Rapid Method for Determination of Nitrate in Plant and Soil Extracts

R. H. LOWE AND J. L. HAMILTON

A rapid automated method for determination of nitrate in plant and soil extracts is described. The method utilizes soybean nodule bacteroids for reduction of nitrate to nitrite. A portion of the nitrite is removed from the sample and bacteroids by continuous flow dialysis and is measured colorimetrically. The method is sensitive to as little as 0.01 μg. of nitrate nitrogen per ml. in the sample solution and is adaptable to extremely wide ranges of nitrate concentrations. No purification of either plant or soil extracts is necessary. The bacteroids possess an extremely active nitrate reductase and utilize succinate as an exogenous electron donor. No sterile techniques or aseptic culturing procedures are necessary. One 10-inch pot of well-nodulated soybeans will produce sufficient bacteriods for 300 to 500 nitrate analyses.

Numerous chemical methods for determination of nitrate have been described in the literature. None of these, however, have been very satisfactory for determination of nitrates in soil and plant extracts because of the large amounts of interfering substances these extracts may contain. Usually, nitrate is determined after chemical reduction to nitrite. However, the usual reducing reagents are not sufficiently specific, and it is normally difficult to obtain quantitative reduction of nitrate to nitrite.

Nason and Evans (*3*) suggested that the enzyme, nitrate reductase, may be used in analytical procedures to reduce nitrate to nitrite. The enzyme is highly specific and the reduction is quantitative. Garner and coworkers (*1*) showed that intact microbial cells would carry out the reduction, thereby avoiding the tedious purification, preservation, and use of the rather unstable cell-free enzyme. Hill *et al.* (*2*) improved this procedure by using an organism that grows on a nitrate-free medium and does not form a nitrite reductase. However, both these procedures possess at least two serious limitations. Aseptic techniques and procedures for maintaining and culturing the microorganisms are tedious and time consuming. Further, long periods of incubation (1.5 to 5 hours) are required for the reduction of nitrate because these organisms utilize an endogenous electron donor (probably reduced pyridine nucleotide) which must be produced by the organisms during the incubation period.

The present paper describes a simple automated procedure which utilizes soybean nodule bacteroids for reduction of nitrate to nitrite and its subsequent quantitative determination. The bacteroids possess an extremely active nitrate reductase and utilize an exogenous supply of succinate as electron donor.

Materials and Methods

Culture of Soybeans. Soybeans, *Glycine max* (L.) Merr. var. Lee, were grown in a warm greenhouse (30–35° C.) in 10-inch paper pots (Western Pulp Prod. Co., Corvallis, Ore.) containing a 2-to-1 mixture (v./v.) of soil and horticulture grade Perlite (a soil conditioner added to improve aeration of the soil). Soil with a low nitrate content was used, and calcium hydroxide was added to bring the soil pH between 6.5 and 7.0. About one week after seeding, the plants were inoculated by applying a suspension of approximately 3 grams of a commercial nitrogen-fixing inoculum in 2 liters of water to the top of each pot. The plants were watered from the bottom by placing them in trays in which a nitrogen-deficient nutrient solution was maintained to a depth of about one-half inch. The nutrient solution contained the following components per liter: K_2HPO_4, 87 mg.; KH_2PO_4, 68 mg.; K_2SO_4, 258 mg.; $MgSO_4 \cdot 7H_2O$, 246 mg.; iron sequestrene (E. C. Geiger, North Wales, Pa.), 2.5 mg.; H_3BO_3, 0.72 mg.; $MnSO_4 \cdot H_2O$, 0.38 mg.; $ZnSO_4 \cdot 7H_2O$, 0.11 mg.; $CuSO_4 \cdot 5H_2O$, 39 μg.; $Na_2MoO_4 \cdot 2H_2O$, 12 μg. During the winter months supplemental light was used which supplied approximately 800 foot-candles at 2 feet from the light source.

Collection of Bacteroids. Five to 8 weeks after seeding, nodules were removed from the roots and immediately placed in cold distilled water. They were washed repeatedly in cold distilled water, blotted dry, and macerated with a cold mortar and pestle in 0.1*M* potassium succinate at pH 6.8 (5 ml. per gram of nodules). The resulting slurry was squeezed by hand through two layers of cheesecloth and then the liquid portions were centrifuged at 5000 × G for 5 minutes. Sediments containing the bacteroids were resuspended in the original volume of 0.1*M* potassium succinate at pH 6.8 with the aid of a stirring rod. After recentrifuging as described above, the sediments were resuspended with the aid of a Ten Broeck homogenizer in the original volume of 0.1*M* potassium succinate at pH 6.8. The suspension was placed in a small suction flask fitted with a rubber stopper through which was inserted a glass capillary tube to facilitate replacing the air in the flask with nitrogen. This stock suspension of bacteroids was placed under nitrogen and stored in an ice bath in a refrigerator until ready for use. This stock suspension is usable for 2 to 4 weeks if kept at

Crops Research Division, Agricultural Research Service, University of Kentucky, Lexington, Ky.

0° C. and free of atmospheric oxygen. The bacteroids may be recovered and used two to four times with about a 50% loss in activity.

To determine the extent to which the stock suspension of bacteroids should be diluted for use, nitrate reductase activity of the suspension was assayed in a reaction mixture containing: 40 μmoles of $NaNO_3$, 40 μmoles of potassium succinate at pH 6.8, and 0.1 ml. of the bacteroid suspension in a final volume of 2 ml. The reaction mixture was incubated for 10 minutes at 45° C. The reaction was stopped by adding 0.5 ml. of each of the diazo-coupling reagents [1% sulfanilamide in 3*N* HCl and 0.01% *N*-(1-napthyl)ethylenediamine hydrochloride]. The absorbance in a 1-cm. light path was determined after 10 minutes, using a Gilford spectrophotometer at a wavelength of 540 mμ. A duplicate reaction mixture stopped at zero time was used as a reference solution. The following formula was used to determine the dilution of the working suspension of bacteroids:

Ml. of working suspension =

absorbance × ml. of stock suspension × 10

The absorbance, when determined in this manner, was usually between 1 and 2. Suspensions were not used when the absorbance fell below 0.5. If the spectrophotometer being used is not accurate at high absorbances, the suspension should be diluted to an absorbance between 0.5 and 1.0 and the formula adjusted accordingly. The working suspension of bacteroids was made to a potassium succinate concentration of 0.02*M* and placed under a nitrogen atmosphere.

Apparatus. The analytical system consists of the modules of a Technicon *Auto*Analyzer illustrated in Figure 1. The sampling rate was 30 per hour with a 2-to-1 sample-to-wash ratio. The time-delay coil was 20 feet long by 2 mm. in i.d. The time-delay coil and mixing coil were placed in the dialyzer bath which was maintained at 45° C. The dialyzer is comprised of a matching pair of plastic plates whose mating surfaces are mirror grooved to provide two continuous channels separated by a cellophane membrane sandwiched between the two plates. It serves to remove a portion of the nitrite from the bacteroids and sample. The colorimeter flow cell had a 15-mm. light path and absorbance was measured at 533-mμ wavelength. Nitrogen was used to segment the incubation stream because atmospheric oxygen competes with nitrate for electrons from the succinate. The same nitrogen line is attached to the working suspension of bacteroids and is kept at a pressure of approximately $^1/_4$ pound per square inch. The bacteroids are stirred gently with a magnetic stirrer to keep them suspended.

Sample Preparation. Nitrate was extracted from 0.2 gram and 1 gram of burley and flue-cured tobacco, respectively, by shaking vigorously for 30 minutes in a 25 × 200 mm. test tube with 50 ml. of 0.1% Brij-35 (Atlas Powder Co., Wilmington, Del.). Fresh tobacco leaves were extracted by sonifying 5 grams of leaf tissue in 200 ml. of 0.1% Brij-35 for 5 minutes on a Branson Sonifier at full intensity. Soil was extracted by vigorously shaking 10 grams of dry soil for 30 minutes in a 500-ml. Erlenmeyer flask with 200 ml. of 0.1% Brij-35. Brij-35 is a detergent added to facilitate uniform flow through the *Auto*Analyzer. The extracts were filtered through Whatman No. 1 filter paper and an aliquot diluted to a nitrate-nitrogen concentration of 1 to 3 μg. per ml.

Results and Discussion

Figure 2 shows the relationship between nitrate concentration in standard solutions of KNO_3 and the absorbance recorded by the analyzer. The relationship is linear over a wide range of concentrations. The procedure is sensitive to as little as 0.01 μg. of nitrate nitrogen per milliliter in the sample solution. The sensitivity of the system can be increased approximately fivefold by switching tubes for the sample and bacteroids. This necessitates using a more concentrated suspension of bacteroids because of the dilution effect when the sample is pumped through the larger tube. Samples high in nitrates can be diluted automatically on the proportioning pump.

Nitrate was added to extracts of several plant and soil samples. Table I shows the recovery of nitrate. The samples of flue-cured grades X4L and B4R contained 0.0023 and 0.0030% nitrate nitrogen, respectively, in the leaf tissue. Flue-cured tobacco has high levels of substances (pigments, sugars, etc.) that usually cause interference with chemical procedures for nitrate. However, even with these extremely low levels of nitrate in the leaf tissue, the recovery of nitrate added to extracts of this tissue was excellent.

Very good precision was obtained with this method of analysis. The data in Table II show the reproducibility which can be obtained when a sampling rate of 30 per hour is used. Slightly better precision can be obtained with a sampling rate of 20 per hour. However, in most instances the slight improvement in precision would not warrant the loss of time necessary for the slower analysis rate.

The method described allows quantitative determinations of nitrate in the plant and soil extracts used. Tobacco was selected for the recovery studies because nitrate determinations on tobacco have been difficult owing to large amounts of interfering substances in tobacco extracts, especially in flue-cured tobacco extracts. The reduction of nitrate to nitrite is quantitative and extremely rapid. The bacteroids for the reduction can be obtained easily in large quantities and no aseptic procedures are necessary. One 10-inch pot of well-nodulated soybeans will produce sufficient nodules for 300 to 500 nitrate analyses. Thus, one or two pots seeded each week will supply sufficient bacteroids for the nitrate analysis needed by most laboratories.

The method is simple and easily carried out by technicians. However a few criteria must receive careful attention if accurate and reproducible results are to be obtained. Night temperature of the greenhouse in which the soybeans are grown should not fall below 75° F. if a high level of nitrate reductase is to be obtained, and it is desirable that the temperature be maintained above 80° F. Soil with a low level of

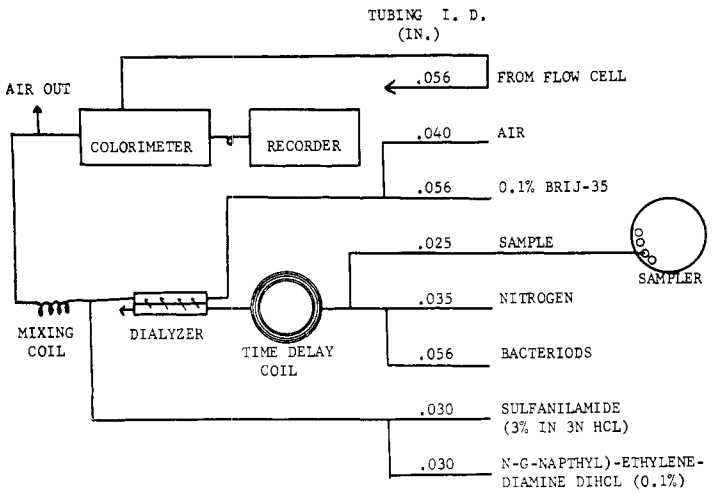

Figure 1. Flow diagram of apparatus used in automated method for determining nitrate

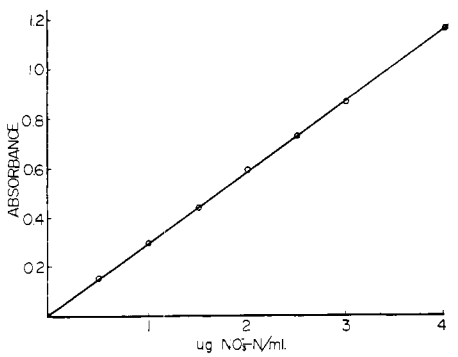

Figure 2. Relation of absorbance to concentration of nitrate

Table I. Recovery of Nitrate Added to Plant and Soil Extracts

Sample	NO_3^--N, µg./Ml. Analyzed[a]	Added	Total recovered[a]	Recovery, %
Burley				
N1L	1.26	1.10	2.37	100
T4R	1.32	1.10	2.44	101
Leaf composite	1.23	1.10	2.32	100
Fresh leaf	0.72	1.10	1.81	100
Flue-cured				
X4L	0.46	1.10	1.55	99
B4R	0.59	1.10	1.67	99
Soil	0.50	1.10	1.59	99

[a] Average of three analyses.

nitrate must be used because nitrate inhibits nodulation and formation of the nitrate reductase. A crop of sudan grass will rapidly deplete soil of nitrate if a low-nitrate soil is not available. Tubing and coils through which the reaction mixture and reagents are pumped must be kept clean so that a uniform bubble pattern and flow rate are maintained. Good tubing and coil connections are essential to prevent mixing between samples. Flow rates through the two sides of the dialyzer must be uniform or resolution will be decreased

Table II. Precision of Replicate Determinations of Nitrate in Cured Burley Tobacco Extracts

Sample No.	Nitrate Nitrogen Concn., P.P.M.
1	2.63
2	2.63
3	2.64
4	2.63
5	2.64
6	2.62
7	2.63
8	2.64
9	2.64
10	2.64
11	2.64
12	2.64

and a slower sampling rate will be required. The bacteroids should be passed through the lower side of the dialyzer to prevent plugging of the membrane over long periods of use. If the bacteroids are to be reused, they should be collected in an ice bath, removed from the reaction mixture by centrifuging, washed once with 0.1*M* potassium succinate at pH 6.8, and stored under nitrogen.

For routine analysis, a set of standard solutions of KNO_3 (in 0.1% Brij-35) containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µg. of nitrate nitrogen per ml. is included for each revolution of the sampler tray (40 sample cups per tray). If nitrite is present in the samples, it must be measured and subtracted from the nitrate determinations. Nitrite can be determined separately by use of the analytical system without the bacteroids and the incubation coil.

The soybean nodule bacteroids would be useful in a nonautomated method for nitrate determination similar to that described by Hill *et al.* (*2*). No sterile culture techniques are needed. The extremely high level of nitrate reductase activity and the utilization of succinate as an exogeneous electron donor would enable considerable shortening of the incubation period. Also, it should allow bacteroid suspensions sufficiently dilute that good precision could be obtained without centrifuging at the end of the incubation.

Literature Cited

(1) Garner, G. B., Baumstark, J. S., Muhrer, M. E., Pfander, W. H., *Anal. Chem.* **28,** 1589 (1956).
(2) Hill, R. M., Pivnich, H., Engelhard, W. E., Bogard, M., J. AGR. FOOD CHEM. **7,** 261 (1959).
(3) Nason, A., Evans, H. J., *J. Biol. Chem.* **202,** 655 (1953).

Received for review August 5, 1966. Accepted October 17, 1966. The investigation reported in this paper is in connection with a cooperative project of the Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, and the Kentucky Agricultural Experiment Station, and is published with the approval of both agencies. Presented in part at the 20th Tobacco Chemists' Research Conference, 1966. The mention of commercial products and industrial companies anywhere in this paper does not imply their endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.