PREFERENTIAL ASSIMILATION OF AMMONIUM ION BY *CHLORELLA VULGARIS*

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Introduction

It is generally known that, when the culture medium contains ammoniacal nitrogen as well as nitrate and nitrite nitrogen, green algae utilize the ammoniacal nitrogen. However, when the supply of ammoniacal nitrogen is limited, there is a question as to the selectivity of the organism with regard to the nitrogen available. This situation arises when algal cells deplete the medium of the ammoniacal nitrogen originally supplied and are forced to rely on nitrogen in other forms, including those compounds arising from the metabolic activities of the cells.

A series of experiments has been performed by the authors as a part of a general program concerning the nitrogen utilization of algae, using heavy nitrogen as a tracer and the mass spectrometer as an instrument for its assay. Like many experiments in which the newer techniques have been used, the results are not unusual but merely confirm, by a relatively independent technique, what has already been known. For example, PRATT and FONG (**7**) showed that cultures of Chlorella preferentially utilize ammonium as long as it is present, and then absorb nitrate. An advantage of the method employing a tracer and the mass spectrometer is that it is possible to get data concerning the source of the cell nitrogen when the nitrogen is presented to the organism as both ammoniacal and nitrate nitrogen simultaneously. No precautions were taken to prevent the algae from fixing atmospheric nitrogen during the experiments.

Methods and materials

The medium for the growth of the alga consisted of inorganic salts and glucose. The only source of nitrogen in the medium was ammonium nitrate. Five liters of medium containing 0.20 gm. NH_4NO_3, 0.08 gm. KH_2PO_4, 0.08 gm. $MgSO_4 \cdot 7\,H_2O$, 0.04 gm. $CaCl_2 \cdot 2\,H_2O$, 0.4 mg. $FeSO_4 \cdot 7\,H_2O$, and 20.0 gm. glucose per liter were prepared with distilled water. The ammonium nitrate used had been obtained from Eastman Kodak Co. and the specified atom per cent. of N^{15} in the ammonium radical was 61.5. A determination of this value using the atomic masses for the ratio 15/14 was 61.1 atom per cent. which was accepted as the standard for all of the other ratio measurements. For convenience in handling, the five liters of medium were divided

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into two portions of 2500 ml. and each portion put into a four-liter serum bottle. The bottles were plugged and autoclaved at 15 pounds for 30 minutes.

A pure culture of *Chlorella vulgaris* Beijerinck was used for inoculation. This organism had been isolated as a unialgal culture free of bacteria and fungi in this laboratory and had been identified by Dr. Francis Drouet of the Chicago Natural History Museum. The inoculum culture was made by putting a loop of the cells from a slant culture into a tube containing 15 ml. of liquid medium of the composition described previously, except that non-labeled NH_4NO_3 was used. The culture was incubated at 21° C for seven days under white fluorescent lamps which provided an illumination of 250 foot-candles. Five milliliters of this inoculum culture were used for inoculation of each of the two serum bottles.

After seven days of growth under the temperature and light conditions listed above, the bottles were removed from the incubator, the cells centrifuged from the medium, washed twice with distilled water, and then frozen. The medium was resterilized in two four-liter serum bottles and reinoculated. The same process of removing cells, resterilizing medium, and reinoculation was repeated twice again, giving in all, four experiments. The time of growth for each of these was 7, 6, 10, and 16 days respectively.

Analyses for ammoniacal nitrogen were made on the media, and the cells were analyzed for total nitrogen. Total nitrogen determinations were done by the Kjeldahl method, modified to include the nitrogen of nitrates (**1**). The ammoniacal nitrogen determinations on the media were done by means of an alkaline distillation into standardized HCl solutions.

Samples for the mass spectrometer were prepared by the same methods except that the excess standard acid into which the NH_4 had distilled was not titrated with standard NaOH. The contents of the receiving flask were boiled down, and then the ammonium oxidized to molecular nitrogen by means of the hypobromite method and apparatus suggested by RITTENBERG (**8**).

A Nier-type mass spectrometer (**2, 3, 4, 5, 10**) was used to measure the relative abundance of atomic nitrogen, defined as

$$\frac{N^{15}}{N^{14} + N^{15}} \times 100 = \text{atom per cent. of } N^{15}.$$

The atomic masses (N^{15}/N^{14}) were chosen in preference to the conventional molecular masses (N^{29}/N^{28}) because this procedure gives freedom from interference by residual carbon monoxide, better spectral separation for smaller masses, and quicker and more simple computation of results since molecular combinations of N^{14} and N^{15} involve additional statistical calculations. Since the change in the abundance ratio was of primary concern, all ratio measurements were compared with the standard sample. Thus any isotope discrimination which occurs during dissociation at the ion source is eliminated in the relative abundance ratios. The instrument used was constructed (**9**) in the Physics Department of the University of Cincinnati

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TABLE I

MASS SPECTROMETRIC DATA CONCERNING NITROGEN METABOLISM IN *Chlorella vulgaris*. THE AVERAGE VALUES SHOWN ARE RELATIVE TO THE ATOM PER CENT. OF N^{15} IN THE AMMONIUM RADICAL OF NH_4NO_3 IN THE CULTURE MEDIUM AT THE BEGINNING OF EXPERIMENT 1 (61.1).

Experiment	Age of medium	Material	Atom per cent. N^{15} in total N
	days		
1	0	Medium at start of experiment	30.5
		Cells at end of experiment (7 days)	55.5
2	7	Medium at start of experiment	24.4
		Cells at end of experiment (6 days)	55.4
3	13	Medium at start of experiment	18.1
		Cells at end of experiment (10 days)	13.9
4	23	Medium at start of experiment	7.9
		Cells at end of experiment (16 days)	5.4
	39	Medium at end of all experiments	8.0

essentially according to the description of NIER (**3**). The magnetic analyzer and the FP-54 electrometer circuit are patterned fundamentally after those described earlier by NIER (**4, 6**). From a sample of ammonium chloride containing approximately 1.5 mg. of nitrogen, obtained from the Kjeldahl process and oxidized by a hypobromite solution (**8**), sufficient molecular nitrogen was obtained to fill a 15 ml. bulb at a pressure of 10 cm. Hg. This sample was introduced into the mass spectrometer with the technique used by NIER (**3**).

Results and discussion

The original medium contained equal proportions of ammoniacal and nitrate nitrogen as NH_4NO_3 in a concentration of 0.2 mg./ml., and no other form of nitrogen. In this environment the algal cells appear to have had a surplus of NH_4 available at the beginning of their growth. In consequence, the cells harvested at the end of the first experiment (seven days) had 55.5 atom per cent. of N^{15} and the resulting medium had 24.4 atom per

TABLE II

CHEMICAL DATA CONCERNING NITROGEN METABOLISM IN *Chlorella vulgaris*. ALIQUOTS OF THE SAME MEDIUM FOR WHICH THE MASS SPECTROMETRIC ANALYSES APPEAR IN TABLE I WERE USED FOR THE CHEMICAL DETERMINATIONS.

Experiment	Age of medium	Ammoniacal nitrogen	pH
	days	*μg./ml.*	
1	0	35	4.6
2	7	23	3.2
3	13	6	3.0
4	23	5	3.3
	39	3	3.5

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cent. of N^{15}. These figures show that the cells definitely preferred NH_4 to NO_3. However, the value of 55.5 atom per cent. of N^{15} (instead of 61.1 atom per cent. of N^{15}) in the harvested cells, seems to indicate that even with a supply of NH_4 available, the cells were utilizing a form of nitrogen other than NH_4. A situation similar to that of experiment 1 is present in experiment 2, except that, by the end of the latter, the medium has been depleted of NH_4 due to preference for it over all other forms of nitrogen. This depletion is reflected in the 55.4 atom per cent. of N^{15} in the harvested cells.

At the beginning of experiment 3, practically no NH_4 was available in the medium and so the cells grew according to a new selection of nitrogen from additional sources other than the labeled ammoniacal nitrogen. The medium now contains nitrogen compounds of N^{15} as by-products of cell growth and decay. The cells contain the atom per cent. of the medium, but apparently do so according to two different mathematical factors. At the very beginning of the first experiment the only N^{15} is in the NH_4 radical of the NH_4NO_3 in the medium. Therefore the total nitrogen in the medium has an atom per cent. of N^{15} equal to one half that of the ammonium radical. When the cells take up NH_4 predominately, their atom per cent. should, and did, equal approximately twice that of the medium. However, when N^{15} in the medium is in compounds other than ammonium (presumably resulting from algal metabolism) and when, at the same time, very little NH_4 is available, the cells have an atom per cent. of N^{15} approximately equal to that of the medium.

The fourth experiment seems to be an extension of the third. The atom per cent. of N^{15} in the starting medium, in the final medium, and in the harvested cells was approximately the same which indicates that a type of dynamic equilibrium has been reached, in that the cells are removing representative nitrogen samples from the medium (removing N^{14} and N^{15} in the same proportion as these are found in the medium) and returning the same proportions to the medium in metabolic products.

Summary

A pure culture of *Chlorella vulgaris* was grown in an organic medium containing NH_4NO_3 having 61.1 atom per cent. of N^{15} in the ammonium radical. After a period of growth, the cells were harvested and the cell-free medium resterilized and reinoculated. This process was repeated three more times. At the end of each experiment, analyses for N^{15} in the medium and cells were performed using a mass spectrometer.

It was found that the algal cells definitely preferred ammoniacal nitrogen to the nitrate form since their atom per cent. of N^{15} approximated twice that of the medium. However there was evidence that nitrogen from a source other than the ammonium supplied in the medium was retained by the cells even when a plentiful supply of ammoniacal nitrogen was present. When practically no NH_4 was available in the medium, the cells grew

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according to a new selection of nitrogen from sources other than the labeled ammoniacal nitrogen. The medium now presumably contained nitrogen compounds of N^{15} as by-products of cell growth and decay. It was found that the cells had an atom per cent. of N^{15} which equalled that of the medium.

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