UPTAKE AND UTILIZATION OF ALANINE-1-^{14}C BY FRESHWATER ALGAE

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

in

Biology

by

David Bennett Campbell

Waco, Texas

August, 1969

ABSTRACT

Experiments were conducted with Chlorella pyrenoidosa, Cosmarium formosulum, and Gloeocapsa sp. to determine their relative heterotrophic capacity at various conditions. Cultures were incubated in nutrient media with 0.5 uc DL-alanine-1-^{14}C added at various temperature (10C, 20C, 30C) and illumination (0 ft-c, 2137 ft-c, 8547 ft-c) conditions. A temperature increase resulted in an increase in alanine uptake in C. pyrenoidosa and C. formosulum. Uptake decreased in Gloeocapsa at 20C but increased at 30C. High light intensity and darkness stimulated uptake in C. pyrenoidosa and C. formosulum and uptake was minimal at 2137 ft-c. Uptake occurred at all illumination conditions with Gloeocapsa, but was maximum at 2137 ft-c.

Alanine was incorporated into cytoplasmic protein in C. pyrenoidosa and C. formosulum but a significant amount was incorporated into protein in Gloeocapsa only at 2137 ft-c. These species were concluded to be facultative heterotrophs.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Owen T. Lind for his suggestions, instructive criticism, and enthusiasm in directing this research. My gratitude is extended to Dr. Floyd F. Davidson for his assistance in providing the necessary materials for this research and to Dr. T. J. Bond for his critical review and suggestions.

I also wish to thank the Baylor Chemistry Department for the use of the Beckman scintillation counter, and John Coulter for his assistance and suggestions in operating this equipment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Plants of lake, stream, and river communities have long been assumed to be entirely autotrophic-assimilating only inorganic nutrients. A heterotrophic role among algal organisms with the assimilation of organic as well as inorganic carbon was thought to be present only in sewage and waters contaminated by it, as found in numerous algal organisms (Wiedeman, 1964).

There is a considerable amount of dissolved organic matter in natural bodies of water (Birge and Juday, 1934; Watt, 1966), being derived from runoff of the drainage area, autolysis of organisms in the system, excretion and secretion of living organisms, defecation, and from sediments through diffusional processes. This dissolved organic matter is composed of several photosynthetic and metabolic intermediates.

The relationship between dissolved organic matter in natural bodies of water and the organisms that produce, transform, and use it remains one of the important unsolved problems in aquatic biology. Several investigators (Bristol Roach, 1928; Skinner and Gardner, 1930; Lewin, 1953; Samejima and Myers, 1958; and Hoare and Moore, 1965) have shown that many algal organisms of supposed autotrophic nutrition can make use of soluble carbohydrates, fat soluble organic compounds, and sodium and potassium salts of various organic acids. However, the possibility that autotrophic organisms might utilize nitrogenous organic compounds has been considered very little.

Organic substrate utilization varies widely among species and

strains, with the exception of the utilization of glucose and acetate. Utilization of these compounds has been investigated extensively and found to occur among most organisms studied. I investigated the amino acid, alanine, utilization by phytoplankton species and the conditions under which this occurs. Since Rodhe (1955) reports large algal masses in lakes under snow cover for long time periods, it seemed important to measure relative heterotrophy among certain species of freshwater algae under light variations as well as temperature variations. The hypotheses were that heterotrophy increases with a decrease in photosynthesis and increases with an increase in temperature.

REVIEW OF THE LITERATURE

Evidence For The Presence Of Dissolved Organic Matter

Indirect evidence exists for the presence of dissolved organic matter in natural bodies of water. In order to culture many algae in the laboratory, extracts from soil, lake water, and yeast or peptone are necessary in addition to the synthetic media (Bold, 1942; Pringsheim, 1946; Provosoli and Pinter, 1953). This indicates there are certain organic growth factors necessary for the maintenance of the algal cells. If these cells require organic growth factors for reproducing in the laboratory, then it seems reasonable that these same organic growth factors will be necessary for growth in natural bodies of water.

Direct evidence is obtained by biochemical analysis. Total dissolved solids, in general, in fresh waters range from about 15 to 300 ppm. Total dissolved organic matter for Wisconsin lakes ranges from 2.9 to 39.6 ppm. with an average of 12.8 ppm. (Welch, 1952). Total dissolved organic matter for Lake Waco ranges from 6.2 to 9.3 ppm. with an average of 7.9 ppm. (O. T. Lind, personal communication).

Some of the initial work with dissolved organic material was by Birge and Juday (1934) working in the Highland Lake District of northeastern Wisconsin. These lakes contained considerable amounts of dissolved organic matter consisting of crude protein 24%, ether extract 2.5%, and carbohydrate 73.5%. Vallentyne and Whittaker (1956) found sucrose and glucose in two lakes of eastern Ontario. Watt (1966),

working with *in situ* productivity experiments on four freshwater ponds, found glutamate, serine, and alanine present. Vallentyne (1957) found the following dissolved organic matter in freshwater and marine sources: biotin, glucose, niacin, sucrose, thiamin, vitamin B_{12}, alanine, aspartic acid, cystine, glycine, and tryosine.

Many of these photosynthetic and metabolic intermediates are actively or passively excreted or secreted from living, healthy algal cells and therefore have special significance in the flux of energy through an aquatic system. Aleyev (1934) found indications that algal cell autolysis plays an important part in organic substance accumulation in surroundings inhabited by algae. The most extensively studied extracellular product is glycolic acid. Tolbert and Zill (1956) found that 3 to 10% of the total carbon fixed by photosynthesis was excreted as glycolic acid.

Previous Studies On Heterotrophy

The role of heterotrophy among autotrophic algae has been studied to some extent with soluble carbohydrates, fat soluble organic compounds, and sodium and potassium salts of various organic acids. Rodriguez-Lopez (1966), studying the uptake and utilization of several carbohydrates by a strain of *Chlorella pyrenoidosa* under various conditions, found glucose, fructose and mannose to be utilized. The carbohydrates were polymerized to starch, with light being very stimulatory for their utilization.

A switch from photosynthesis in the light to a heterotrophic mode of nutrition in the dark by *Chlorella vulgaris* and *Scenedesmus obliquus* by utilizing glucose and acetate has been found by Minerva (1962).

Bristol Roach (1928) measured the growth rate of a species of Scenedesmus in the light, in the dark with added glucose, and in the light with added glucose. At low light intensities, the specific growth rate was 0.15, 0.25, and 0.38, respectively. He concluded that autotrophic and heterotrophic metabolism are additive in supporting growth under these conditions. Neish (1951) found that glucose, fructose, glactose, alloboise, and lactose are good sources of carbon and energy for Chlorella vulgaris in the dark. Illumination was found to stimulate growth in the presence of glucose and fructose. Lewin (1953) stated that the diatom Navicula pelliculosa can metabolize such substrates as lactic, pyruvic, citric, and succinic acids but cannot utilize these for growth. Ability to metabolize these substrates was determined by their respiratory response to these substrates and although no increase in the number of cells occurred, it is possible that part of these compounds could have entered the metabolic pathway. It is also possible that the death rate was equal to the growth rate, resulting in the conclusion that no growth occurred. Of a large number of possible carbon sources only glucose, galactose, and acetate supported continued growth of Chlorella pyrenoidosa in darkness (Samejima and Myers, 1958). Thus, Chlorella pyrenoidosa is limited in its ability to utilize organic carbon sources as contrasted to Chlorella vulgaris which is able to utilize glucose, fructose, galactose and B-glucosides for growth in darkness.

Algeus (1948a, b; 1949; 1950; and 1951a, b) investigated the possibility of several organic compounds as nitrogen sources for algal organisms. Glycocoll was a good source of nitrogen for Scenedesmus obliquus and Chlorella vulgaris when grown in the light, but growth in the dark was insignificant due to glycocoll being a poor source of carbon. The difference between the two species is of a quantitative nature in

that the mechanism of deamination in S. obliquus is over proportional and an excess of nitrogen is surrendered to the nutrient solution in the form of ammonia. In C. vulgaris, this mechanism is underproportional and it has a limiting effect upon the nitrogen assimilation. Glutamine was superior to asparagine and succinamide in supporting Scenedesmus obliquus growth. B-alanine was an unsuitable nitrogen source for S. obliquus, whereas DL-alanine, although inferior to glycocoll as a nitrogen source, proved to be relatively effective in supplying nitrogen to S. obliquus.

Previous Use Of Radioisotopes

In previous studies on the heterotrophic capacity in autotrophic organisms, utilization was determined by measuring the growth rate by cell count or by turbidity readings (Arnov, Oleson, and Williams, 1953). One of the more recently used and improved techniques has been to add labeled organic compounds directly to the water sample and follow their uptake. Parsons and Strickland (1962) described a method for studying heterotrophy by measuring the radioactive organic compound uptake by natural plankton populations. This work has been improved and given support by Wright and Hobbie (1965) who studied the uptake of radioactive glucose and acetate in lake water.

Presence Of Alanine And Its Physiological Significance

The amino acid alanine, is an intermediate product of photosynthesis, and plays an important role in protein formation in certain algae. Zak and Nichiporovish (1964) found, by paper chromatography, that carbon is incorporated into serveral amino acids, alanine being one of the major

products. Glutamine and alanine were found in filtrates from 28 day old Anabaena cultures, indicating these amino acids must be present within the cell (Fogg, 1952). Fowden (1951) established that the amino acids in the free state in algae are compounds very similar to those in higher plants when he found alanine to be one of the compounds in the free state in Chlorella vulgaris. Alanine has also been found in the marine algae Griffithsia flosculosa, Pelvetia canaliculata, and Corallina officinalis (Fowden, 1962). As well as being found in the free state of algal cells, alanine is also known to be incorporated into protein of whole cells. By chromatography, the knowledge of the amino acid composition of proteins isolated from whole cells has been improved. Protein analysis in which all the component amino acids have been assayed has established the presence of alanine in Fucus vesiculosa, Chlorella vulgaris, Anabaena cilindrica, Navicula pelliculosa, and Tribonema aequale (Fowden, 1954; Smith and Young, 1953). Thus, alanine plays a major role in protein composition as well as in being a free-state constituent of the cell.

Ecological Significance

Since algae were long considered to be entirely autotrophic organisms, their heterotrophic role is of interest in community energetics and the cycling of organic material through an aquatic system. Hobbie and Crawford (1968), from their study of the amino acid flux in a New York estuary, concluded that the total amino acid flux represented from one to ten per cent of the daily photosynthetic carbon fixation. Although heterotrophy was not considered to be the sole source of energy, Rodhe, Hobbie, and Wright (1966) suggested that a heterotrophic

role may be partially responsible for the survival of planktonic species in high mountain lakes during winter ice cover. In addition to being important in the energy flow through a community, the heterotrophic role of certain species of algae may determine their prominance in organic-laden water. Provasoli and Pinter (1953) stated that photosynthetic organisms endowed with heterotrophic abilities may flourish in waters high in organic content.

MATERIALS AND METHODS

Experiments were made on three freshwater unicellular species of algae obtained from the Culture Collection of Algae at Indiana University, and on a natural plankton sample collected from Waco Reservoir. Collection #26 *Chlorella pyrenoidosa* Chick, #LB 795 *Cloeocapsa sp.* Markel, and #LB 1046 *Cosmarium formosulum* Hoff, were used since they represent the two main divisions of phytoplankton found in freshwater, are easily maintained in the laboratory, and their unicellular form permits a fairly uniform and homogeneous sample of the culture. Natural plankton (mixed phytoplankton and zooplankton) from Waco Reservoir was used to correlate the results with natural situations.

Stock cultures of *C. pyrenoidosa* and *C. formosulum* were maintained in 250 ml of modified Chu's 10 solution (Gerloff, Fitzgerald, and Skoog, 1952), while *Gloeocapsa sp.* was maintained in 250 ml of modified Beneke's solution (De, 1939). Each was maintained at 25C with a 16 hour photoperiod. Stock cultures were maintained by periodically pouring off half the culture and replenishing the volume with fresh medium. Stock cultures were periodically checked microscopically for contamination.

Experiments were conducted at three different temperatures and three different light intensities. The temperatures were 10C, 20C, and 30C with 2137 foot-candles illumination. The light intensities were 0 ft-c, 2137 ft-c. and 8537 ft-c at 25C. All experiments were conducted in a constant temperature chamber (Honeywell, model 706AHD) with a

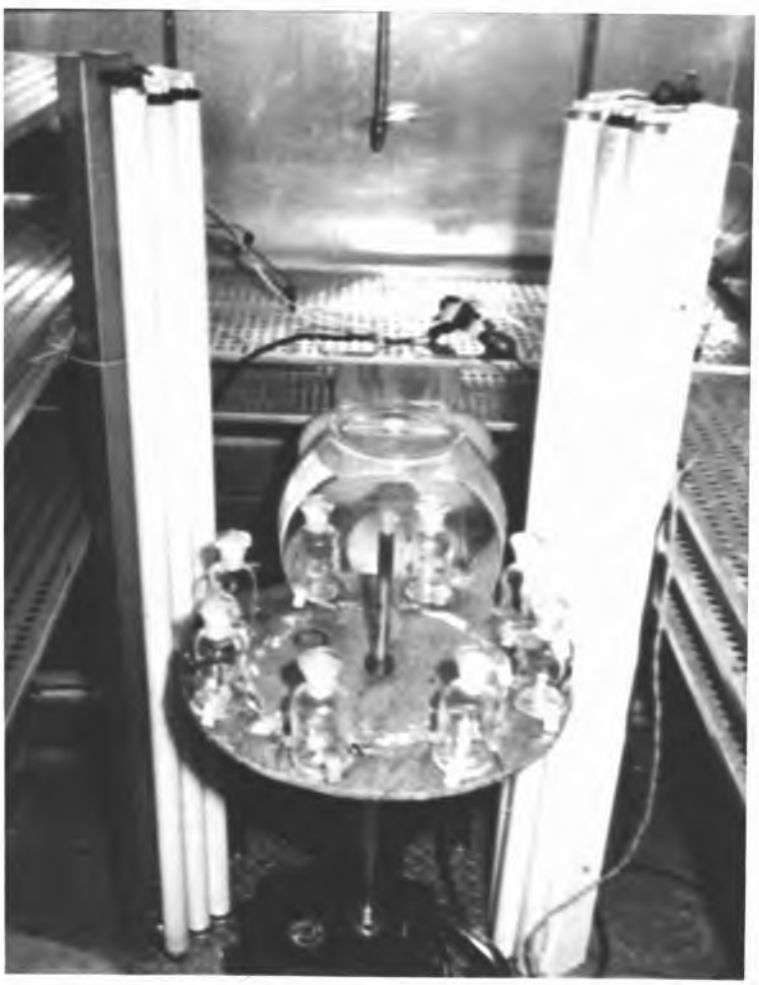
temperature deviation of 0.1C. Light intensity was determined with a foot-candle meter (Welch Scientific Company, model 3588). Illumination was by a light bank of three 250 watt General Electric incandescent bulbs and six 40 watt General Electric flourescent tubes (cool white). The nine bulbs collectively produced 8547 ft-c. The 2137 ft-c illumination was obtained by removing one of the 250 watt incandescent bulbs and repositioning the entire apparatus. A glass water container in front of the incandescent bulbs filtered out excess heat. In order that each sample receive the same amount of light during the eight hour incubation period, samples were equally spaced around the outer perimeter of a turntable making six revolutions per hour (Fig. 1).

Fifty uc of DL-alanine-1-^{14}C with specific activity of 3.26 mc/mM were obtained in granular form from New England Nuclear Corporation. This was dissolved in sterile distilled water at a concentration of 0.5 uc/ml, and stored in a sterilized glass container at 3C.

After each stock culture was shaken to obtain a homogenous cell suspension, one ml of this suspension was removed and placed in a 125 ml glass container in 120 ml of its respective nutrient solution. One ml of DL-alanine-1-^{14}C was added to each sample to make a final concentration of 4.099×10^{-3} uc/ml or 1.11×10^{-4} mg/ml. Incubation was for 8 hours. Preliminary runs showed that 15 minutes were required for processing the samples at the end of the incubation period. Therefore, samples were placed on the turntable at 15 minute intervals and removed consecutively after an eight hour period for each.

Each sample was filtered through a dry, weighed membrane filter (pore size = 0.45u) and washed with four 25 ml portions of distilled water. Preliminary tests made to check the amount of alanine adsorption to the cell walls showed that distilled water was sufficient to remove most

Fig. 1. Lighting apparatus used to allow equal illumination of all samples.



of the alanine. To eliminate any possible error with adsorption, an additional sample was treated in the same manner, with the exception of the incubation period. This sample was filtered immediately after addition of alanine, then washed four times with 25 ml of distilled water. Corrections were made for adsorption on a per mg dry weight basis by taking the difference of the radioactivity on this filter and that of the incubated samples.

To correct for loss of weight by washing detergent (wetting agent) from the filter, the same volume of nutrient media (without algae) and distilled water was filtered through a membrane filter. The filter was dried in the desiccator for 24 hours and weighed. The difference in weight represented that amount of detergent on the membrane lost by the filtration process.

After filtering, wet filters were dried in a desiccator for 24 hours, weighed and placed vertically in a 20 ml glass scintillation vial. Twenty ml of scintillation solution consisting of toluene as the primary solvent, 0.4% PPO-(2,5-Diphenyloxazole), and 0.01% Dimethyl POPOP 1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene were added to each vial.

It is possible that a certain amount of alanine entering the metabolic pathway of the organisms within the system will be respired and recycled as $^{14}CO_2$. To correct for this recycling, carbon dioxide in the filtrate was precipitated with barium hydroxide. An additional $Ba(OH)_2$ trap in the filtration apparatus precipitated CO_2 diffusing into the air from the filtrate. These two solutions were combined, filtered through a Whatman #44 filter paper, and dried in the oven at 60C for 12 hours. A fraction of the precipitate was weighed and placed in a 20 ml glass scintillation vial. To this vial was added 20 ml of

scintillation solution consisting of toluene as the primary solvent, 0.4% PPO-(2,5-Diphenyloxazole), 0.01% Dimethyl POPOP 1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene, and 4% Cab-O-Sil.

Counting-efficiency variations caused by $BaCO_3$ sample quenching were corrected by use of a quench curve. Eight samples of $BaCO_3$, with different aliquots of ^{14}C labeled toluene of known activity, were counted. The first sample was unaltered. Each additional sample had progressively increasing amounts of $BaCO_3$ added. The final concentration of $BaCO_3$ was far in excess of any counted during the experimental period. The system's counting efficiency of each sample was determined by dividing the counting rate by the DPM (disintigrations per minute) of the sample. The efficiency was plotted against the external standard ratio. The counting efficiency of all $BaCO_3$ samples could then be calculated on the graph from the external standard ratio.

A test was made for adsorption of alanine onto the $BaCO_3$ complex by adding 0.5 uc of DL-alanine-1-^{14}C to a suspension of $BaCO_3$. After 24 hours, the same filtering and counting procedures were followed as on all $BaCO_3$ samples. Less than 0.1% adsorption of alanine occurred.

Due to the flourescing nature of the scintillation solution, all samples were stored in the dark for at least 24 hours prior to counting. This enabled flourescent energy picked up by the scintillation solution to be lost before counting, thus eliminating the possibility of erroneous counts (this procedure was found to be necessary by John Coulter, Baylor Chemistry Department, when he found background counts to decrease over a period of 24 hours). Samples were counted automatically for three 10-minute counts in a Beckman LS-100 liquid scintillation system.

The eight replicates of each condition were used to calculate the mean and standard deviation. An analysis of variance was calculated tc compare temperature and illumination variations within species and to compare species at specific temperature and illumination conditions.

Alanine incorporation into the cell contents was determined by assaying the soluble cytoplasmic protein for radioactivity. Different 12 mg portions dry weight of each species of algae were incubated for eight hours under 0 ft-c, 2137 ft-c, and 8547 ft-c at 25C. Samples were centrifuged, the supernatant fluid poured off, and the cells washed four times with distilled water to remove residual alanine. Cell structure was disrupted by a tissue homogenizer and by sonic oscillation (Raytheon Sonic Oscillator, model OF101, 10Kc/s) for 30 minutes. Centrifugation at 6000 RCF for ten minutes separated the cell wallsfrom the cytoplasmic contents. The supernatant fluid was collected and the proteinprecipitated from this supernatant fluid with cold trichloroacetic acid. This material was centrifuged and the protein pellet washed three times with cold trichloroacetic acid to remove any free amino acid. The protein was placed in a scintillation vial, dried, and counted after addition of 20 ml of scintillation solution. Eight replicates were made of each species under the three light conditions. Samples were automatically counted for three 10-minute periods. the mean and standard deviation of the eight replicates were recorded as percentage of alanine absorbed by the respective species.

RESULTS

Alanine was taken up by all species under all conditions (Figs. 2 and 3). Activity hereafter will refer to uptake of alanine. Cosmarium formosulum was the most active at each temperature under 2137 ft-c illumination (Fig. 2). The natural plankton sample was relatively active at the same conditions but since an unknown portion of this could possibly be due to zooplankton utilization, no comparison can be made with the other species. In general, Chlorella pyrenoidosa was the least active.

Figure 3 illustrates a comparison of species at 25C under various light intensities. Cosmarium formosulum was again the most active. Gloeocapsa sp. was the least active except under 2137 ft-c where it was more active than C. pyrenoidosa.

Activity increased with an increase in temperature for all species except for C. pyrenoidosa. Units of uptake are recorded as mg alanine hr^{-1} per mg dry wt algae. However, for simplicity, uptake will hereafter be referred to as mg alanine. Activity in C. pyrenoidosa decreased from 1.625×10^{-4} mg alanine to 6.34×10^{-5} mg alanine with an increase of 10C. Activity then reached a maximum of 1.86×10^{-4} mg alanine with an additional increase of 10C (Fig. 4). Activity in C. formosulum increased progressively with an increase in temperature, the increase being much greater from 20C to 30C than it was from 10C to 20C (Fig. 5). Gloeocapsa sp. also had a greater increase in activity from 20C to 30C than it did from 10C to 20C (Fig. 6). The maximum activity at 30C however, was less

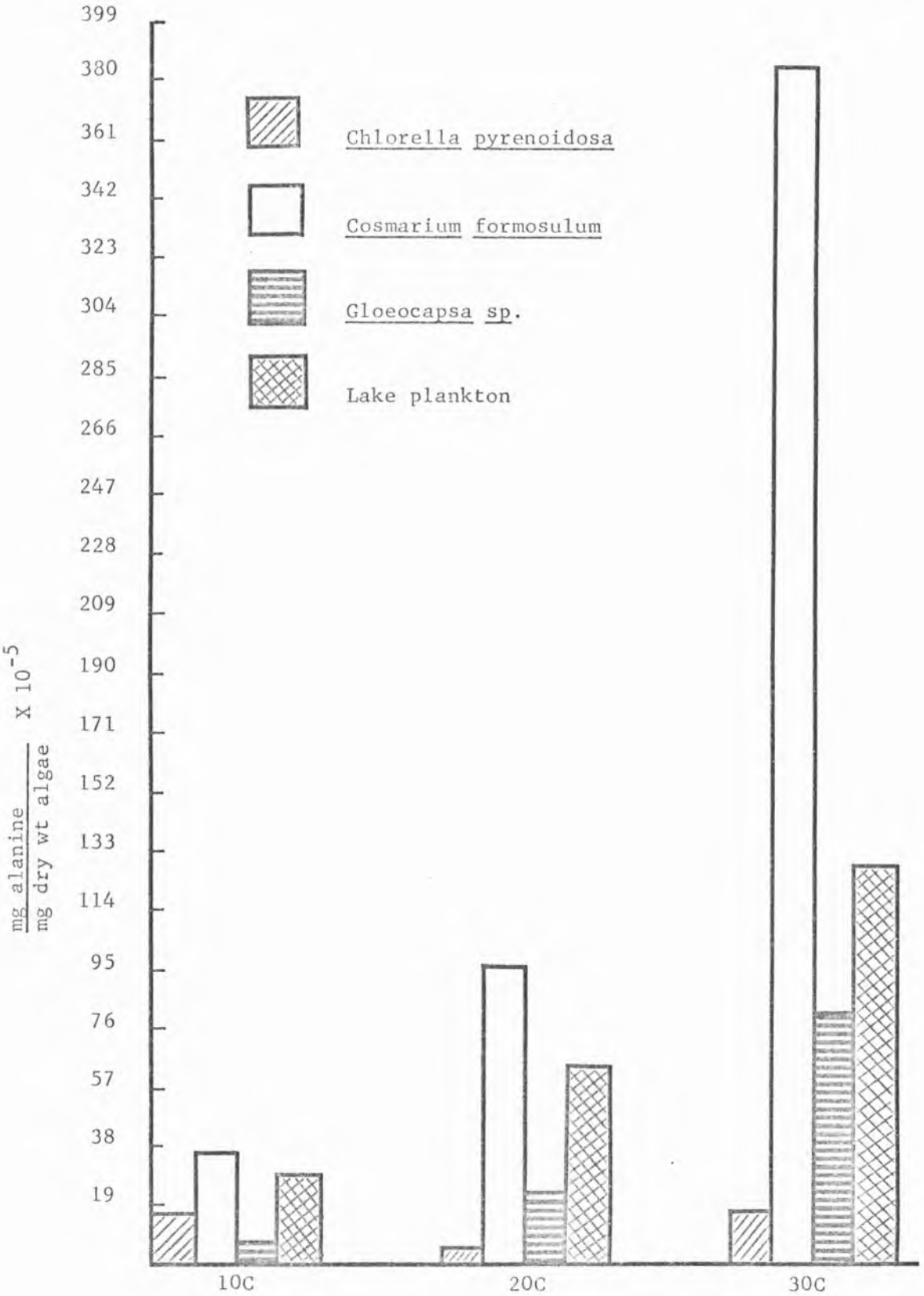


Fig. 2. Uptake of alanine at various temperatures with 2137 ft-c illumination.

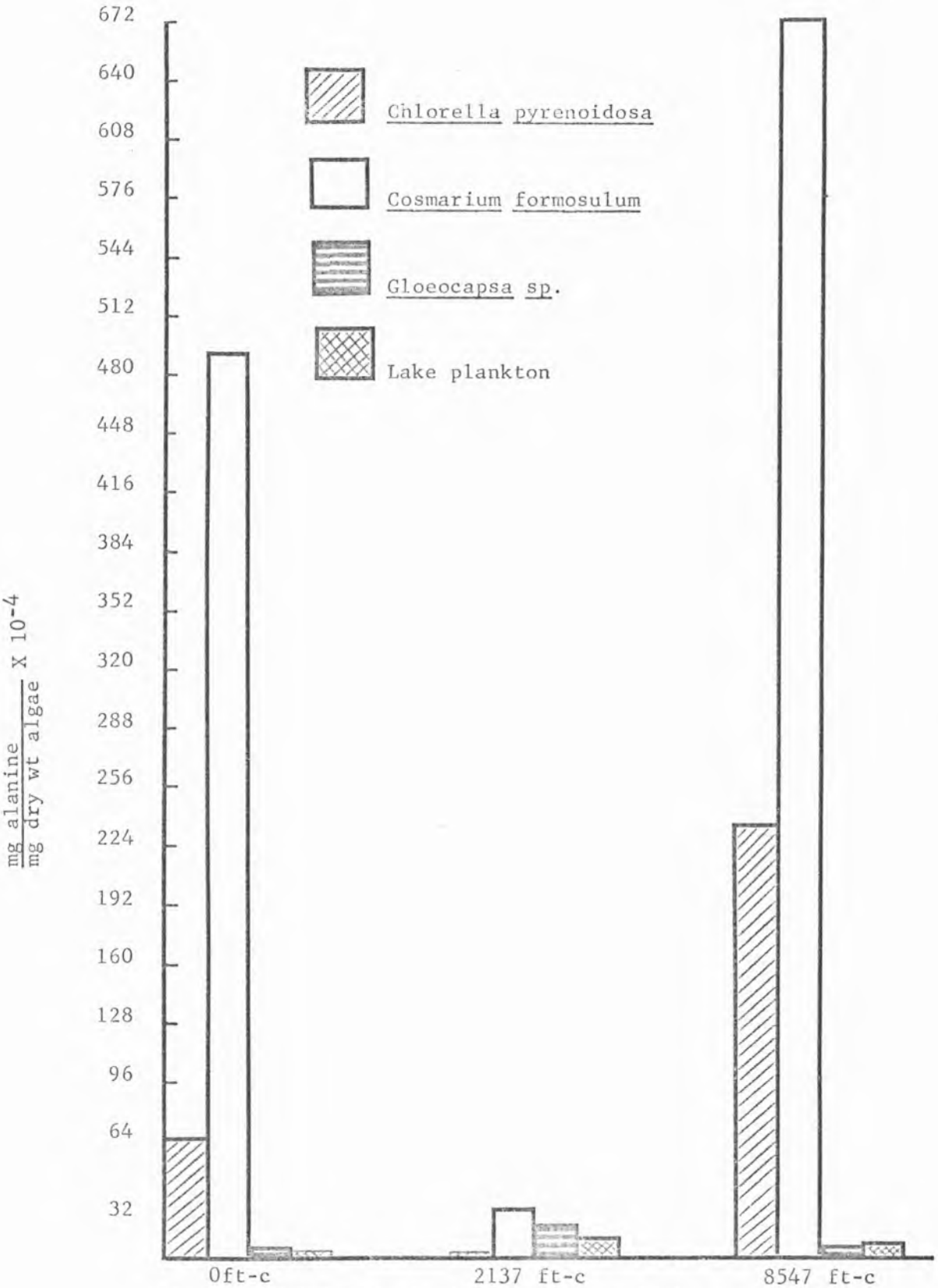


Fig. 3. Uptake of alanine at various light intensities at 25C.

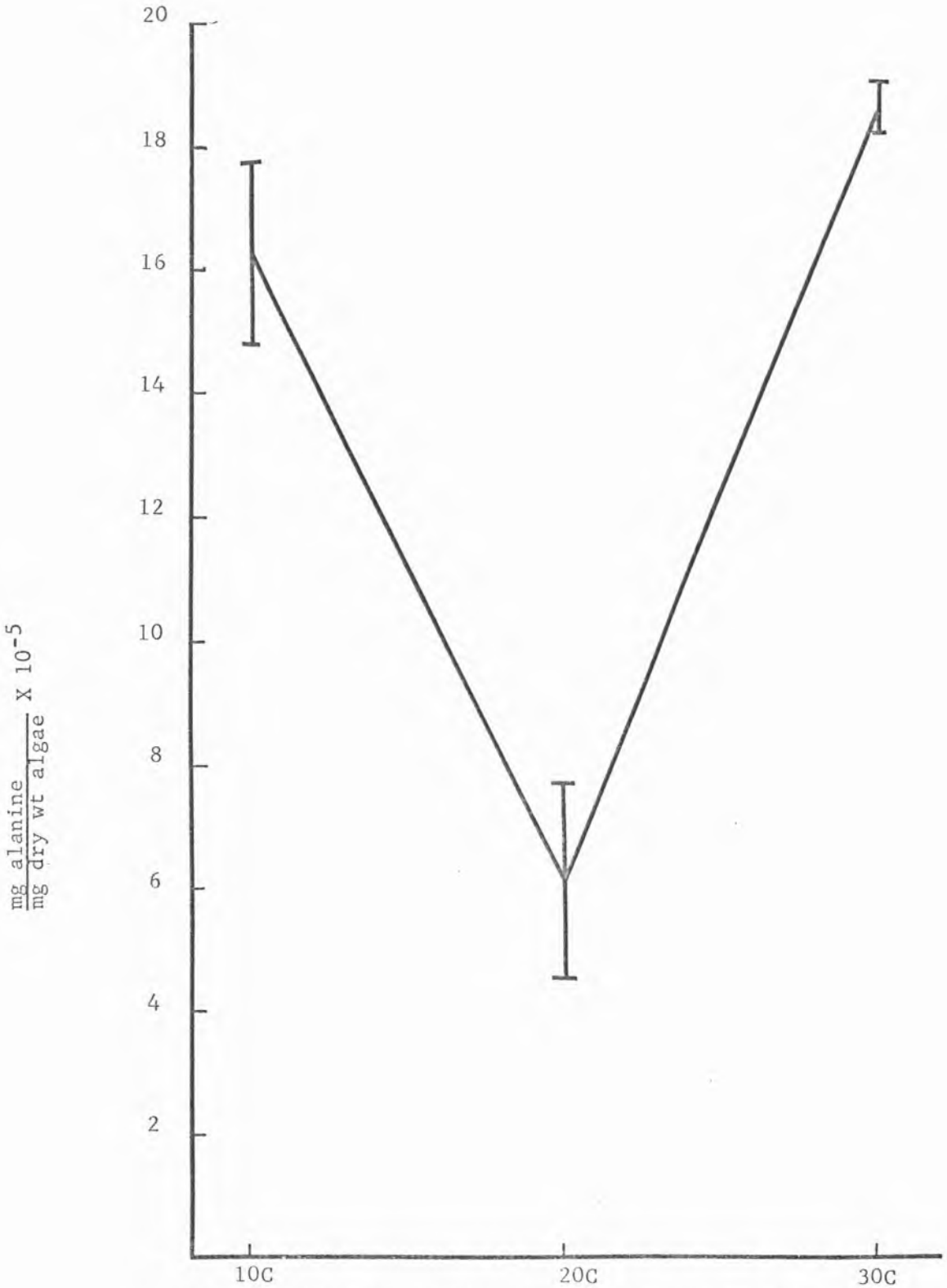


Fig. 4. Mean (± 1 SD) uptake of alanine by Chlorella pyrenoidosa at various temperatures with 2137 ft-c illumination.

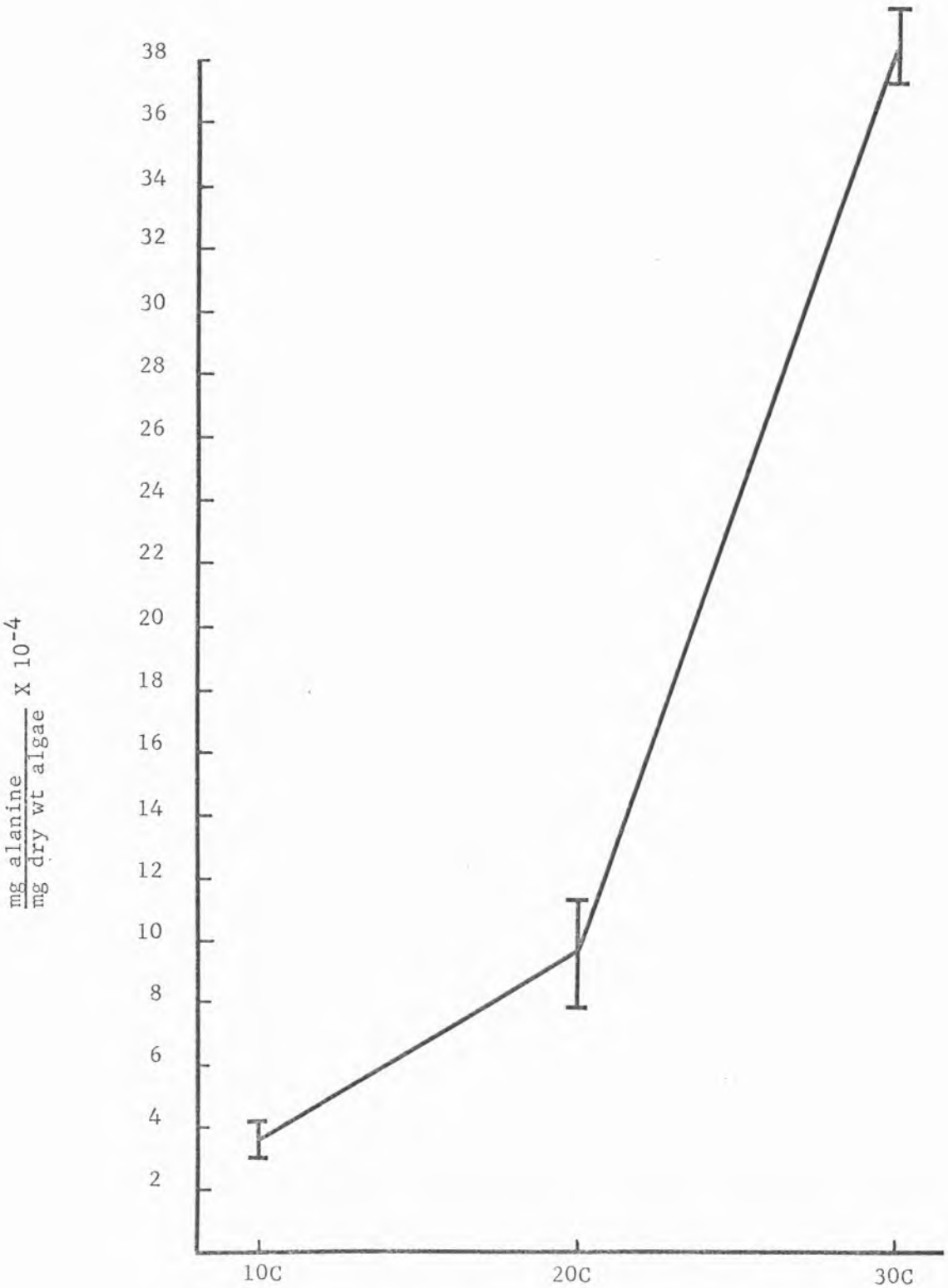


Fig. 5. Mean (± 1 SD) uptake of alanine by Cosmarium formosulum at various temperatures with 2137 ft-c illumination.

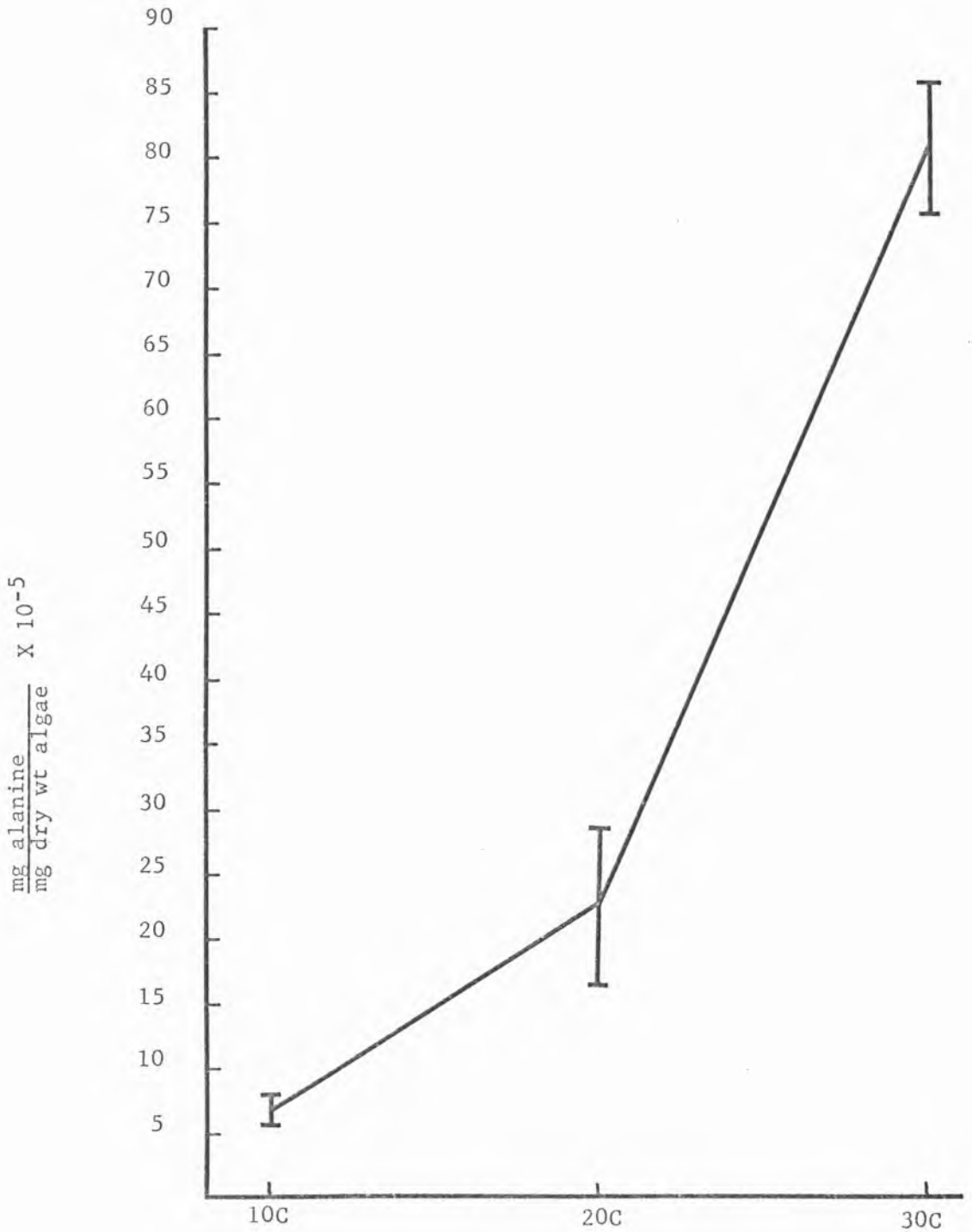


Fig. 6. Mean (± 1 SD) uptake of alanine by Gloeocapsa sp. at various temperatures with 2137 ft-c illumination.

than the maximum activity at 30C for C. formosulum.

Uptake in C. pyrenoidosa decreased from 6.41 X 10^{-3} mg alanine at 0 ft-c to 3.03 X 10^{-5} mg alanine at 2137 ft-c. Maximum uptake occurred at 8547 ft-c (Fig. 7). Cosmarium formosulum decreased in uptake from 49.3 X 10^{-3} mg alanine at 0 ft-c to 2.69 X 10^{-3} mg alanine at 2137 ft-c. Maximum uptake also occurred at 8547 ft-c (Fig. 8). Uptake by Gloeocapsa sp. followed the opposite pattern with illumination variations. A minimum uptake of 5.98 X 10^{-4} mg alanine occurred at 0 ft-c and a maximum uptake of 17.64 X 10^{-4} mg alanine occurred at 2137 ft-c. Uptake then decreased to 6.98 X 10^{-4} mg alanine at 8547 ft-c (Fig. 9).

The minimum quantities of alanine incorporated into protein were recorded as percentage of alanine taken up by each species (Table 1). Chlorella and Cosmarium incorporate into protein a significant amount of alanine taken into the cell. The amount utilized by Gloeocapsa sp. however, is too small to indicate any significance. Although the extraction technique was not a quantitative method, the same procedure was followed for each species at each experimental condition and a trend toward greater utilization at 8547 ft-c can be seen. However, before any definite statements can be made about relative incorporation into protein, a more quantitative method must be used.

An analysis of variance was calculated for temperature and illumination variations within each species. These calculations showed that the variations in mean uptake at the three temperature and illumination conditions varied significantly ($P = 0.05$) among species and within each species.

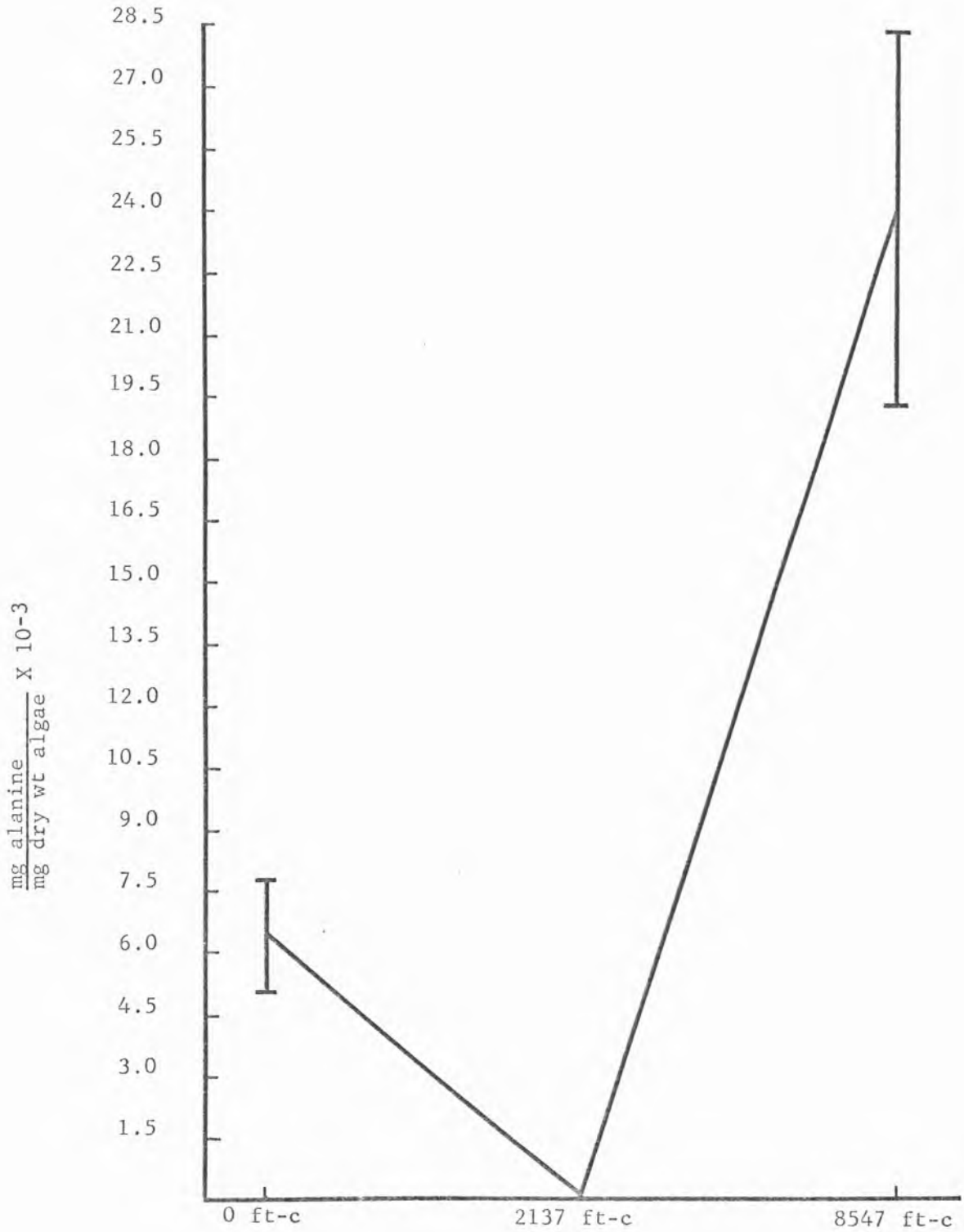


Fig. 7. Mean (± 1 SD) uptake of alanine by Chlorella pyrenoidosa at various light intensities at 25C.

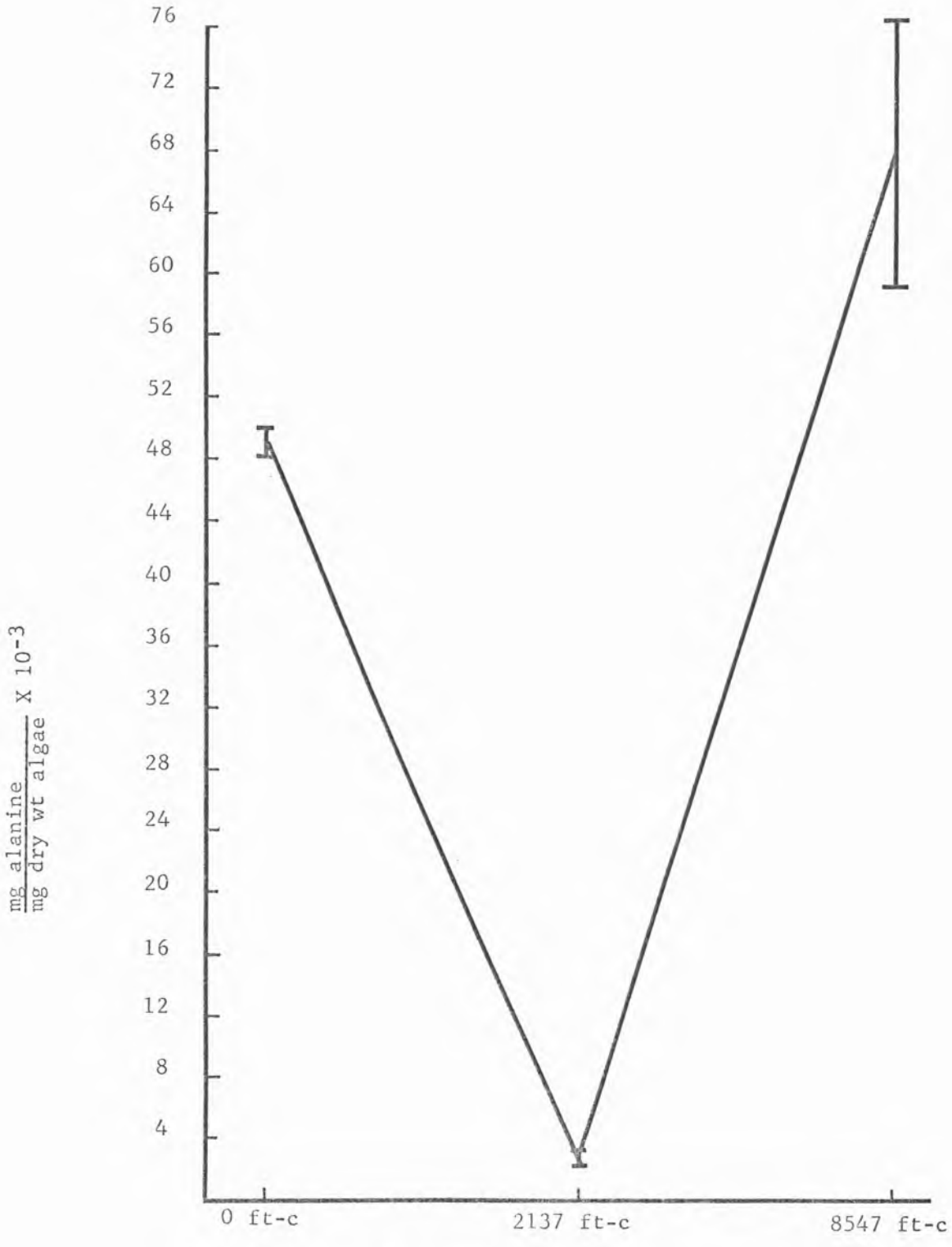


Fig. 8. Mean (± 1 SD) uptake of alanine by Cosmarium formosulum at various light intensities at 25C.

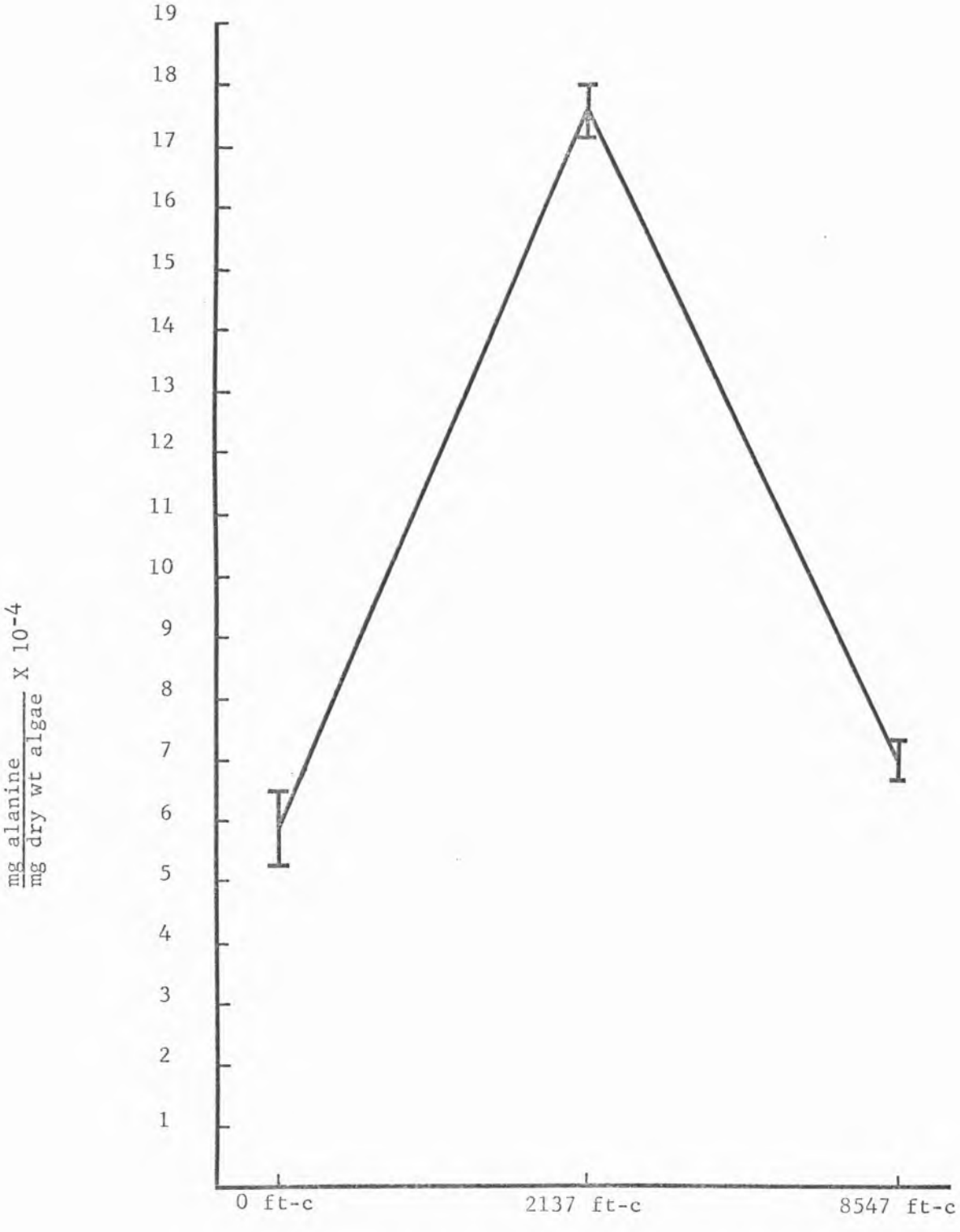


Fig. 9. Mean (± 1 SD) uptake of alanine by Gloeocapsa sp. at various light intensities at 25C.

Table 1. Minimum percentage alanine taken up that is incorporated into soluble cytoplasmic protein.

Illumination	Chlorella	Cosmarium	Gloeocapsa
0 ft-c	7%	17%	<1%
2137 ft-c	4%	11%	2%
8547 ft-c	32%	27%	1%

DISCUSSION AND CONCLUSIONS

Before discussing the results, the various categories of heterotrophy into which freshwater algae may be placed should be established. Several investigators (Bristol Roach, 1927; Neish, 1951; Killam and Myers, 1956; and Samejima and Myers, 1958) found that the spectrum of nutritional characteristics among algae ranges widely with substrates and with species. According to Hutchinson (1967), the nutritional requirements of phytoplankton can be divided into five categories. He has classified as autoauxotrophic phototrophs those organisms which need only materials produced by photosynthesis and are unable to use, as major sources of energy or matter, the organic compounds that may be available. Facultative heterotrophic phototrophs are able to live indefinitely, in some cases, on various organic substrates. Alloauxotrophic but obligate phototrophs are unable to use organic compounds as major sources of carbon or energy but depend on an external source of some vitamins. Organisms which require an external source of at least one vitamin and are able to use, though in the light are not dependent on, an external organic carbon source are classified as alloauxotrophic and facultative heterotrophic. Those organisms that carry on photosynthesis but do not meet all the major nutrient requirements by this process are placed in the category of alloauxotrophic and partly obligate heterotrophic.

The results of this investigation demonstrate that the amino acid alanine can be included in this spectrum of organic substrates. Uptake

of alanine by Chlorella pyrenoidosa, Cosmarium formosulum, and Gloeocapsa sp. was apparent (Figs. 2 and 3) and although varied significantly among species with temperature and illumination variations, these species can be placed among those organisms which Hutchinson classifies as alloauxotrophic and facultatively heterotrophic. The categorizing of these species is naturally based upon results obtained in this investigation and does not exclude them from another category. It is entirely possible that they may be facultative heterotrophic phototrophs. However, experiments were not conducted with alanine as the sole nutrient for an indefinite time period. Although Algeus (1949) found alanine to be a poor source of carbon for Scenedesmus obliquus, this does not mean that it would be an unsuitable carbon source for the species used in this investigation since several investigators (Bristol Roach, 1927; Niesh, 1951; Killam and Myers, 1956; and Samejima and Myers, 1958) have found that the ability to utilize organic substrates is subject to wide variation among species and strains.

With uptake confirmed, a second question arose. Was this alanine taken up actually metabolized by the cell or was it simply stored in the cell as the free amino acid? According to Table 1, a significant fraction of alanine was incorporated into cytoplasmic protein by Chlorella and Cosmarium. The low percentages detected in Gloeocapsa indicates that an insignificant fraction of alanine is incorporated into protein by this species. However, one must be cognizant of the fact that due to the inefficiency of the extraction method, these values represent minimum percentages of utilization. At the end of the extration procedure, a large fraction of Gloeocapsa remained intact as compared to Chlorella and Cosmarium cells. Therefore, it is possible that a greater fraction than was detected is incorporated into protein by Gloeocapsa.

Two of the major algal divisions are represented in this investigation and it is interesting to observe their heterotrophic capacities in relation to evolutionary tendencies. The two algal divisions, Chlorophyta and Cyanophyta, exhibit certain conspicuous morphological tendencies which, in so far as they affect the photosynthetic apparatus, are bound to be reflected in a changed nutritional pattern. Provasoli and Pinter (1953), in reviewing the evolutionary trends in algae leading to the development of the animal forms, indicate that there is an increased tendency towards phagotrophy as the organisms become more advanced. One would therefore expect a greater heterotrophic capacity among the Chlorophyta than among the Cyanophyta. The results of this investigation partially coincide with this tendency since Cosmarium, a member of the Chlorophyta, has the greatest uptake at each temperature and illumination condition. The clearest index of an adequate heterotrophy is the ability to grow in the darkness. Chlorella and Cosmarium had a greater rate of uptake with the absence of photosynthesis, indicating a greater heterotrophic capacity than Gloeocapsa. The fact that Chlorella did not have a greater heterotrophic capacity than Gloeocapsa at each condition indicates that heterotrophy not only varies among species and with substrates, but also with environmental conditions. This corresponds with results obtained by Algeus (1948a, b; 1949; 1950; and 1951a, b,), Samejima and Myers (1958), and Rodriquez-Lopez (1966). Although Cosmarium exhibits a greater heterotrophic capacity at each condition, the heterotrophic capacity of Gloeocapsa increases over that of Chlorella at the warmer temperatures. One would therefore expect to find a greater heterotrophic capacity in Gloeocapsa in warmer waters than Chlorella would have in the same environment.

Although these data do not point toward the mechanism of uptake, mechanisms resembling simple diffusion (Wright and Hobbie, 1966) and active uptake (Taylor, 1959) have been suggested for the uptake of various organic substrates by algae. If the uptake occurring here is a diffusional process, it would be difficult to translate the rate of uptake into biomass in the natural samples because the surface to volume ratio, important in diffusion, varies among species. Difficulty in interpretation of the natural samples also rests with the presence of zooplankton, which, as indicated by Provasoli and Shiraishi (1959), may utilize dissolved organic matter. However, comparisons of the three unialgal species can be made, regardless of the uptake mechanism. If diffusion is the process involved, then added support is given to C. formosulum as a better adapted heterotrophic organism. Cells were measured microscopically and Cosmarium had a diameter approximately sixty times that of Chlorella. A greater surface area would exist with Chlorella than with Cosmarium, providing there was an equal mass of the two species, therefore one would expect a greater diffusion rate with Chlorella. However, the uptake of Cosmarium ranged from 2 to 24 times that of Chlorella, indicating that either Cosmarium is more permeable to alanine or possesses a faster rate of deamination.

The comparative heterotrophic capacity of Chlorella and Gloeocapsa cannot be stated in general. Each possesses a greater heterotrophic capacity than the other under various conditions, due to temperature and illumination effects that will be discussed later.

The increase in uptake by Cosmarium and Gloeocapsa produced by an increase in temperature paralleled the reactions expected for this environmental condition. This increase in uptake may be the result of

one or both of two factors. Enzyme activity increases with a rise in temperature up to an optimum. The enzyme activity involved in the deamination of alanine would therefore increase resulting in a more rapid rate of deamination. Protein synthesis may also increase due to an increased enzyme activity involved in this synthesis. If the uptake mechanism is diffusional, the increased metabolic rate will result in a higher diffusion gradient due to the more rapid breakdown of alanine or the more rapid synthesis of protein or both. Therefore, there will be a more rapid rate of diffusion into the cell. Since temperature affects metabolic processes, it is expected to influence the rate of active transport. Therefore, if the uptake mechanism is an active process, the rate of liberation of energy by respiration is likely to be the limiting factor in the passage of alanine through the cell membrane.

Cosmarium activity has Q_{10} of 2.3 from 10C to 20C and a Q_{10} of 4.0 from 20C to 30C. There are two possible reasons for the higher Q_{10} values from 20C to 30C. A certain amount of energy is required for a molecule to pass across the barrier of the cell membrane (Giese, 1966). The ten degree rise in temperature will increase by several fold the number of molecules with this required energy of activation necessary to cross the barrier in the cell membrane. Another possible explanation for the higher Q_{10} value is a change in viscosity of the cell membrane, which will lower this required level of energy to pass through the membrane. Gloeocapsa has a Q_{10} of 3.3 from 10C to 20C and a Q_{10} of 3.5 from 20C to 30C. This indicates that the energy required to pass across the cell membrane is greater than that in Cosmarium which could be due to a greater permeability, a greater diffusion gradient, or a more rapid rate of deamination of alanine and synthesis of protein in Cosmarium. The maximum uptake of

Cosmarium was 4.8 times as great as the uptake by Gloeocapsa at 30C, which is possibly due to a greater permeability of the cell membrane in Cosmarium or the sheath that surrounds each Gloeocapsa cell (Smith, 1950) may retard the absroption of alanine. The data in Fig. 4 do not follow a characteristic response to temperature and cannot be interpreted at this time.

The results presented in Figs. 7-9 partially support the second hypothesis of this investigation. The decrease in uptake by Chlorella and Cosmarium at 2137 ft-c supports the hypothesis that this heterotrophic role takes over in the absence of photosynthesis. The decrease in uptake at 2137 ft-c indicates that in the presence of light the photosynthetic process provides sufficient organic compounds for the chemical machinery of the cells. However, at 0 ft-c the cell no longer synthesizes organic compounds and the cell must depend on reserve supplies or other substrates to carry out metabolic and growth processes. In this case, the cells partially rely on alanine as an external source of energy. This corresponds with data supplied by Bristol Roach (1928), Neish (1951), and Samejima and Myers (1958) where it was found that heterotrophic nutrition increased with a decrease in photosynthesis.

Although it was not the scope of this investigation to provide mechanisms of incorporation of alanine into the cell, one may speculate as to the fate of this compound in the absence of photosynthesis. Alanine may be directly incorporated into the cell and synthesized into protein. Energy-requiring processes for synthesizing alanine may thus be by-passed. The incorporation of a fraction of this amino acid into protein in the absence of photosynthesis is evident from Table 1. Upon deamination, alanine yields pyruvic acid (Giese, 1966) which goes directly into the

Kreb's cycle. Thus, alanine may also supply the cell as a carbon source, although this does not agree with results obtained by Algeus (1949) in which he found alanine to be a poor carbon source for Scenedesmum obliquus. His conclusions were based on an increase in number of cells and although inconsiderable growth was observed with alanine as the substrate, this does not delete the possibility of part of the alanine molecule entering the metabolic pathway as a source of carbon in other species.

Steeman Nielson (1952) found that in bright light photo-oxidation, besides enzymes, attacks part of the photochemical mechanism. The twenty-fold increase in uptake at 8547 ft-c could possibly be due to an inhibition of photosynthesis by photo-oxidation, or an increase in permeability of the cell membrane, or a combination of the two. The increase in uptake at 2137 ft-c by Gloeocapsa sp. corresponds with the additive effect of autotrophic and heterotrophic metabolism in Scenedesmus reported by Bristol Roach (1928). Hoare and Moore (1965) also found the blue-green algae Anacystis nidulans to take up acetate in the light, assimilating it into lipids and four amino acids of the cell proteins. These findings further confirm that species vary in their heterotrophic response to various conditions.

Data on the flux of individual organic substrates, supplemented with their natural concentrations, is important in the study of energy flow through an aquatic system. That the heterotrophic role of the organisms in this study is sufficient to maintain them for long periods of darkness as suggested by Rodhe (1955), cannot be ascertained. However, the uptake of this amino acid has certain ecological implications. An increase in uptake of alanine at low light intensities may have ecological importance when phytoplankton are carried downward by turbulence or are

subjected to overcast weather. The increase in uptake by Chlorella and Cosmarium at high light intensities would also have importance on alanine flux during a mid-summer day. In general, alanine flux can be expected to be greater during the summer months than during the winter months, however when considering alanine flux through a system, one must be cognizant that uptake of this amino acid varies among species.

The work presented here has several limitations. Although evidence is supplied about the relative uptake of alanine among three species of algae, these cultures were not bacteria-free and a certain fraction of the radioactivity was undoubtedly due to ^{14}C fixation in bacteria on the filter. Wright and Hobbie (1966) indicate that the use of ^{14}C isotopes to trace uptake of organic substrates requires the assumption that the organisms are not immediately respiring the newly aquired substrate. However, the recycling of ^{14}C in this investigation was corrected by trapping and analyzing the respired ^{14}C.

In conclusion, it can be stated that Chlorella pyrenoidosa, Cosmarium formosulum, and Gloeocapsa sp. possess the ability to absorb the amino acid alanine and this heterotrophic capacity varies among species as well as with environmental conditions. Although their ability to utilize this amino acid classifies them as facultative heterotrophs, further investigations need to be carried out to determine the ability of the species to exist on alanine as a sole source of energy. Before one can estimate the quantitative role these species play in alanine flux through a natural system, the natural concentration must be obtained and the role of bacteria in this process ascertained.

SUMMARY

1. Alanine is absorbed by Chlorella pyrenoidosa, Cosmarium formosulum, and Gloeocapsa sp. and definitely utilized by the first two species.
2. This absorption varies among species and with environmental conditions.
3. An increase in temperature results in an increase in uptake, except from 20C to 30C by Gloeocapsa sp.
4. Heterotrophy decreases with an increase in light intensity up to 2200 ft-c in Chlorella and Cosmarium. An additional increase in illumination results in an increase in uptake.
5. Heterotrophy in Gloeocapsa increases with an increase in illumination up to 2200 ft-c, then decreases with an additional increase in illumination.

LITERATURE CITED

Aleyev, B. S. 1934. Secretion of organic substances by algae into the surrounding medium. Microbiologiya 3:506-508.

Algeus, S. 1948a. Glycocoll as a source of nitrogen for Scenedesmus obliquus. Physiol. Plant. 1:65-84.

_____. 1948b. The utilization of glycocoll by Chlorella vulgaris. Physiol Plant. 1:236-245.

_____. 1949. Alanine as a source of nitrogen for green algae. Physiol. Plant. 2:266-271.

_____. 1950. The utilization of aspartic acid, succinamide and asparagine by Scenedesmus obliquus. Physiol. Plant. 3:225-234.

_____. 1951a. Effects of B-alanine and pantothetic acid on growth of Scenedesmus obliquus. Physiol. Plant. 4(3):495-497.

_____. 1951b. Notes on the utilization of glutamine by Scenedesmus obliquus. Physiol. Plant. 4(3):459-460.

Arnov, Peter, J. J. Oleson, and J. H. Williams. 1953. The effect of argainine on the nutrition of Chlorella vulgaris. Amer. J. Bot. 40(3):100-104.

Birge, E., and C. Juday. 1934. Particulate and dissolved organic matter in inland lakes. Ecol. Monogr. 4:440-474.

Bold, H. C. 1942. The cultivation of algae. Bot. Rev. 8:69-138.

Bristol Roach, B. M. 1928. On the influence of light and of glucose on the growth of a soil alga. Ann. Bot. 42:317-345.

De, P. K. 1939. The role of the blue-green algae in nitrogen fixation in rice fields. Proc. Roy. Soc. Bot. 127:121.

Fogg, G. E. 1952. The production of extracellular nitrogenous substances by a blue-green alga. Proc. Roy. Soc. Ser. B 139:372-397.

Fowden, L. 1951. Amino acids of certain algae. Nature 167:1030-1031.

_____. 1954. A comparison of the composition of some algal proteins. Ann. Bot. 18:257-266.

_____. 1962. Amino acids and proteins, p. 198. In:R.A. Lewin(ed) Physiology and biochemistry of algae. Academic Press. New York and London.

Gerloff, G. C., G. P. Fitzgerald, and F. Skoog. 1952. The mineral nutrition of Microcystis aeruginosa. Amer. J. Bot. 37:216-218.

Giese, A. C. 1966. Cell physiology. W. B. Saunders Co., Philadelphia. 592 p.

Hoare, D. S., and R. B. Moore. 1965. Photoassimilation of organic compounds by autotrophic blue-green algae. Biochim. Biophys. Acta. 109:622-625.

Hobbie, J. E., and C. C. Crawford. 1968. Amino acid flux in an estuary. Science 159:1463-1464.

Hutchinson, G. E. 1967. A treatise on limnology, Vol. II. John Wiley & Sons., Inc., New York.

Killam, A., and J. Myers. 1956. A special effect of light on the growth of Chlorella vulgaris. Amer. J. Bot. 43:569.

Lewin, J. C. 1953. Heterotrophy in diatoms. J. Gen. Microbiol. 9:305-313.

Minerva, L. A. 1962. The effect of light intensity upon autotrophic and heterotrophic nutrition of Chlorella vulgaris and Scenedesmus obliquus. Mikrobiologiya 31(3):336-340.

Neish, A. C. 1951. Carbohydrate nutrition of Chlorella vulgaris. Canad. J. Bot. 29(1):68-78.

Parson, R. R., and J. D. Strickland. 1962. On the production of particulate organic carbon by heterotrophic processes in sea water. Deep-Sea Res. 8:211-222. (Abstr.)

Pringsheim, E. G. 1946. The biphasic or soil-water culture method for growing algae and flagellates. J. Ecol. 33:193-204.

Provasoli, L., and I. J. Pinter. 1953. Ecological implications of in vitro nutritional requirements of algal flagellates. Ann. N. Y. Acad. Sci. 56:839-851.

Provasoli, L., and K. Shiraishi. 1959. Axenic cultivation of the brine shrimp Artemia saline. Biol. Bull. 117:347-355.

Rodhe, W. 1955. Can plankton production proceed during winter darkness in subarctic lakes? Proc. Int. Assoc. Limnol. 12:117-122.

Rodhe, W., J. E. Hobbie, and R. T. Wright. 1966. Phototrophy and heterotrophy in high mountain lakes. Verh. Internat. Verein. Limnol. 16:302-313.

Rodriques-Lopez, M. 1966. Utilization of sugars by Chlorella pyrenoidosa under various conditions. J. Gen. Microbiol. 43(1):139-143.

Samejima, H., and J. Myers. 1958. On the heterotrophic growth of Chlorella pyrenoidosa. J. Gen. Microbiol. 18:107-117.

Skinner, C. W., and C. E. Gardner. 1930. The utilization of nitrogenous organic compounds and sodium salts of organic acids by certain soil algae in darkness and in the light. J. Bact. 19:161-179.

Smith, G. M. 1950. The fresh-water algae of the United States. McGraw-Hill Book Co., New York. 55 p.

Steeman Nielson, E. 1952. On detrimental effects of high light intensities on the photosynthetic mechanism. Physiol. Plant. 5(3):334-344.

Taylor, F. J. 1959. The absorption of glucose by Scenedesmus quadricauda. I. Some kinetic aspects. Proc. Roy. Soc. Ser. B 151:400-418.

Tolbert, N. E., and L. P. Zill. 1956. Excretion of glycolic acid by algae during photosynthesis. J. Biol. Chem. 222(2):895-906.

Vallentyne, J. R. 1957. The molecular nature of organic matter in lakes and oceans, with lesser reference to sewage and terrestrial soils. J. Fish. Res. Bd. Canada 14(1):33-82.

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

Watt, W. D. 1966. Release of dissolved organic material from the cells of phytoplankton populations. Proc. Roy. Soc. Ser. B 164(997):521-551.

Welch, P. S. 1952. Limnology. McGraw-Hill Book Co., New York, 538 p.

Wiedeman, V. E. 1964. Heterotrophic growth of waste-stabilization pond (green) algae. Amer. J. Bot. 51(6pt. 2):683.

Wright, R. T., and J. E. Hobbie. 1965. The uptake of organic solutes in lake water. Limnol. Oceanogr. 10:22-28.

_____. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology 47:447-464.

Zak, F. G., and A. A. Nichiporovich. 1964. Formation of amino acids during photosynthesis: identification and degredation of glycine, alanine, and serine. Fizol Rast. 11(1):20-30. (Abstr.)

VITA

David Bennett Campbell, the youngest son of Mr. and Mrs. C. T. Campbell, Mission, Texas, was born in Vallejo, California, February 28, 1945. He attended school in Mission, Texas, graduating from Mission High School in June 1963. He began his sutdies at Baylor University in September, 1963, majoring in biology and minoring in chemistry, and graduated with a Bachelor of Science degree in June, 1967. He began his graduate studies at Baylor in September, 1967, and worked as a laboratory coordinator in Biology 101 during the following year. He finished the requirements for the Master of Science degree in the summer of 1969.