Use of a Dissimilatory Nitrate Reductase from *Escherichia coli* and Formate as a Reductive System for Nitrate Assays

Ann L. McNamara,[1] Gabrielle B. Meeker, Paul D. Shaw, and Richard H. Hageman*

An assay for nitrate that utilizes the dissimilatory nitrate reductase from *Escherichia coli*, strain B, and formate to reduce quantitatively nitrate to nitrite is presented. The nitrite formed is measured by the standard diazotization method. Procedures for culture of *E. coli*, extraction of the enzyme, and assay are given. Since the assay is specific and sensitive (0.1 μg of NO_3^--N in 10-ml volume) it can be used to determine nitrate in partially clarified plant extracts or similar preparations.

The advantages of enzymatic rather than chemical reduction of nitrate prior to determination of nitrite by diazotization in estimating nitrate content of plant and soil extracts are specificity and reproducibility (Lowe and Hamilton, 1967). The assay described by Lowe and Hamilton is most satisfactory, with the exception that problems arise in obtaining adequate amounts of usable soybean nodules that can serve as a source of the dissimilatory nitrate reductase. Because of the space and time it takes (6 to 8 weeks) to produce soybeans with nodules, and the inability to obtain reproducibly nodules with the dissimilatory enzyme, or to culture *in vitro Rhizobium japonicum*, it seemed desirable to attempt to use bacteria as a source of the enzyme. Previous work of Taniguchi and Itazaki (1959) and Fewson and Nicholas (1961a,b) suggested *E. coli*, *Pseudomonas aeruginosa*, or *Micrococcus denitrificans* as suitable sources of dissimilatory nitrate reductase. Because stocks of *E. coli* strain B were readily available and preliminary work showed that this bacteria was a good source of the enzyme, it was selected for study. The work of Ruiz-Herrera and DeMoss (1969) indicates that other strains of *E. coli* would also be sources of the enzyme.

The objective of the work was to show that *E. coli* strain B could be readily cultured to provide a convenient source for the dissimilatory nitrate reductase used in quantitative determination of nitrate, as previously described by Lowe and Hamilton (1967).

MATERIALS AND METHODS

Many of the procedures used were adapted from the manuscript of Taniguchi and Itazaki (1960).

Stock Cultures. *Escherichia coli* strain B is stored on nutrient agar slants. The nutrient agar contains (in g/l.): bactopeptone, 5.0; beef extract, 3.0; and bactoagar, 15.0 (obtainable from Difco Labs, Detroit, Mich.). The final pH of this mixture is approximately 7.0. After solubilization of the components by warming and gentle agitation, the material is transferred to test tubes and sterilized for 20 min at 120° C and cooled. The organism is streaked on the solidified slants and allowed to grow for 3 to 4 hr at 37° C prior to storage at 2° C.

Growth Medium. The growth medium contains (in g/l.): bactopeptone, 10.0; beef extract, 1.0; casamino acids (vitamin free), 3.0; yeast extract, 3.0 (all obtainable from Difco Labs); K_2HPO_4, 2.0; KNO_3, 1.0; and glucose, 20.0. The final pH was 7.2. The glucose stock solution (50%) (w/v) is made separately. The necessary amounts of the medium and glucose solutions are autoclaved at 120° C for 20 min, cooled at 22° C, combined, and made to final volume. With the organism used, 1.0 g per l. of KNO_3 gave optimum yields of the dissimilatory enzyme. Phosphate was superior to citrate as a buffer in the growth medium.

Culture Procedure. One hundred milliliters of growth medium is added to a 500 ml Erlenmeyer "starter colony" flask. (This flask should have a minimum surface area to volume ratio of 5 to 1.) After flushing the oxygen from the flask and medium with N_2, the *E. coli* from a freshly prepared (grown at 37° C for 24 hr) slant is washed into the flask. After again flushing the oxygen from the flask, N_2 is bubbled through the medium at a rate that is just sufficient to maintain anaerobic conditions during the growth of the organism. If N_2 is not available, the *E. coli* can be grown in an evacuated vacuum filter flask conveniently fitted with a three-way valve. The "starter colony" is grown at 37° C with continuous gentle agitation. Growth is determined by observing the change in absorbancy at 660 mμ on aliquots removed at periodic intervals. At the end of log phase growth (usually 3 to 4 hr after transfer), enough (usually 50 ml is adequate) of the "starter colony" is transferred to an "inoculum" flask to give an initial absorbancy at 660 mμ of 0.05. The "inoculum" flask is a 2-l. Erlenmeyer flask that contains 1 l. of sterile growth medium. The "inoculum" colony is grown under the same conditions as the "starter colony." Three to four hours after transfer (end of log growth phase) the "inoculum" colony can be harvested or the entire contents of the flask transferred to a fermenter tank (Model FS-314, New Brunswick Sci. Co., New Brunswick, N.J.) containing 10 l. of growth medium, if a larger amount of enzyme is desired. If the fermenter method is used, the "inoculum" colony can be stored at 2° C overnight without noticeably changing the amount of dissimilatory enzyme obtained after the final growth period. If the "inoculum" colony is used as the source of the enzyme, it should be harvested immediately, since the enzyme deteriorates with storage in medium.

The culture in the fermenter is kept anaerobic by bubbling N_2 through at a minimal rate, and it is mechanically agitated at a rate (100 to 250 rpm) that causes minimal foaming. An antifoaming agent such as P-2000 (Dow Chemical Co., Midland, Mich.) is recommended. At the end of the log growth phase, the culture is ready for harvest.

It is important to measure both absorbancy and pH of the cultures at intervals during the growth of the "starter," "inoculum," and "fermenter" colonies. Harvest time (end of the log growth phase) is indicated when the absorbance

Department of Agronomy, University of Illinois, Urbana, Ill. 61801

[1] Present address: Pathology Department, Northwestern Medical School, Chicago Campus, Chicago, Ill. 60611

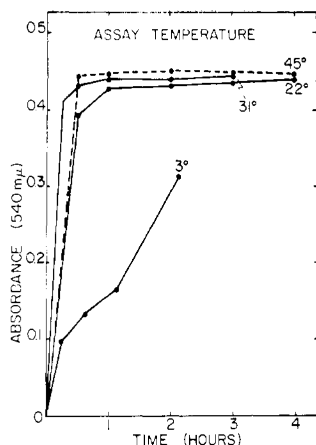

Figure 1. The effect of incubation temperature on the enzymatic reduction of nitrate to nitrite

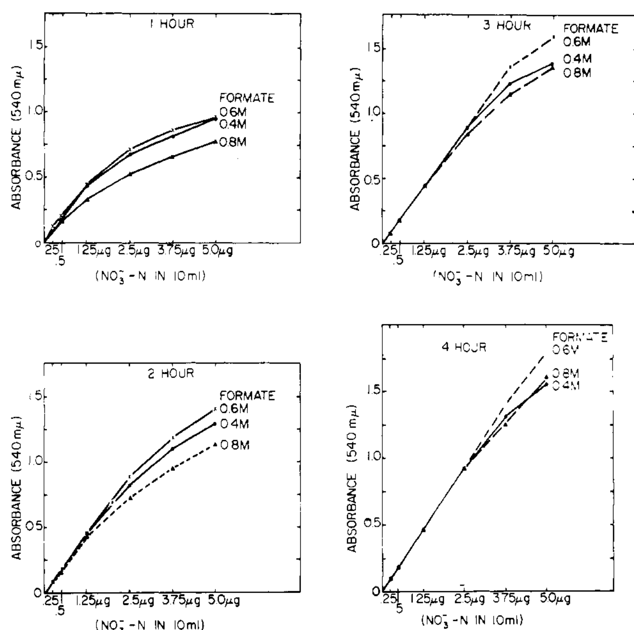

Figure 2. The effect of incubation time at 45° C and sodium formate concentration on the reduction of nitrate to nitrite

values fail to increase in a linear manner. An unexpected sharp decrease in absorbance values occurring prior to the expected harvest time (3 to 4 hr after inoculation) may indicate contamination (phage). Occasionally *E. coli* cultures may produce an excess of lactic acid which can be toxic to the cells. In these instances the pH may decrease to 5.0 to 5.5, and the pH should be readjusted to 7.2 by addition of K_2HPO_4.

Harvest. The cells are most conveniently harvested by continuous flow centrifugation (Model KSB-R, I. Sorvall Inc., Norwalk, Conn.) or by batch centrifugation at 4000 × *g* for 5 min. The precipitate is resuspended in a small volume of 1% NaCl (w/v), and recentrifuged (4000 × *g* for 5 min) and repeated if necessary to remove all nitrite. The resultant precipitate (whole cells) can be stored at −15° C for periods up to 6 months.

Enzyme Extraction. The purpose of this operation is to separate the dissimilatory nitrate reductase (reduction of nitrate to nitrite) from the assimilatory system (reduction of nitrate to ammonium).

The collected cells (fresh or from the freezer) are weighed and then suspended in potassium phosphate, 0.1 *M* pH 7.25, at a 1 to 10 (w/v) ratio. A homogeneous suspension can be achieved, along with partial cell disruption, by means of a glass homogenizer, or mortar and pestle. The resultant suspension is then passed through a French press (20,000 psi) and repeated if necessary. If a French press is not available, the cells may be disrupted by prolonged hand grinding in the presence of fine silica sand. Presumably the cells can be disrupted by sonification; however, this method did not prove satisfactory in this laboratory. The resultant homogenate is centrifuged at 2000 × *g* for 20 min and the precipitated cell debris discarded. The supernatant is recentrifuged at 20,000 × *g* for 40 min, and the resultant supernatant is discarded. The precipitate which contains the dissimilatory nitrate reductase is resuspended in 5 volumes of potassium phosphate (0.1 *M*, pH 7.25) for each unit of weight of fresh wet cells (initial harvest). A glass homogenizer is used to facilitate resuspension. Since variation in activity exists from batch to batch, it is desirable to determine the proper dilution (activity) of each enzyme preparation before subdividing (2.0 ml per tube) and storage at −15° C. The dilution is based on assays of the original solution and a series of dilutions ranging from 1 to 2, to 1 to 10. Based on the results of these assays, the desired dilution of the entire batch is made so that 0.05 ml of enzyme will quantitatively reduce, in a linear manner, 4.0 μg of NO_3^--N per assay tube under the standard assay conditions.

Assay: The reaction mix for 40 assays is made as follows:

		ml
Potassium phosphate	0.1 *M* pH 7.25	17.0
Sodium formate	1.0 *M*	41.0
Enzyme solution		2.0

After mixing, 1.5 ml of the mixture is pipetted (automatic pipette is recommended) into each tube. The desired standard or sample containing from 0.25 to 2.5 μg of NO_3^--N is then pipetted into each tube, and the volume adjusted to 1.7 ml with water and mixed. After 4 hr of incubation at 45° C, the reaction is stopped by pipetting (automatic pipette) into each tube 2.0 ml of reagents for diazotization of the nitrite. The freshly mixed diazotization solution is composed of equal volumes of 1% (w/v) sulfanilamide in 1.5 *N* HCl and 0.02% (w/v) *N*-naphthylethylene diamine dihydrochloride. Each tube is then diluted to 10.0 ml and allowed to stand at room temperature for 15 min before reading at 540 mμ. The final volume (1.7 ml) of the reaction mixture and the final volume (10.0) after color development were chosen for convenience and may be altered.

Experimental Data. The effect of temperature on the enzymatic reduction of nitrate to nitrite is shown in Figure 1. Each tube in this experiment contained 1.25 μg NO_3^--N. The higher temperature was chosen for assay because it tends to speed up the reaction slightly, and was not detrimental to the enzyme.

The effects of incubation time at 45° C and the sodium formate concentrations on the reduction of nitrate to nitrite are shown in Figure 2. From these data it was concluded that 0.6 *M* formate in the complete reaction mix and 4 hr of incubation were optimal for the widest range of nitrate concentrations in sample or standard. Although the data show that the nitrate concentration in standard or sample could range from 0.25 (or less) to 5.0 μg NO_3^--N, an upper limit of 2.5 μg NO_3^--N is suggested as most suitable.

The plant extracts used for assay (Table I) were prepared by macerating fresh leaf material with a homogenizer (MSE Inc., 811 Sharon Drive, Westlake, Ohio) for 3 min, with water as

Table I. Nitrate Assays of Leaf Material from Two Plant Species and the Recovery of a Standard Amount of Nitrate Added to the Samples

Plant Material	Assays: aliquot (ml)	Assays: NO_3^--N (μg/tube)	Assays: NO_3^--N (μg/tube)[a]	Recovery (%)	NO_3^--N content of tissue (μg/g dry wt)
Corn leaves (young)	0.05	0.086	1.326		
	0.05	0.086	1.305	100	540
	0.05	0.086	1.378		
	0.10	0.185	1.404		
	0.10	0.177	1.378	98	565
	0.10	0.177	1.417		
Corn leaves (mature)	0.15	0.083	1.305		
	0.15	0.070	1.326	99	27
	0.15	0.086	1.362		
Dandelion leaves	0.01	0.785	2.106		
	0.01	0.775	2.015	105	20,400
	0.01	0.801	2.106		
	0.05	3.822	4.680		
	0.05	3.588	4.810	92	18,700
	0.05	3.718	5.070		

[a] In addition to sample aliquot, 1.25 μg NO_3^--N, as KNO_3, was added to each tube.

the extractant. The ratio of fresh tissue (w/v) ranged from 1 to 5 (mature corn leaves) to 1 to 30 (young corn and dandelion leaves). The clarified supernatant liquid was used for assay. Plant or soil extracts for assay can also be prepared as described by Woolley *et al.* (1960) or Lowe and Hamilton (1967).

The data of Table I show the reproducibility and range of the assay and the recovery of added nitrate. The low recovery obtained with the 0.5 ml aliquot of the dandelion extract is due to the high amount of nitrate in the sample. The enzyme used in these experiments was from a different preparation than used for the experiments shown in Figure 2. This particular preparation was unable to reduce completely this amount of nitrate in a 4 hr period. Complete recoveries (20,350 μg NO_3^--N/g dry wt) were obtained by increasing the incubation time to 5 hr. These data show that by appropriate selection of extraction volumes and aliquot size, tissue ranging from 27 to 20,400 μg NO_3^--N can be assayed satisfactorily.

ACKNOWLEDGMENT

This manuscript is a contribution from the Agronomy and Plant Pathology Departments, University of Illinois, Urbana-Champaign, Ill. 61801, and supported in part by USDA Cooperative Agreement 12-14-100-8455 and Hatch funds. The *E. coli* strain B was provided by Lowell Hager, Biochemistry Department, University of Illinois, Urbana-Champaign Campus.

LITERATURE CITED

Fewson, C. A., Nicholas, D. J. D., *Biochim. Biophys. Acta* **48**, 208 (1961a).
Fewson, C. A., Nicholas, D. J. D., *Biochim. Biophys. Acta* **49**, 335 (1961b).
Lowe, R. H., Hamilton, T. L., J. Agr. Food Chem. **15**, 359 (1967).
Ruiz-Herrera, J., DeMoss, J. A., *J. Bacteriol.* **99**, 720 (1969).
Taniguchi, A., Itazaki, E., *Biochim. Biophys. Acta* **31**, 294 (1959).
Taniguchi, A., Itazaki, E., *Biochim. Biophys. Acta* **44**, 263 (1960).
Woolley, J. T., Hicks, G. P., Hageman, R. H., J. Agr. Food Chem. **8**, 481 (1960).

Received for review July 30, 1970. Accepted November 23, 1970.