SHORT-TERM N_2^{15}-INCORPORATION BY *AZOTOBACTER*[1]

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ABSTRACT

BULEN, W. A. (Charles F. Kettering Research Laboratory, Yellow Springs, Ohio), J. R. LECOMTE, AND H. E. BALES. Short-term N_2^{15}-incorporation by *Azotobacter*. J. Bacteriol. **85**:666–670. 1963.—Short-term N_2^{15}-incorporation measurements were used to determine which of the growth requirements were necessary for nitrogen fixation by *Azotobacter agilis* (*A. vinelandii*). Normal cells required neither added iron nor molybdenum, but a marked stimulation by Na^+ and a minor stimulation by Mg^{2+} were observed. The Na^+ stimulation was not accompanied by an increase in O_2 uptake. A lag period preceded the response of molybdenum-deficient cells to added Mo. In systems employing 10 and 20% O_2 with 10% N_2^{15} in the gas phase, O_2 appeared to be both required and inhibitory. These observations may be helpful in attempts to fractionate cell-free nitrogen-fixing systems from this aerobe.

The mineral requirements for nitrogen fixation by *Azotobacter* have been determined primarily by growth measurements or by measuring the increase in total nitrogen after relatively long incubation periods. The mineral requirements for growth of *A. agilis* (*A. vinelandii*) utilizing atmospheric nitrogen are basically those contained in Burk's sucrose mineral salts medium (Burk and Lineweaver, 1930). Using specially purified media, Esposito and Wilson (1956) confirmed the requirements of *A. agilis* for calcium and for micro amounts of molybdenum and iron.

The competitive inhibition of nitrogen fixation by oxygen in *A. agilis* was recently re-examined by Parker and Scutt (1958, 1960). Data supporting their conclusions were obtained with 3- to 4-hr growth periods of cells initially in the logarithmic phase.

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The requirements for growth and for short-term nitrogen fixation are not necessarily identical. This investigation was concerned with short-term nitrogen fixation, examined as part of an effort to increase the rate of fixation by cell-free extracts to levels suitable for investigating the mechanism of nitrogen fixation by this aerobe. Short-term (30-min) N_2^{15}-incorporation measurements were used to examine the response of normal cells to the addition of those minerals required for growth and the effect of the partial pressure of oxygen. The rate of N_2^{15}-incorporation by molybdenum-deficient cells in response to externally added molybdenum was also investigated.

MATERIALS AND METHODS

A. agilis was cultured on Burk's nitrogen-free mineral salts medium (Burk and Lineweaver, 1930) containing (per liter): K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.05 g; and sucrose, 20 g. Mo (1 ppm) and Fe (3 ppm) were added, the latter as the citrate complex. Molybdenum-deficient cells were the $-$Mo #2 cultures described previously (Bulen, 1961).

For the mineral-salt experiments, cells from a normal 16-hr culture were separated by centrifugation, the tubes drained and wiped, and the cells suspended in one-half the original volume of 0.006 M potassium phosphate buffer (pH 7.2). A series of salt solutions containing (mg/ml) NaCl, 6.0; KCl, 7.68; $MgSO_4 \cdot 7H_2O$, 6.0; Na_2SO_4, 7.32; and sucrose, 300, were also prepared in the buffer. For most experiments, Specpure brand NaCl obtained from Johnson, Matthey and Co., Ltd., London, was used. Other salt solutions were prepared from reagent-grade compounds. A reaction mixture contained 0.3 ml of resuspended cells (approximately 0.16 mg of cell N), 0.1 ml of the desired salt solutions, 0.2 ml of sucrose solution, and buffer to give a total volume of 3 ml.

For experiments with different oxygen pres-

sures, cells from normal 16-hr cultures were resuspended in fresh medium from which the iron was omitted. As shown below, iron is not required for maximal short-term N_2^{15}-incorporation by normal cells and, in these experiments, was omitted to minimize variations resulting from cell aggregation. Flasks contained the desired amount of cell suspension plus fresh medium to give a total volume of 3 ml.

Experiments with molybdenum-deficient cells employed cells from 16-hr −Mo #2 cultures resuspended in one-half the original volume of filtered, low-molybdenum medium (Bulen, 1961). Flasks contained 0.6 ml of cell suspension, 0.1 ml of Na_2MoO_4 solution to give 1 ppm of Mo when desired, and fresh medium to give a total volume of 3 ml.

N_2^{15}-incorporation experiments were conducted in Warburg flasks containing the desired constituents and 0.05 ml of 40% KOH in the center wells. Four flasks attached to a capillary manifold were gassed simultaneously. Flasks were flushed twice with helium by evacuation, gassed with an atmosphere of 20% O_2 and 80% He, and the gases mixed with a Toepler pump attached to the manifold. After a 5-min preincubation for the removal of dissolved N_2, the flasks were gassed by the same procedure, with an atmosphere of 10% N_2 containing 98% N^{15}, 20% O_2, and 70% He, and incubated for 30 min at 30 C with a shake rate of 120 oscillations/min. When atmospheres containing 10% O_2 were required, this O_2 pressure was also used during the preincubation period. Premixing of the gases was required to obtain the desired reproducibility between duplicate flasks. At the end of the reaction period, the contents of the flasks were subjected to Kjeldahl digestion and distillation, using the mercuric oxide catalyst recommended by Burris and Wilson (1957). Ammonia nitrogen was converted to N_2 by hypobromite oxidation (Glascock, 1954), and the isotope content determined with a mass spectrometer (model 21-401, Consolidated Engineering Corp.). When different cell populations were used, as in experiments with different oxygen pressures, carrier nitrogen was added as $(NH_4)_2SO_4$ before digestion, to bring all samples to approximately the same total nitrogen value.

Oxygen-uptake experiments were conducted manometrically in Warburg flasks by standard procedures (Umbreit, Burris, and Stauffer, 1957). Flasks contained the contents indicated in Table 3 in a total volume of 3 ml. The gas phase was air, and temperature was 30 C, with a shake rate of 120 oscillations/min.

Results

Preliminary experiments revealed several variables influencing short-term N_2^{15}-incorporation. N_2^{15}-incorporation was not linear with time, especially after 30 min. For example, normal cells (0.15 mg of cell N/flask) in an atmosphere of 20% O_2, 10% N_2^{15}, and 70% He incorporating 43 μg of N^{15}/mg of cell N in 30 min would incorporate 127 μg of N^{15}/mg of cell N in 1 hr; 30-min incubation periods were, therefore, selected for short-term experiments to obtain resting- or lag-phase conditions in which the factors influencing the nitrogen fixation preceding cell reproduction would be most prevalent. The amount of N_2^{15}-incorporation per mg of cell N was also found to increase with the period of preincubation with 20% O_2 and 80% He. This was observed with both normal cells and molybdenum-deficient cells supplied with 1 ppm of Mo. Preincubation periods were, therefore, limited to 5 min.

Molybdenum is not required in the external medium by normal cells for short-term N_2^{15}-incorporation (Bulen, 1961). The response of Mo-deficient cells to the addition of 1 ppm of Mo was examined, and Fig. 1 shows the results of a typical experiment. Molybdenum did not im-

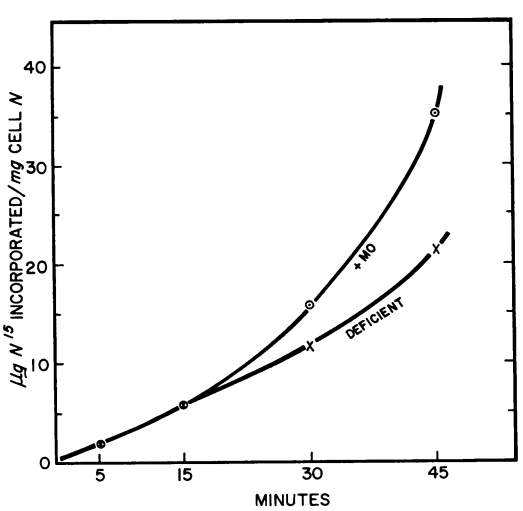

FIG. 1. *Rate of N_2^{15}-incorporation by Mo-deficient cells supplied with 1 ppm of Mo.*

TABLE 1. *Effect of iron on short-term N_2^{15}-incorporation*

Medium	Incorporation*
Complete	43.1
Complete, filtered	45.2
EDTA	42.3
Minus Fe	44.4

* Expressed as μg of N^{15} per mg of cell N in 30 min. Average of two to four determinations.

TABLE 2. *Influence of sodium and magnesium ions on short-term N_2^{15}-incorporation*

Additions*	Incorporation†
None	8.4
$MgSO_4$	12.1
NaCl	21.4
Na_2SO_4	22.4
$MgSO_4$ + KCl	11.3
$MgSO_4$ + NaCl	31.6

* To 0.006 M potassium phosphate buffer (pH 7.2) containing 2% sucrose. Final concentrations (mM): Na^+ and K^+, 3.44; Mg^{2+}, 0.81.

† Expressed as μg of N^{15} per mg of cell N in 30 min.

TABLE 3. *Influence of sodium and magnesium ions on oxygen uptake*

Additions*	O :uptake (μliters per 30 min)	
	Cell N, 0.020 mg	Cell N, 0.038 mg
None	53.0	125.4
$MgSO_4$	63.4	135.7
NaC	48.8	116.1
$MgSO_4$ + NaCl	59.4	133.3

* To 0.006 M potassium phosphate buffer (pH 7.2) containing 2% sucrose. Final concentrations (mM): Na^+, 3.44; Mg^{2+}, 0.81.

mediately stimulate N_2^{15}-incorporation. The response became measurable only after a 15-min incubation period, after which the difference became increasingly greater with time.

The effect of iron in the external medium was examined by use of complete medium, complete medium filtered just before use, ethylenediaminetetraacetate (EDTA) medium to which iron was added as sodium ferric EDTA, and medium prepared without added iron. The data in Table 1 demonstrate the lack of stimulation of N_2^{15}-incorporation by the presence of iron. With cells in regular unfiltered medium, where cell aggregation occurs, considerable variation of results was observed; this can be eliminated by filtering the medium or by sequestering the iron.

The effects of sodium and magnesium were examined by adding these ions to normal cells suspended in phosphate buffer containing 2% sucrose. The data presented in Table 2 are typical of a number of similar experiments. The Na^+ and Mg^{2+} concentrations used are identical to those found in Burk's medium. Mg^{2+} always induced a small but significant stimulation of short-term N_2^{15}-incorporation. In control experiments, $MgCl_2$ was as effective as $MgSO_4$. The greatest single stimulation resulted from the addition of Na^+. Na_2SO_4 was as effective as NaCl, and the addition of KCl at the same concentration as NaCl was without effect. The use of Specpure NaCl minimized the possibility that a contaminant in the reagent elicited the stimulation. An additive effect was observed upon the addition of Na^+ and Mg^{2+} together.

Magnesium is known to be involved in several of the enzymatic conversions of carbohydrate metabolism. To determine whether the Mg^{2+} and Na^+ stimulation of short-term N_2^{15}-incorporation was merely a reflection of their effect on carbohydrate metabolism, oxygen-uptake experiments

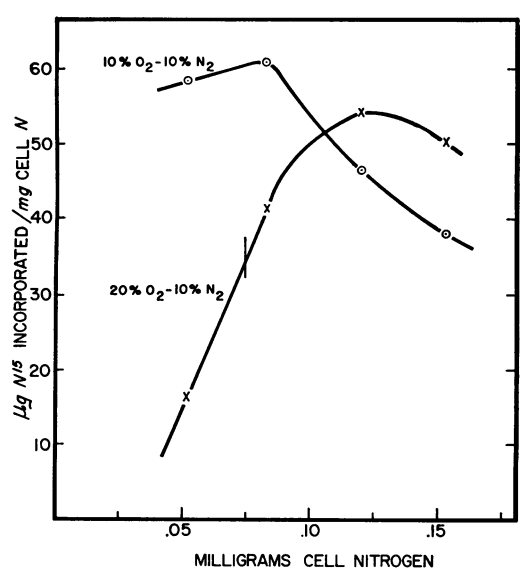

FIG. 2. *Effect of the partial pressure of oxygen on short-term (30-min) N_2^{15}-incorporation.*

with a sucrose medium were conducted, yielding the data recorded in Table 3. Mg^{2+} caused a small but measurable increase in O_2 uptake. Na^+, either alone or in the presence of Mg^{2+}, produced no such increase; in fact, a slight decrease, although of questionable significance, was observed in all experiments.

Preliminary 30-min N_2^{15}-incorporation experiments using 20% O_2 and 10% N_2^{15} indicated that the mg of N^{15} incorporated/mg of cell N increased with increasing cell densities. Since such an increase could result from an oxygen inhibition at low cell densities (Parker and Scutt, 1960), an examination of N_2^{15}-incorporation as a function of cell density was made, using 10 and 20% oxygen in the gas phase. The data from a typical experiment (Fig. 2) reveal an increase in N_2^{15}-incorporation with increasing cell N when 20% O_2 is used, and a decrease when 10% O_2 is used. The vertical line on the curve obtained with 20% O_2 represents the approximate cell density at which the amount of oxygen available to the cells (about 700 μliters/hr) is limited by the diffusion rate, with air as the gas phase and with the same conditions of incubation. By necessity, reactions were conducted in a closed system, and no oxygen was admitted during the course of the reaction.

Discussion

Neither iron nor molybdenum, which are required in micro amounts for growth of *Azotobacter* on nitrogen gas, stimulates short-term N_2^{15}-incorporation when added to normal cells. In their functional form, these metals are probably not significantly dissociated from enzyme proteins. Mo^{99} bound to protein has been found in cell-free preparations from *Azotobacter* in an ammonium sulfate-precipitated fraction by Magee and Burris (1956), and in a particulate fraction by Keeler, Bulen, and Varner (1956). Iron is known to be a constituent of a number of enzymes. Two-thirds of the Fe^{59} incorporated by *Azotobacter* was found in the membrane fraction (Keeler, Carr, and Varner, 1958).

The rate of response of Mo-deficient cells to added Mo does not indicate the existence of a preformed protein that is "activated" by the addition of Mo. Some metabolic activity is apparently associated either with the uptake of Mo or its incorporation into a functional system. Since as much N^{15} is incorporated in the absence of Mo as in its presence for about the first 15 min of incubation, the data could reflect the incorporation of N_2 to the level of some intermediate whose reduction requires Mo. This interpretation seems unlikely. It has not been possible to detect free intermediates preceding ammonia in the cell-free nitrogen-fixing system from *Clostridium pasteurianum* (Garcia-Rivera and Burris, 1962), and an HI reduction pretreatment prior to Kjeldahl digestion, which insures recovery of N from oxidized forms, has not increased the amount of N^{15} recovered in our experiments. In addition, Mo-deficient cells do contain some molybdenum.

Both Mg^{2+} and Na^+ stimulate short-term N_2^{15}-incorporation, the greatest stimulation being induced by Na^+. The magnesium stimulation is probably a reflection of its role in carbohydrate metabolism, increasing the supply of electrons for nitrogen reduction. No explanation for the sodium ion stimulation is readily apparent. A possible role in the cellular uptake of substrate is not substantiated by any increase in cellular respiration. Its function might be ascribed to its ability to inhibit enzymes activated by potassium. The potassium concentration of the buffer, however, was more than three times that of the sodium added.

Experiments on the effects of the partial pressure of oxygen on short-term N_2^{15}-incorporation indicate both an oxygen requirement and an oxygen inhibition. Pertinent to this interpretation is the fact that cells under these conditions demonstrate a $Q_{O_2}^N$ in air of about 9,000 μliters per hr per mg of N, when determined for the first 30 min of incubation. The diffusion rate of oxygen, which reaches a maximum in air of about 700 μliters/hr, determines the amount of O_2 available to the cells. As the cell population increases, the ratio of the oxygen available to the oxygen-uptake capacity of the cells decreases. Only about 12% of the O_2 in the flasks was utilized during the course of the experiment. The oxygen requirement is probably associated with the uptake and transformation of the sucrose preceding the transfer of electrons to N_2. The inhibition is possibly associated with the N_2-incorporation process, as suggested by Parker and Scutt (1960). Oxygen pressures above 20% O_2 were not used, since respiration is inhibited above about 30% O_2, apparently by a suppression of the pyruvic oxidase system (Dilworth, 1962).

The highest levels of N_2 fixation by cell-free preparations from *Azotobacter* reported to date were obtained under aerobic conditions by Nicholas and Fisher (1960*a*, *b*) and Nicholas, Silvester, and Fowler (1961), using extracts prepared by sonic oscillation of cells in the medium in which they grew. Although a specific role for sodium in the N_2-incorporating system is not claimed, it may be well to provide the system with sodium ions during attempted purifications, at least until the requirements are better defined. If the oxygen inhibition is, as suggested, an inhibition of the N_2-incorporating system, cell-free experiments employing an electron-donor system not linked to O_2-requiring reactions should yield higher values if conducted anaerobically.

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