Photosynthetic Assimilation of ^{15}N-Ammonia and ^{15}N-Nitrate in the Marine Diatoms *Bellerochea yucatanensis* (von Stosch) and *Skeletonema costatum*

Günter Döhler and Hans-Jürgen Roßlenbroich

Botanisches Institut der Universität Frankfurt am Main, Siesmayerstr. 70, D-6000 Frankfurt a. M.

Z. Naturforsch. **36 c**, 834–839 (1981); received July 3, 1981

^{14}C- and ^{15}N-Assimilation, ^{15}N-Labelled Amino Acids, Marine Diatoms

The marine diatoms *Bellerochea yucatanensis* and *Skeletonema costatum* were grown at + 20 °C in 0.03 vol.% CO_2 with nitrate or ammonia. The ^{15}N-ammonia and ^{15}N-nitrate assimilation and ^{15}N-incorporation into various amino acids were studied of both diatoms during exponential growth phase in dependence of different nitrogen conditions. In all experiments the ^{15}N-ammonia uptake was lower than the ^{15}N-nitrate assimilation rate up to 20–40 min photosynthesis. Nitrate limitation – cells grown in nitrate followed by growth in nitrogen-free medium for 24 h – caused a strong ^{15}N-label into aspartate after adding $^{15}NH_4Cl$ (1 mM). In cells grown in nitrate highest enrichment of ^{15}N was found in glutamine. Results were discussed with reference to the operating of the GS/GOGAT system and glutamic acid dehydrogenase pathway. Photosynthetic $^{14}CO_2$ fixation experiments showed a very high labelling of aspartate which was interpreted with a phosphoenolpyruvate carboxylation catalysed by phosphoenolpyruvate carboxykinase.

Since a long time bacteria and algae have been used for investigating of nitrate and ammonia assimilation [1]. Recently Canvin and Atkins [2] reported on light dependend $^{15}NH_4^+$- and $^{15}NO_3^-$-incorporation into several amino acids. Bassham and Kirk [3] observed in *Chlorella* a strong labelling in glutamic acid after 5 minutes photosynthesis using ^{14}C and ^{15}N and concluded that glutamic acid was the first product. In short-term kinetics experiments the primary products of biological nitrogen fixation have been studied using ^{13}N-labelled compounds. These results showed that glutamine was the first ^{13}N-labelled compound and glutamic acid the second [4–6]. These data favour the operation of the glutamine synthetase/glutamate synthase (GOGAT) pathway in nitrogen-fixing microorganisms. Several workers studied the assimilation of nitrate and ammonia, but only few used ^{15}N as a tracer for the nitrogen assimilation mechanism. ^{15}N-kinetic analysis of nitrate and ammonia assimilation in roots of rice showed a rapid nitrogen incorporation into glutamine and other amino acids [7, 8].

In the present report the nitrate and ammonia assimilation and ^{15}N-incorporation into various acids in marine diatoms during short-term experiments under steady state conditions is demonstrated using ^{15}N-analysis.

Reprint requests to Prof. Dr. G. Döhler.

0341-0382/81/0900-0834 $ 01.00/0

Materials and Methods

The marine diatoms *Bellerochea yucatanensis* (von Stosch) and *Skeletonema costatum* obtained from Prof. Dr. H. von Stosch, Marburg, and Dr. G. Drebes, Sylt, were grown in glass tubes at + 20 °C, a light-dark-regime of 14: 10 h and bubbled with normal air (0.03 vol% CO_2). Light intensity of 1 mW/cm² (5000 lux) and a nutrient medium described by von Stosch [9] were used. Nitrogen source was varied during growth and experiments: nitrogen-free medium, nitrate or ammonia supply. Marine diatoms were harvested two hours after the beginning of the light period and concentrated by filtration. For quantitative estimation of chlorophyll a and c we used the procedure of Jeffrey and Humphrey [10] after sonification for 90 sec with a Branson Sonifier (Model S-75) at 20 KHz.

Kinetic experiments were carried out in a special assimilation chamber of plexiglass at + 20 °C, continuously illumination with a light intensity of 1 mW/cm² and under normal air conditions. In all experiments algae were resuspended in a nitrogen-free medium and illuminated for 15 minutes before adding $^{15}NH_4Cl$ (96 atom%) or $K^{15}NO_3$ (96,8 atom-%). The concentration of ^{15}N-labelled compounds in the medium of 15 ml was in all cases 1 mM. Samples of 2 ml were collected by a syringe and filtrated on glass filters (Whatman GF/C) for measurements of total incorporation rate or extrated with 80%

ethanol for estimation of ^{15}N enrichment into the amino acids. In some experiments we have added both ^{14}C-bicarbonat (20 µCi/ml; 56,8 mCi/mM) and $^{15}NH_4Cl$ (96 atom%) for estimating the label into the carbon skeletts and into the amino acids. For analysis of ^{15}N enrichment we used a Zeiss Statron NOI-5 atomic emission spectrophotometer. Combustion of samples was carried out by the Dumas method in sealed tubes according to Faust [11]. The sample was introduced into a special discharge tube of pyrex or duran glass according to Schröder [12] with a CuO and CaO mixture (1:1) and evacuated to 5×10^{-5} Torr. For estimation of ^{15}N enrichment into various amino acids argon (2–3 mbar) was introduced before sealing off [13]. For more details see [14] and [15].

The alcohol extracts were evaporated below 40 °C and chlorophylls removed by partitionning in chloroform. The amino acids and amides were separated by two-dimensional thin-layer chromatography on cellulose plates (Machery & Nagel, MN 300 HR). Solvents were ethanol/butanol-2/water/propionic acid (10:10:5:2, v/v) in the first dimension and propanol-1/water (7:3, v/v) in the second dimension (see Fig. 1). After identification by spraying ninhydrin solution the spots were scraped, eluated in methanol, concentrated by evaporation at 60 °C and collected into a capillary tube, which was brought into the discharge tube. Estimation of protein content were carried out according to the procedure of Bradford [16]. Pool sizes of several amino acids were measured after the method described by Bieleski and Turner [17]. Details of procedure for ^{14}C-method see [18] and Roßlenbroich and Döhler (in press).

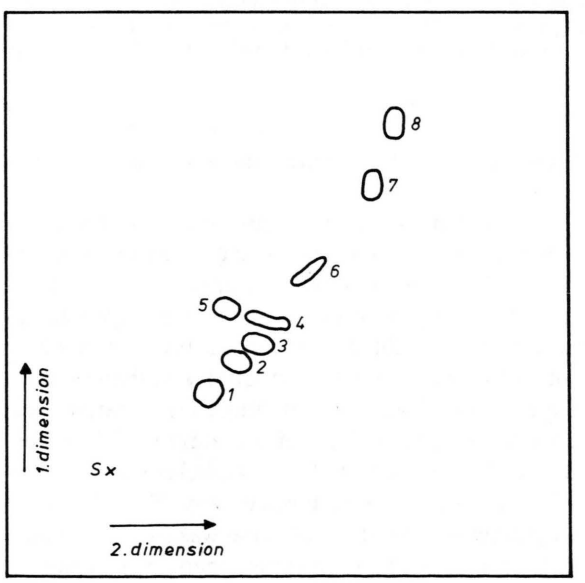

Fig. 1. Distribution of various free amino acids and amides of a *Bellerochea* extract on a thin-layer plate (cellulose powder of Machery & Nagel, MN 300 HR) using two-dimensional chromatography. Solvents in 1. dimension were ethanol/butanol-2/water/propionic acid (10:10:5:2 v/v) and in 2. dimension propanol-1/water (7:3 v/v). Symbols: S x start point, 1 asparagine, 2 glutamine, 3 aspartate, 4 glycine/serine, 5 glutamate, 6 alanine, 7 valine and 8 isoleucine.

Results and Discussion

No effect of nitrogen source (NH_4Cl or KNO_3) during growth of *Bellerochea yucatanensis* could be detected in the exponential and steady phase of culture. Similar results are described on *Skeletonema costatum* [19] and *Phaeodactylum tricornutum* [20]. The chlorophyll a content was constant independent on the nitrogen sources. But chlorophyll c_1 and c_2 amounts decreased by 20% in ammonia grown diatoms. In agreement with other authors [21] we observed no influence of nitrate or ammonia on the protein values of *Bellerochea* estimated by Bradford method.

Pool sizes of free amino acids and amides increased in ammonia grown cells (see Table I): Glutamina 38-fold and asparagine 5,6-fold. On the other hand, the amino acid pools were enhanced 3–4-fold only. Aspartate showed in NH_4^+-cultures a higher pool size than glutamate. In nitrate grown cells nearly identically values of both amino acid pools could be found.

Cells of *Skeletonema costatum* and *Bellerochea yucatanensis* grown under different nitrogen conditions were tested for their capacity to assimilate nitrate or ammonia in a nitrogen-free medium after

Table I. Pool sizes of free amino acids and amides of *Bellerochea yucatanensis* grown under different nitrogen conditions. Values expressed in nmol/µg Chl a; average of 3 independent determinations.

Compound	NO_3^-	NH_4^+ grown cells
aspargine	9.36	52.28
glutamine	1.26	48.60
aspartate	8.80	33.16
glutamate	9.07	26.32
alanine	7.69	22.96

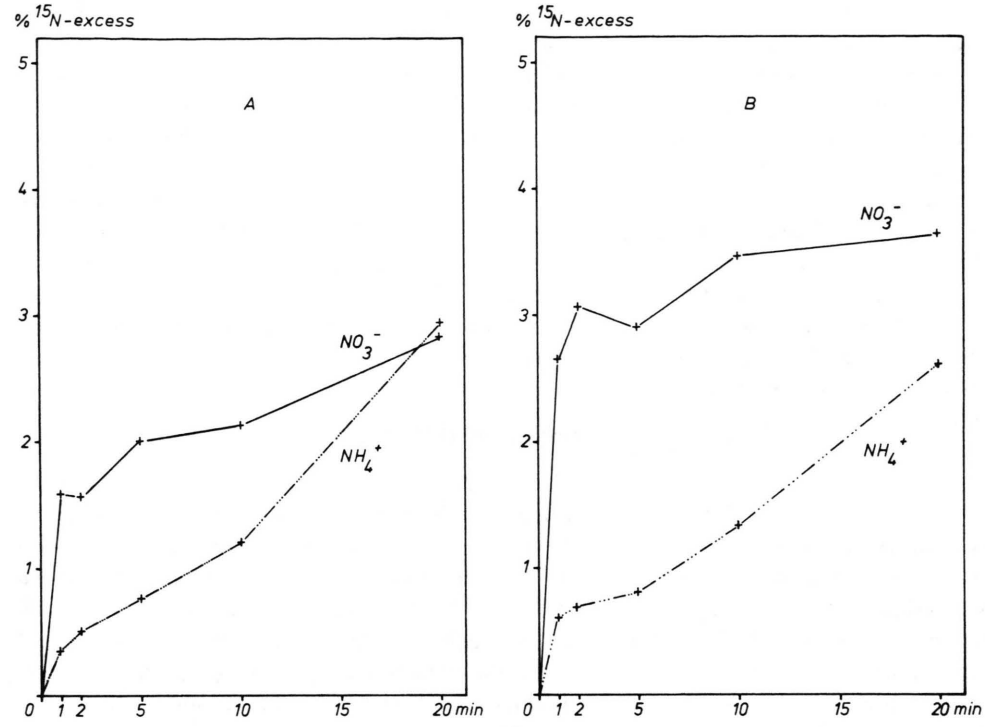

Fig. 2. Ammonia (NH_4^+) and nitrate (NO_3^-) assimilation in cell suspensions of *Skeletonema costatum* grown in nitrate (A) and 2 days in a nitrogen-free medium (B) at + 20 °C. Cells were separately resuspended in a nitrogen-free medium at a density of 20×10^6 cells/ml, bubbled with air (0.03 vol% CO_2) and 1 mM $^{15}NH_4Cl$ (96.0 atom%) or 1 mM $K^{15}NO_3$ (96.8 atom%) was added after 15 min photosynthesis. Chlorophyll a content 4.491 µg/ml and chlorophyll c content 0.963 µg/ml.

15 minutes photosynthesis. As shown in Figs. 2 and 3 the assimilation of both nitrogen compounds was rapidly immediatly after the additions of ^{15}N-labelled nitrate or ammonia independent on the culture conditions. In all cases ammonia uptake was lower than assimilation rate of nitrate; nitrogen assimilation is repressed by ammonia (1 mM). After 20–40 minutes practically no differences could be observed between both assimilated substances when algae were grown in nitrate (Fig. 2 part A) or in ammonia followed by 24 h culture without any nitrogen source (Fig. 3, part B). Nitrate limitation – cells grown in nitrate during exponential phase and without nitrogen for 24 or 48 h – caused a strong depression of ^{15}N-ammonia uptake by *Skeletonema* (Fig. 2 part B) and *Belllerochea* (Fig. 3 part A). Cells grown in ammonia (until 3 weeks) can assimilate nitrate, which is in agreement with the results obtained with *Skeletonema* [22]. This can be interpreted by a rapid induction of nitrate reductase [23, 24]. *Bellerochea* suspensions provided with both ammonia and nitrate favoured the assimilation of ammonia.

Figure 4 shows the time course of ^{15}N incorporation into various free amino acids after exposing intact cells of *Bellerochea yucatanensis* to $^{15}NH_4Cl$ (1 mM) during photosynthesis. Algae grown in nitrate were brought during exponential growth phase for 24 h in a nitrogen-free medium and then used for experiments. The ^{15}N enrichment of aspartate was consistently the highest of all amino acids tested. 30 sec after feeding $^{15}NH_4Cl$ ^{15}N label could be detected in aspartate and glutamate, only. The ^{15}N excess in glutamine and glycine/serine was found at 2 minutes and showed lower values than in aspartate. A very low ^{15}N content we observed in alanine after 10 minutes photosynthesis. Under these conditions – nitrogen limitation of nitrate grown cells – a more rapidly nitrate uptake occured (see Fig. 3 part B), but a ^{15}N enrichment in free amino acids could be obtained after 10 minutes photosynthesis, only (compare Table II). A ^{15}N content in aspartate and

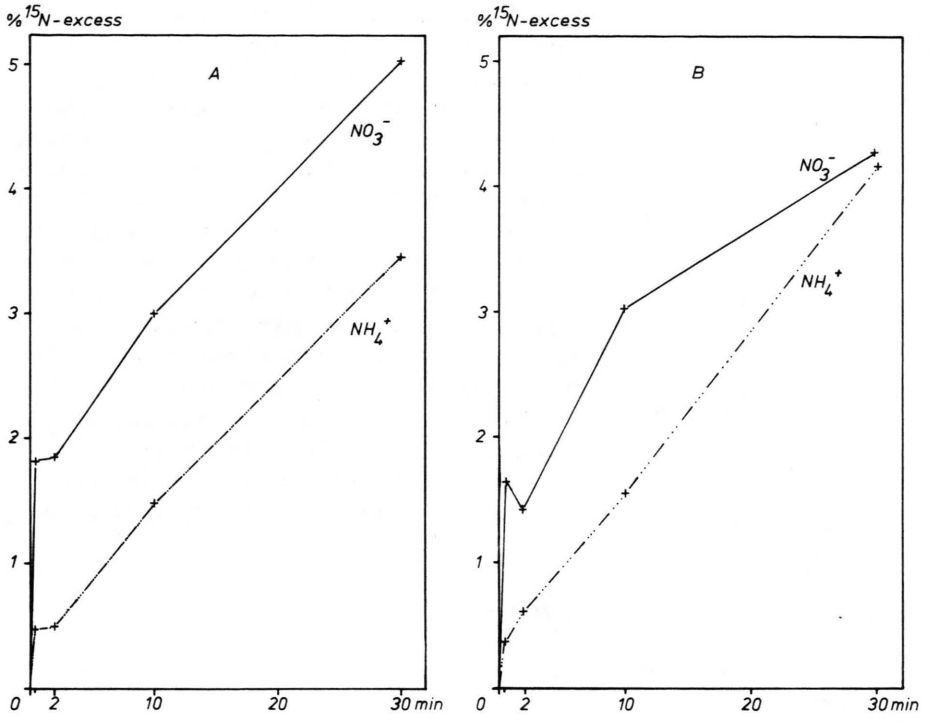

Fig. 3. Ammonia (NH_4^+) and nitrate (NO_3^-) uptake in cell suspensions of *Bellerochea yucatanensis* grown under nitrate (A) and 24 h in a nitrogen-free medium (B) at + 20 °C and continuously illumination (1 mW/cm²: light intensity). Cells resuspended in a nitrogen-free medium at a density of 25 x 10^6 cells/ml were supplied with 1 mM $^{15}NH_4Cl$ (96.0 atom%) or 1 mM $K^{15}NO_3$ (96.8 atom%) after 15 min photosynthesis. Chlorophyll a content 7.838 µg/ml and chlorophyll c content 1.678 µg/ml.

glycine/serine was detectable after 30 minutes photosynthesis. Relatively high values we found in glutamate and alanine at 10 minutes photosynthesis after adding $K^{15}NO_3$ (1 mM). It was impossible to measure ^{15}N enrichment in free amino acids or glutamine up to 10 minutes. No ^{15}N label could be observed in asparagine. These findings are in agreement with other reports on *Chlorella* [3, 25] and indicate that the main route in the initial assimilation of ammonia was the ammonia-scavenging mechanism with the key enzyme glutamate dehydrogenase. In the nitrogen-starved *Bellerochea* cells the intracellular NH_4^+ concentration after adding 1 mM $^{15}NH_4Cl$ can cause a toxical effect and therefore the glutamate dehydrogenase many be important in removing ammonia. The high ^{15}N content of aspartate and glycine/serine might be considered that there was a pathway through which amino nitrogen was introduced directly from ammonia. Baker and Thompson [25] could not prove the direct synthesis of alanine from ammonium. Kinetic experiments of $^{14}CO_2$ fixation showed that the carbon skeletons of aspartate and glycine/serine were formed at the early phase of photosynthesis (see Table III). Recently it was concluded that glutamic acid was the primary product of ammonia assimilation and that the other amino acids and amides were produced secondarily by transamination from

Table II. ^{15}N excess of various free amino acids and amides in cell suspensions of *Bellerochea yucatanensis* grown in nitrate followed by 24 h culture in a nitrogen-free medium. $K^{15}NO_3$ (1 mM) was added after 15 min photosynthesis under continuous illumination (1 mW/cm²) at + 20 °C. Values expressed in atom% ^{15}N excess.

Amino acid	^{15}N excess after 10 min	^{15}N excess after 30 min photosyn.
glutamine	0.117	0.138
aspartate	–	0.216
glutamate	0.256	1.152
alanine	0.229	0.660
glycine/serine	–	0.240

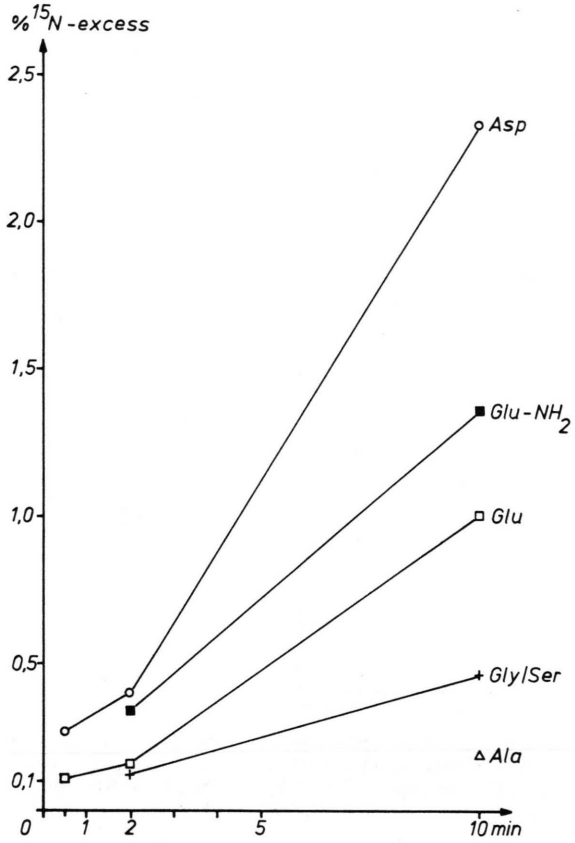

Fig. 4. Kinetics of the distribution of ^{15}N into various free amino acids and glutamine (glu-NH_2) of *Bellerochea yucatanensis*. Algae grown in nitrate were cultured 24 h in a nitrogen-free medium at + 20 °C and 1 mW/cm² light intensity under normal air conditions (0.03 vol% CO_2). Cells resuspended in a nitrogen-free medium at a density of 25 × 10^6 cells/ml were supplied with 1 mM $^{15}NH_4Cl$ (96.0 atom-%) under steady state conditions after 15 minutes photosynthesis. Chlorophyll a content 7.838 µg/ml and chlorophyll c content 2.054 µg/ml. Symbols: Ala alanine, Asp aspartate, Glu glutamate, Glu-NH_2 glutamine and Gly/Ser glycine/serine.

glutamate [3, 26]. Our data of in nitrogen-free medium grown *Bellerochea* support this mechanism (see Fig. 4).

In an other series of short-term kinetic experiments with nitrate (1 mM) grown marine diatoms $^{15}NH_4Cl$ (1 mM; 96.0 atom%) and ^{14}C-bicarbonate were fed immediatly into the same algae suspension during steady state photosynthesis. Figure 5 show the ^{15}N incorporation into various free amino acids and amides under continuous illumination of *Bellerochea yucatanensis*. The slope of the curve of glutamine was much steeper than that of the other amino acids and the ^{15}N content was also much higher. ^{15}N excess in glutamine could be found 10 sec after adding $^{15}NH_4Cl$ to the cell suspension. ^{15}N label was observed in glutamate and alanine after 1 minutes and in aspartate and glycine/serine after 2 minutes. On the other hand, the kinetic analysis of distribution of ^{14}C-labelling into the same amino acids and amides showed different results (see Table III). After 5 sec photosynthetic $^{14}CO_2$ fixation radioactivity was found in alanine, aspartate, glutamate and glycine/serine. Glutamine was ^{14}C-labelled at 2 minutes photosynthesis. ^{14}C- and ^{15}N-labelling of asparagine could be estimated simultaneously after 5 minutes illumination. The very early and high ^{14}C-labelling of aspartate can be interpretated by the operation of a phosphoenolpyruvate carboxylation catalysed by phosphoenol-

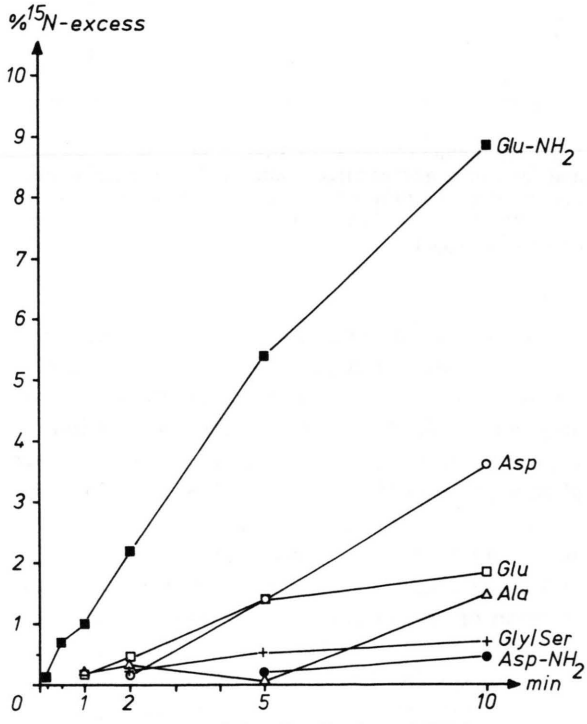

Fig. 5. Time course of the distribution of ^{15}N into various free amino acids and amides of *Bellerochea yucatanensis* extracts. Algae were grown in the normal nutrient medium with (1 mM) nitrate at + 20 °C under normal air conditions (0.03 vol% CO_2) and resuspended in a nitrogen-free medium before starting the experiments. 1 mM $^{15}NH_4Cl$ (96.0 atom%) was added after 15 min photosynthesis. Chlorophyll a content 6.248 µg/ml, chlorophyll c content 1.603 µg/ml and cells density 20 × 10^6 cells/ml. Symbols: Ala alanine, Asp aspartate, Asp-NH_2 asparagine, Glu glutamate, Glu-NH_2 glutamine and Gly/Ser glycine/serine.

Table III. Distribution of ^{15}N- and ^{14}C-label into various amino acids and amides of *Bellerochea yucatanensis* during photosynthesis at + 20 °C under normal air conditions (0.03 vol% CO_2). Cells grown in limited nitrate (1 mM) and resuspended in a nitrogen-free medium at a density of 20×10^6 cells/ml and chlorophyll a content of 6.248 µg/ml and chlorophyll c content of 1.603 µg/ml. $^{15}NH_4Cl$ (1 mM; 96.0 atom%) and ^{14}C-bicarbonate (56.8 mCi/mM) were added simultaneously after 15 min preillumination. Values expressed of ^{14}C as dpm $\times 10^3$/µg Chl a and of ^{15}N as ^{15}N excess in (···). – * No estimation of ^{15}N enrichment.

Amino acid	5 sec	10 sec	30 sec	1 min	2 min	5 min	10 min
aspartate	0.13	0.26	0.95	4.56	13.27 (0.174)	34.78 (1.427)	104.28 (3.610)
glutamate	0.02	0.02	0.11	0.47 (0.154)	1.73 (0.465)	6.19 (1.427)	10.91 (1.833)
asparagine	–	–	–	–	–	0.15 (0.209)	0.51 (0.463)
glutamine	–	– (0.144)	– (0.711)	– (1.052)	0.13 (2.206)	0.82 (5.410)	4.28 (8.874)
alanine	0.09	0.18	0.54	2.16 (0.177)	4.48 (0.254)	8.43 (0.103)	20.26 (1.468)
glycine/serine	0.06	0.09	0.25	1.03	2.67 (0.230)	7.94 (0.513)	27.94 (0.702)
threonine *	–	–	–	–	0.30	0.99	5.03
valine *	–	–	–	0.17	0.53	1.05	2.68
isoleucine *	–	–	–	0.03	0.12	0.33	1.06

pyruvate carboxykinase. This could be shown by the pattern of ^{14}C-labelled photosynthetic products and enzym studies (Roßlenbroich and Döhler, in press).

Our results from nitrate grown *Bellerochea* provide exidence that in marine diatoms the GS/GOGAT system is operating (see Fig. 5) which was suggested by Falkowski and Rivkin [27] investigating the Km values of glutamate dehydrogenase and glutamine synthetase in extracts of *Skeletonema costatum.* The incorporation of ^{15}nitrogen into glutamine shown by short-term ^{15}N analysis (Fig. 5) indicates that the main route of the assimilation of ammonia occurs through the glutamine synthetase/glutamate synthase pathway in *Bellerochea.*

Acknowledgements

This work was supported by a grant from "Deutsche Forschungsgemeinschaft". We wish to thank Prof. Dr. von Stosch, Marburg, for his generous gift of *Bellerochea yucatanensis* and Dr. Hentschel, Hohenheim, for his helpful support by ^{15}N-analysis. We thank Miss Erbelding for her work with *Skeletonema.*

[1] O. Warburg and E. Negelein, Biochem. Z. **110,** 66–115 (1920).
[2] D. T. Canvin and C. A. Atkins, Planta **116,** 207–224 (1974).
[3] J. A. Bassham and M. Kirk, Biochim. Biophys. Acta **90,** 553–562 (1964).
[4] C. P. Wolk, S. M. Austin, J. Bortins, and A. Galonsky, J. Cell. Biol. **61,** 440–453 (1974).
[5] J. Thomas, C. P. Wolk, and P. W. Shaffer, Biochem. Biophys. Res. Comm. **67,** 501–507 (1975).
[6] W. D. P. Stewart, A. Haystead, and M. W. N. Dharmawardene, in: Nitrogen fixation by free-living micro-organism. (W. D. P. Stewart, ed.), p. 129–158, Cambridge Univ. Press, 1975.
[7] T. Yoneyama and K. Kumazawa, Analyt. Biochem. **67,** 327–331 (1975).
[8] O. Ito and K. Kumazawa, Soil Sci. Plant Nutr. **24,** 299–303 (1978).
[9] H. A. v. Stosch, Nova Hedwigia **8,** 113–168 (1977).
[10] S. W. Jeffrey and G. F. Humphrey, Biochem. Physiol. Pfl. **167,** 191–194 (1975).
[11] H. Faust, Isotopenpraxis **3,** 100–103 (1967).
[12] M. Schröder, Diss. Hohenheim 1980.
[13] G. B. Cook, J. A. Goleb, and V. Middelboe, Nature **216,** 475–476 (1967).
[14] T. Yoneyama and Kumazawa, Plant Cell Physiol. **16,** 21–26 (1975).
[15] H.-J. Roßlenbroich, Diss. Frankfurt/M. 1981.
[16] M. M. Bradford, Analyt. Biochem. **72,** 248–254 (1976).
[17] R. L. Bieleski and N. A. Turner, Analyt. Biochem. **17,** 278–293 (1966).
[18] G. Döhler, Planta **107,** 33–42 (1972).
[19] Y. Collos and G. Slawyk, J. Phycol. **15,** 186–190 (1979).
[20] N. J. Anita, B. R. Berland, D. J. Bonin, and S. Y. Maestrini, J. mar. biol. Ass. U. K. **55,** 519–539 (1975).
[21] J. D. Strickland, O. Holm-Hansen, R. W. Eppley, and R. J. Linn, Limnol. Oceanogr. **14,** 23–34 (1969).
[22] J. M. De Manche, H. C. Curl, M. D. Lundy, and P. L. Donaghay, Mar. Biol. **53,** 323–333 (1979).
[23] J. L. Serra, M. J. Llamma, and E. Cadenas, Plant Physiol. **62,** 987–990 (1978).
[24] C. Rigano, V. D. M. Rigano, V. Vona, and A. Fuggi, Arch. Microbiol. **129,** 110–114 (1981).
[25] J. E. Baker and J. F. Thompson, Plant Physiol. **40,** 208–212 (1961).
[26] J. Ingversen and S. Ivanko, Physiol. Plant. **24,** 199–204 (1971).
[27] P. G. Falkowski and R. B. Rivkin, J. Phycol. **12,** 448–450 (1976).