Estimation of Nitrogenase Using a Colorimetric Determination for Ethylene

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ABSTRACT
Ethylene is measured by oxidizing it to formaldehyde and determining the formaldehyde colorimetrically. The assay is applied to estimation of nitrogenase in nodulated legume roots by measuring the ethylene produced from acetylene.

In 1966, Dilworth (2) reported that nitrogen-fixing extracts of Clostridium pasteurianum reduced acetylene to ethylene. It was soon apparent that this provided a convenient and sensitive assay of nitrogenase, for ethylene and acetylene are readily separated and determined by gas chromatography. The acetylene reduction assay is now well established in the study of biological nitrogen fixation. Its utility is attested to by its reported use in over 200 publications (3).

Until now gas chromatography has been the only means of measuring ethylene in the presence of acetylene. This publication reports a colorimetric assay for ethylene sufficiently sensitive to estimate nitrogenase in nodulated legume roots. In designing the procedure, stress was placed on economy, safety, and simplicity. All reagents are inexpensive, none are particularly toxic or dangerous to the operator, and no heating steps are necessary.

MATERIALS AND METHODS
Distilled water should be used in making up all reagents. Sodium metaperiodate: 0.05 m NaIO₄, 16.5 g/l; store in a dark bottle for no more than 4 weeks. Potassium permanganate: 0.005 m KMnO₄, 0.79 g/l; store in a dark, well stoppered bottle. A slight precipitate usually forms after 1 to 2 days; if there is much precipitate, use another lot of KMnO₄. Sulphuric acid: 4 N. Sodium arsenite: 4 m, NaAsO₂, 52 g/100 ml. Oxidant solution: 80 ml of 0.05 m NaIO₄, 10 ml of 0.005 m KMnO₄, adjust pH to 7.5 with KOH, dilute to 100 ml. Acetyl acetone (2, 5-pentanediene): purity by distillation. Nash reagent (1, 6): 150 g of ammonium acetate, 3 ml of acetic acid, 2 ml of acetyl acetone diluted to 1 liter.

Procedure. Oxidant solution (1.5 ml) is placed in a 10-ml conical flask which is then sealed with a rubber serum bottle cap. From 1 to 5 ml of gas containing up to 1 μmole of C₂H₄ are transferred by syringe from a chamber in which plant roots are incubated with C₂H₄. If necessary, some of the atmosphere in the conical flask is removed first with a syringe. The conical flask is agitated vigorously on a rotary shaker at 300 rpm for 90 min at room temperature (22°C). One-fourth ml of 4 m NaAsO₂ and one-fourth ml of 4 N H₂SO₄ are added, mixing to destroy excess oxidant. One ml of Nash reagent is added, and the absorbance at 412 nm is determined after 60 min. Standards containing known amounts of C₂H₄ are carried through the analysis at the same time as the samples.

RESULTS
The absorbance of the final solution is proportional to ethylene from 0.1 μmole to 1 μmole per sample. This sensitivity permits the estimation of nitrogenase activity of peas as early as 3 weeks after planting. The nitrogenase activity of field peas shows the typical exponential increase until pod filling starts when there is a rapid drop (Fig. 1). The ethylene concentrations are the same as those determined by gas chromatography.

Fig. 1. Field peas, grown in a greenhouse with nitrogen-free nutrient medium, were harvested at 9:00 AM. Individual roots were sealed in 140-ml jars, and 3 ml of C₂H₄ were injected through a serum bottle stopper. After 30 to 90 min, 1 to 5 ml of gas phase were removed for analysis as described in the text (●). One ml was analyzed by gas chromatography (▲). Each point is the average of four plants. Plant nitrogen (○) was determined with a Hewlett-Packard CHN analyzer.
DISCUSSION

Chemistry of the Reaction. Although their chemical properties are otherwise similar, acetylenes are not as readily oxidized as olefins. Moreover, the resistance to oxidation by acetylenes increases with decreasing chain length, and acetylene is oxidized only slowly.

Olefins are oxidized by permanganate to vicinal diols or ketols (9), and the decolorization of a permanganate solution is an established test for unsaturated compounds. Vicinal diols are cleaved by periodate (the Malaprade [5] reaction) to acids and aldehydes. This reaction has been used extensively for characterizing carbohydrates by the nature and amounts of products produced. Lemieux and von Rudloff (4, 7, 8) combined the reagents to hydroxylate and cleave olefins in one step. They noted that periodate regenerated permanganate from the hypomanganate produced in the first step of the reaction, and that permanganate was therefore necessary only in catalytic amounts.

In the case of ethylene, the probable first products of oxidation are ethylene glycol and some glyxol. These are oxidized to formaldehyde, formic acid, and CO₂. Because sensitive assays exist for formaldehyde, even low levels of ethylene can be detected.

The Nash (6) assay for formaldehyde is based on the Hantzsch reaction between acetylacetone, ammonia, and formaldehyde which form 3,5-diacetyl-1,4 dihydrolutidine. The reaction is specific for formaldehyde; the product formed with acetaldehyde absorbs only slightly at 412 nm.

Acetylene is oxidized by permanganate, but does not yield formaldehyde. Excess acetylene will interfere with the reaction by reducing the amount of permanganate available for oxidation of ethylene. In the procedure described here, more than 0.3 ml of acetylene interferes with the estimation of ethylene.

Specificity and Sensitivity. The permanganate-periodate reagent will yield formaldehyde not only from ethylene but from compounds with a terminal olefin group. However, such compounds are not expected to appear in the gas phase from roots. Nitrogenase is the only biological agent known which converts acetylene to ethylene.

Periodate will yield aldehydes from many naturally occurring compounds, including most carbohydrates. Even 1 μg of mannitol, for example, will yield detectable amounts of formaldehyde. Therefore, the operator must take care not to introduce into the oxidant any nongaseous matter from his sample. Glassware used in the assay must be clean and should be washed and stored separately from other laboratory equipment.

The sensitivity of the assay is sufficient to measure nitrogenase activity over most of a legume’s growth. Although we have not used it for free living nitrogen fixing bacteria, it should detect nitrogenase in actively growing cultures. However, it would not be sensitive enough to measure fixation by algae in lake water or bacteria in unamended soils. The low levels of ethylene produced by plants cannot be detected by this procedure.

Time and pH. Formaldehyde is destroyed slowly by the oxidant, and oxidation for more than 90 min results in a decrease in absorbance of the final mixture. The nature of the products and the speed of oxidation varies with conditions of temperature and pH. In agreement with Lemieux and von Rudloff (4, 7, 8), we found that a slightly alkaline pH produced a good compromise between speed and sensitivity.

Speed. We have analyzed 40 plants per operator day. This compares to 80 samples per day using a gas chromatograph.

Reagents. All chemical reagents used are readily available and stable in storage. With the exception of 2,4-pentanediene, chemicals were sufficiently pure for the analysis. If necessary, a recrystallization should remove interfering impurities. The calculated cost of reagents is $6.00 (U.S.) per 1000 assays.

The procedure described is not as sensitive, rapid, or convenient as the gas chromatographic assay. However, it has the advantage of economy and should find use in laboratories which do not have gas chromatographs or where the need for only an occasional nitrogenase assay does not warrant setting up gas chromatograph facilities.

Modifications of this procedure will no doubt occur to users. Moreover, the extensive literature on the chemistry of ethylene may suggest alternative procedures for colorimetric determinations.

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LITERATURE CITED