporting heterotrophic growth of *Nitzschia angularis* (++: dense growth; +: fair growth; -: absence of growth)

Compound	Concentration	Light	Dark
Used singly			
Tryptone	1.0, 0.5, 0.25, 0.125 (%)	++	-
Yeast extract	1.0, 0.5, 0.25, 0.125 (%)	++	-
Glucose	28, 14, 7, 3.5 (mM)	++	-
Na acetate	120, 60, 30, 15, 7.5 (mM)	++	-
Na lactate	44, 22, 11, 5.5 (mM)	++	-
Na succinate	30, 15, 7.5, 3.8 (mM)	++	-
Glycerol	108, 54, 27, 13.5, 6.8 (mM)	++	-
Mannitol	54, 27, 13.5, 6.8, 3.4 (mM)	++	-
Na glutamate	60, 30, 15, 7.5 (mM)	++	++
Glutamine	17, 8.5, 4.3 (mM)	-	-
Aspartic acid	18, 9, 4.5 (mM)	++	-
Asparagine	18, 9, 4.5 (mM)	++	-
Alanine	2, 0.2 (mM)	++	+
16 additional amino acids tested singly	2 (mM)	++	-
Combinations			
Tryptone + glucose	0.1% + 5 mM	++	++
Glutamate + glucose	2 + 0.6 (mM)	++	++
Aspartate + glucose	2 + 0.6 (mM)	++	++
Asparagine + glucose	2 + 0.6 (mM)	++	++
Alanine + glucose	2 + 0.6 (mM)	++	++
Glutamate + aspartate	0.08 + 0.2 (mM)	++	-
Glutamate + alanine	0.08 + 0.2 (mM)	++	++
16 additional amino acids each tested in combination with glucose	2 + 0.6 (mM)	++	-

for 4 days were determined by first washing the cells with fresh medium, then resuspending the cells in distilled water and homogenizing them by 2-min sonication with a Biosonic (Bronwill) sonicator. Subsamples of the resulting suspension were injected into a Beckman Model 915 Total Carbon Analyzer. Corrections were made for the presence of small amounts of inorganic carbon.

Results

Compounds Capable of Serving as Substrates for Dark Growth

The various compounds tested, ranges of concentrations used in the experiments, and the resulting qualitative assessment of the final yield both in the light and in the dark are summarized in Table 1. Cultures in the light were run as controls for the dark cultures to determine whether or not the concentrations used were inhibitory.

Among the single compounds tested, only glutamate and alanine could serve as substrates for growth in the dark; the growth rate on alanine was slower than on glutamate. In addition to these, glucose in the presence of tryptone allowed for vigorous growth in the dark at the most rapid rate of any of the substrates. Growth also took place with glutamate combined with glucose (faster than with glutamate alone) and also with alanine, aspartate, or asparagine, when combined with glucose. Aspartate inhibited growth on glutamate in the dark, but not in the light.

Growth Rate in the Light

Cells growing photoautotrophically in the light (intensity 4000 lux) multiplied with a generation time of 10 h.

Growth Rates in the Dark with Varying Concentrations of Na Glutamate

A positive response to glutamate could be demonstrated at a concentration as low as 0.0032 mM (Fig. 1). It appeared that the growth rates with the two lowest concentrations tested (0.0032 and

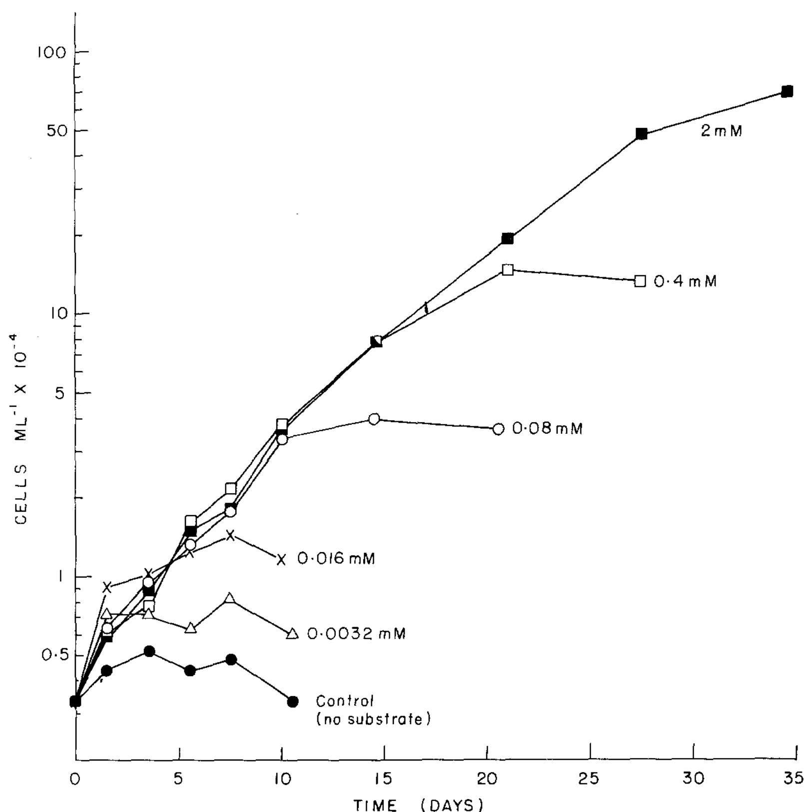

Fig. 1. *Nitzschia angularis*. Growth in the dark in presence of different concentrations of Na glutamate

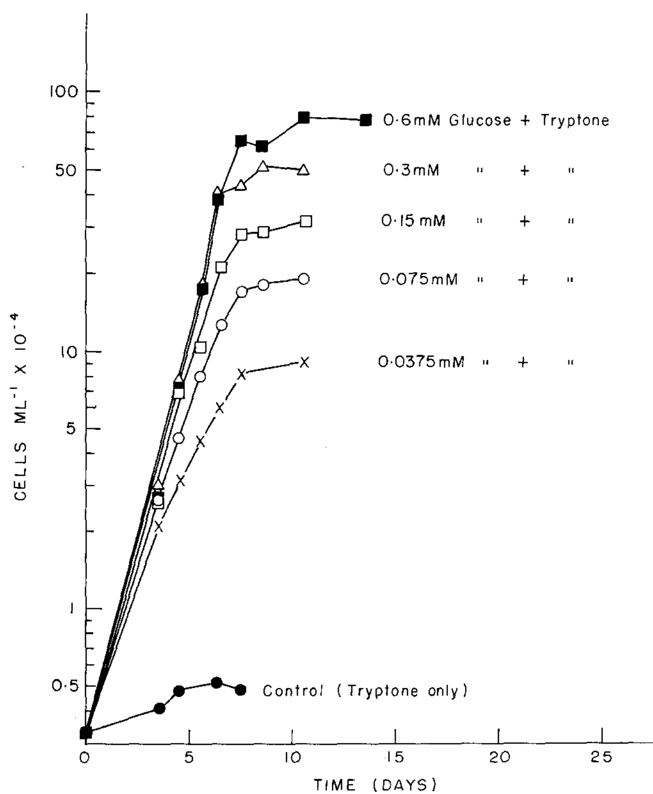

Fig. 2. *Nitzschia angularis*. Growth in the dark in presence of 0.1% tryptone plus different concentrations of glucose

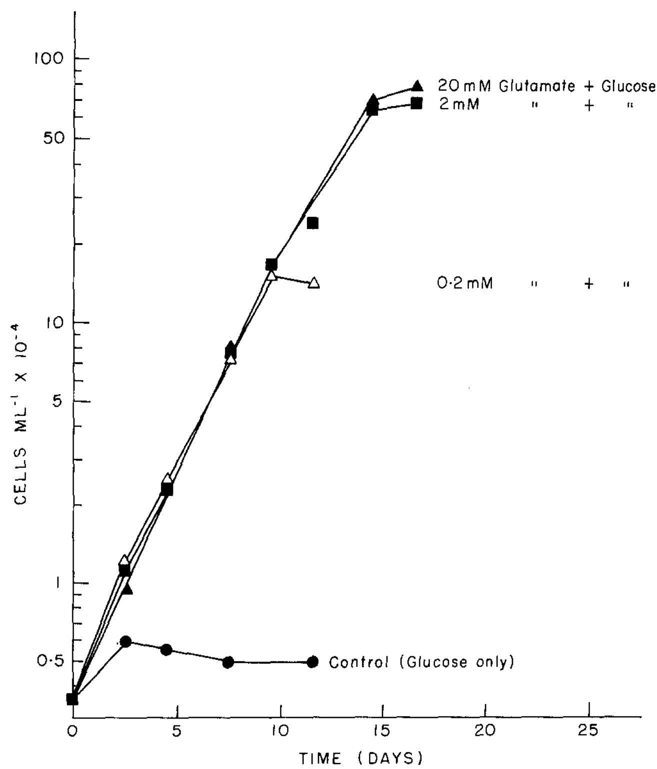

Fig. 3. *Nitzschia angularis*. Growth in the dark in presence of 0.6 mM glucose plus different concentrations of Na glutamate

0.016 mM) were somewhat more rapid during the first day of growth than with the higher glutamate concentrations (Fig. 1). Otherwise, there was no obvious effect of substrate concentration on the growth rate. The generation time observed with a glutamate concentration of 2 mM (or greater) was 78 h for the first 10 days followed by a slower rate of 120 h until the stationary phase was reached.

Growth Rates in the Dark in Presence of Tryptone (0.1%) with Varying Concentrations of Glucose

The cells did not multiply in the presence of tryptone alone (Fig. 2; see also Table 1) or in any of the glucose concentrations alone (Table 1), confirming the results of an earlier study (Lewin, 1963). A positive response to glucose could be demonstrated at a concentration as low as 0.0375 mM when it was present with 0.1% tryptone (Fig. 2). There was some effect of glucose concentration on growth rates in that the rates were somewhat slower at the lower concentrations (Fig. 2). The cells had a generation time in the dark of 16 h with glucose concentrations ranging from 0.15 to 10 mM.

Growth Rates in the Dark in Presence of Glucose (0.6 mM) with Varying Concentrations of Na Glutamate

Glutamate concentrations of 0.2, 2 and 20 mM were tested in the presence of glucose (0.6 mM). The generation time in the presence of all three glutamate concentrations was around 42 h (Fig. 3).

Absence of Lag Phase

No lag phase was detectable in any of the dark-growth experiments (Figs. 1, 2, and 3). When cells were taken from the light and used as inoculum for the dark experiments, they were apparently able to shift their metabolic pathways almost immediately for utilization of glutamate alone or for utilization of glucose plus glutamate or glucose plus tryptone.

Growth Experiments with Glutamine, Aspartic Acid, and Asparagine

Experiments were carried out to study the effects of the amino acids and amides most closely related to glutamate. Glutamine was toxic in the light at the concentrations tested (Table 1). Aspartic acid and asparagine could not serve as substrates when supplied alone in the dark (Table 1); in this respect they both differed from glutamate. However, when supplied in the presence of glucose (at 0.6 mM), both were able to support growth of *Nitzschia angularis* in the dark.

Efficiency of Conversion of Glutamate Carbon to Cell Carbon

From the cell yields obtained with 0.08, 0.4 and 2 mM glutamate (according to Fig. 1), it could be determined that it took 147, 177 and 150 pg glutamate carbon at these concentrations, respectively, to produce one cell, or an average value of 155 pg cell^{-1}. Analytical determinations of the actual carbon content of cells grown for several generations on glutamate in the dark resulted in an average value of 66 pg C cell^{-1}. From these two values it can be calculated that the efficiency for converting glutamate carbon to cell carbon must have been 43% (assimilation efficiency = carbon in cells x 100/carbon taken up by cells).

Calculations of Relative Contribution of Carbon from Glucose, when in Combination with Glutamate or with Tryptone

From the cell yield obtained with 0.2 mM glutamate combined with 0.6 mM glucose (Fig. 3), if one assumes that all of the glutamate has been consumed with an efficiency of 43%, it can be estimated that each cell must contain 34 pg C from glutamate. Therefore, the remaining 32 pg C (again assuming a total carbon content of 66 pg C cell^{-1}), or about 50%, must have been derived from glucose.

It was determined from the cell yields obtained with 0.0375 and 0.075 mM glucose combined with 0.1% tryptone (according to Fig. 2) that, assuming that all the glucose was used up by the cells, each cell had in both cases consumed 30 pg of glucose carbon. If one further assumes that glucose is converted to cell carbon with 50% efficiency, approximately 15 pg C, or 23% of the total cell carbon (assumed to be approximately 66 pg C cell^{-1} in this case also) was derived from glucose. Therefore, about 75% of the cell carbon can be presumed to have been derived from tryptone.

Estimation of Uptake Rate of Glutamate Required to Produce Observed Doubling Time of 78 h

It is possible to estimate the required rate of glutamate uptake ($\frac{dx}{dt}$) for the

Table 2. *Nitzschia angularis*. Rates of uptake (µmole cell^{-1} min^{-1} x 10^{-10}) of different radioactively labeled compounds by cells grown in continuous light (3,000 lux), or in continuous light followed by 48 h in the dark in absence or presence of glutamate, glucose or lactate. nd: not determined

^{14}C-labeled compound (0.1 mM)	Light	Dark	Dark glutamate (1 mM)	Dark glucose (1 mM)	Dark lactate (1 mM)
Alanine	0.5	0.9	0.9	0.9	1.0
Aspartate	1.3	2.3	2.1	2.2	3.0
Glutamate	2.1	4.0	3.6	3.7	4.2
Glutamine	0.9	2.0	2.3	1.3	1.1
Isoleucine	0.1	0.2	0.2	0.1	0.1
Glucose	0.0	0.0	2.6	0.0	0.0
Lactate	0.0	0.1	0.1	0.2	0.3
Malate	0.0	0.1	0.1	0.1	0.2
Glycerol	0.1	0.1	nd	nd	0.1

observed doubling time (t_d) of approximately 78 h for cells grown on glutamate in the dark, from the glutamate carbon requirement of 155 pg to produce one cell (x_0). The following relationships derived from the equation for exponential cell growth were used in the estimate of the required uptake rate: (1) $\frac{dx}{dt} = x_0\mu$, where µ is the specific growth constant; and (2) $\mu = \frac{0.693}{t_d}$ (the relation between specific growth constant and doubling time). By introducing the known values for x_0 and t_d in the above equations, it was found that $\frac{dx}{dt} = 2.3 \times 10^{-2}$ pg C cell^{-1} min^{-1}, or 3.8×10^{-10} µmole glutamate cell^{-1} min^{-1}.

Rates of Uptake of Radioactively Labeled Compounds

Cells grown in continuous light, or in the dark in the presence or absence of organic substrates were in all cases able to take up amino acids (Table 2). Glutamate and aspartate were taken up most efficiently of the amino acids; isoleucine was taken up only at very low rates. Uptake rates for dark-incubated cells were higher than for cells grown in continuous light. Glucose was not taken up by cells which had been grown previously in continuous light or had been kept in the dark either without a substrate or in the presence of glucose or lactate (see Table 2). However, cells incubated in the dark with glutamate developed the ability to take up glucose rapidly. The organic acids, lactate and malate were not taken up by light-grown cells, and only at very low rates by cells incubated in the dark with or without organic substrates. Glycerol was taken up by light or dark-incubated cells at very low rates.

Kinetic Constants for Glutamate and for Glucose

Determinations of kinetic constants for uptake of glutamate and glucose at different substrate concentrations by cells incubated in the dark for 2 days in the presence of 1 mM Na glutamate were made from the plots shown in Figs. 4 and 5, respectively, with the following results: (a) for glutamate uptake, K_s = 0.02 mM, and $V_{max} = 3.0 \times 10^{-10}$ µmole cell^{-1} min^{-1}; and (b) for glucose uptake, K_s = 0.03 mM, and $V_{max} = 2.2 \times 10^{-10}$ µmole cell^{-1} min^{-1}.

Discussion

Nitzschia angularis was able to grow only on glutamate or alanine as single substrates. The rate of growth on glutamate (doubling time about 78 h), corresponding to a required rate of glutamate uptake of 3.0×10^{-10} µmole cell^{-1} min^{-1}, can be accounted for by the observed maximum rates of glutamate uptake of 3.6 to 4.2×10^{-10} µmole cell^{-1} min^{-1} ("Results" and Table 2). The similar growth rates observed in the dark with glutamate concentrations ranging from 0.003 to 2 mM can be explained by the high affinity of the uptake system for glutamate (K_s = 0.03 mM). Alanine supported a much slower growth rate in the dark; this can be explained by the slower uptake rate for alanine (0.9×10^{-10} µmole cell^{-1} min^{-1}) than for glutamate by dark-adapted cells (Table 2).

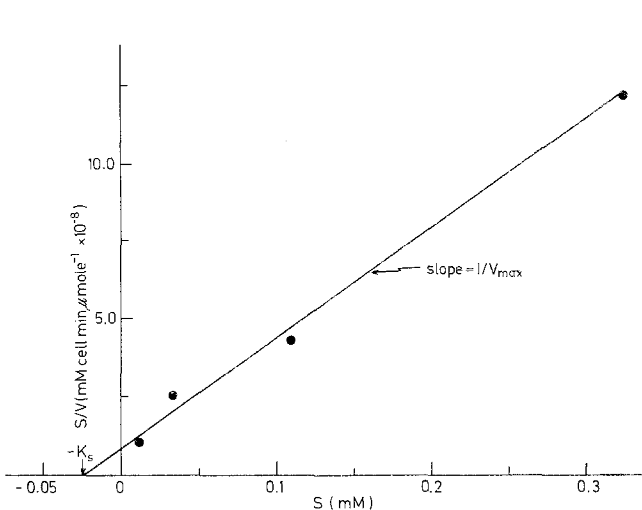

Fig. 4. *Nitzschia angularis*. Rate of uptake (V) of ^{14}C-glutamate measured at different substrate concentrations (S), with S/V plotted against S (Dowd and Riggs, 1965). Cells incubated in the dark in presence of 1 mM glutamate for 2 days prior to uptake experiment

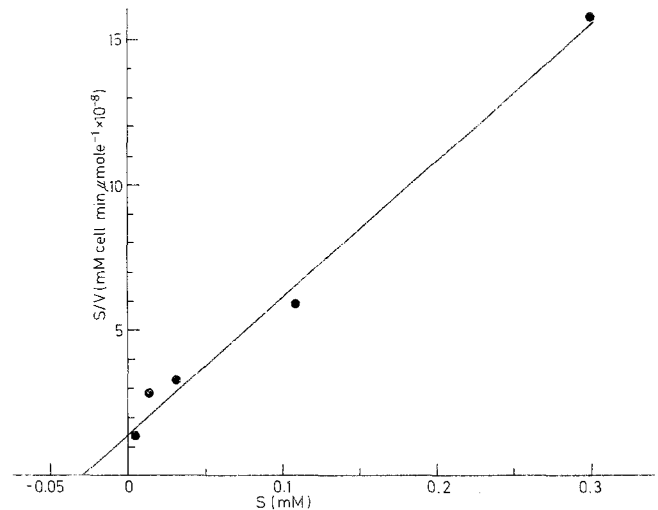

Fig. 5. *Nitzschia angularis*. Rate of uptake (V) of ^{14}C-glucose measured at different substrate concentrations (S), with S/V plotted against S. Cells incubated in the dark in presence of 1 mM glutamate for 2 days prior to uptake experiment

Aspartate did not support growth in the dark when supplied alone, but both aspartate and asparagine supported slow growth when present in addition to glucose. The inhibition by aspartate of growth on glutamate in the dark may in part be due to the commonly observed inhibition of glutamate uptake by aspartate in diatoms (Hellebust, 1970). The presence of 1 mM aspartate inhibited the uptake of 0.1 mM ^{14}C-glutamate by *Nitzschia angularis* by 78%.

It is quite surprising that glucose by itself did not allow growth of *Nitzschia angularis* in the dark but allowed good growth when combined with glutamate, and rapid growth when combined with tryptone (Figs. 2, 3). The lack of growth in the dark on tryptone (a pancreatic enzyme digest of casein) alone is also surprising, but may be due in part to aspartate inhibition of glutamate uptake. It appears that glucose can only supply about 50% of the cell carbon when cells are grown with limiting glutamate concentrations and excess tryptone (Fig. 3 and "Results"). When cells were grown with excess tryptone and limiting glucose concentrations (Fig. 2), about 25% of the cell carbon was derived from glucose and the rest from tryptone (protein hydrolysate). This shows that glucose cannot serve as a sole carbon source, but that an amino acid – glutamate, alanine, aspartate or asparagine – or a combination of amino acids (tryptone) are required for at least 50% of the cell carbon during heterotrophic growth. The biochemical reasons for these observations are currently being investigated.

The regulation of glucose uptake in *Nitzschia angularis* is quite unusual in that while light-grown cells do not take up glucose at significant rates, it is not sufficient to simply incubate the cells in the dark to induce the formation of a glucose-uptake system, as in the case of *Cyclotella cryptica* (Hellebust, 1971b). *N. angularis* requires the presence of a metabolizable substrate, such as glutamate, for this induction to take place.

The rate of uptake of glucose by cells incubated in the dark for 2 days with glutamate, 2.6 x 10^{-10} µmole cell^{-1} min^{-1} (Table 2), is approximately that required to account for the observed doubling time of 42 h in the dark in the presence of glucose and glutamate, assuming that glucose contributes approximately 50% of the cell carbon. This observed rate of glucose uptake would also be sufficient to account for the more rapid growth rate of 16 h observed for cells grown in the presence of glucose and excess tryptone (Fig. 2), since glucose in this case only contributes approximately 25% of the cell carbon. The high affinity of the glucose uptake system for glucose (K_s = 0.03 mM) explains the similar growth rates observed at limiting glucose concentrations in the

range 0.075 to 0.3 mM in the presence of excess tryptone (Fig. 2).

The nutritional pattern of heterotrophic growth of *Nitzschia angularis* shows more complexity than that of any other diatom species studied hitherto. This is the first example of a case where heterotrophic growth has been enhanced by a combination of two substrates present together in the culture medium, with part of the cell carbon coming from each substrate.

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