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Acetylene Reduction Assay for Nitrogenase Activity: Gas Chromatographic Determination of Ethylene Per Sample in Less Than One Minute

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Ammoniacal silver nitrate (10 mg/ml) was added to terminate acetylene reduction assays used to measure nitrogenase activity. Silver nitrate quantitatively precipitated acetylene as the carbide salt, but did not affect the ethylene formed. The vials containing ethylene can be analyzed gas chromatographically at the rate of about 13 samples in 10 min.

The use of nitrogenase-catalyzed reduction of acetylene (C_2H_2) to ethylene (C_2H_4) (2; R. Scholhorn and R. H. Burris, Fed. Proc. **25**:710, 1966) as a sensitive and simple assay to measure biological nitrogen fixation (5, 8) has contributed greatly to progress of research in this area. The biochemical basis of the assay and its application to laboratory and field measurements of nitrogenase activity have been examined and reviewed in detail (1, 3, 4, 7). Although the gas chromatographic C_2H_2 reduction assay is quite convenient because of the high ratio of C_2H_2 to C_2H_4 present, injection of subsequent samples has to wait until all of the C_2H_2 is purged from the column. In this communication we describe a method based on the ability of certain chemicals such as ammoniacal silver nitrate to precipitate acetylene quantitatively, thereby facilitating faster gas chromatographic analysis of samples. Ammoniacal silver nitrate also acts as the agent to terminate the reaction. The modified procedure enables the determination of the ethylene formed per sample in less than a minute.

Aqueous silver nitrate (0.50 g in 5 ml of water) was made ammoniacal by adding ammonia solution (25% [wt/wt] NH_3) dropwise until the precipitate which formed just dissolved. The solution was then made up to 25 ml with water and stored in a dark, cool place. Ammoniacal cuprous chloride was made by dissolving 0.50 g of the freshly prepared salt in minimum volume of ammonia solution and then diluting the solution to 25 ml with water. Mercuric acetate (0.5 g in 15 ml of water) was made up to 25 ml with ammonia solution. Appropriate amounts of these stock solutions were added to yield the final concentrations specified in the various experiments described here. All the chemicals were used at the highest purity available from the British Drug House, Poole, England. The gases were from Indian Oxygen Ltd., Bombay, India, except standard C_2H_4 which was obtained from Matheson Gas Products, Rutherford, N.J. Gas samples were analyzed with a gas chromatograph (type RL04, Toshniwal Instruments, Bombay, India) fitted with flame ionization de-

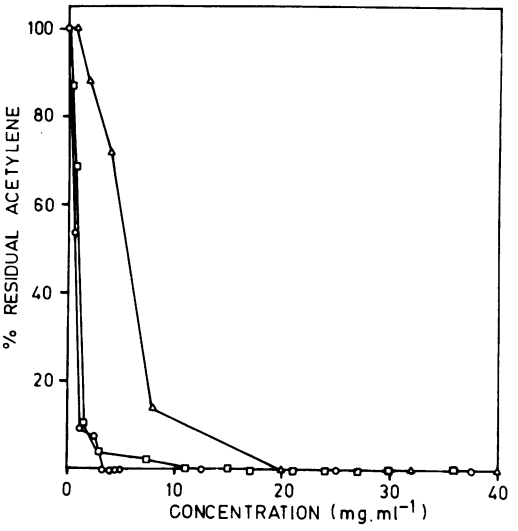

FIG. 1. *Precipitation of acetylene with different concentrations of ammoniacal silver nitrate* (○), *cuprous chloride* (□), *and mercuric acetate* (△). *A 1-ml solution containing appropriate final concentration of the salt was treated with 0.1 atm of acetylene (in air) in a 5-ml sealed vial for 15 min with constant shaking. For convenience, the gas phase from each reaction vial was then transferred to another sealed preevacuated vial and analyzed by gas chromatography. Alternatively, the gas sample from individual vial was withdrawn at the end of a 15-min treatment with the salt and injected directly into the gas chromatograph. Operating conditions for the gas chromatograph were as follows: Column—Porapak T, 6 feet by ⅛ inches (ca. 183 by 0.3 cm); Nitrogen as carrier gas at a flow rate of 30 ml·min^{-1}; Temperatures—column, 60°C; Injection port, 110°C; Flame ionization detector, 120°C.*

tector. Nitrogenase was assayed as described before (8) with the cyanobacterium *Anabaena* L-31. In addition, nodules excised from a local variety of peanut (*Arachis hypogea* var. TG-1) were incubated for 30 min at 25°C in 5-ml serum vials containing a gas phase of air and C_2H_2 (0.1 atm) to determine nitrogenase activity.

Upon treatment with the ammoniacal salt solutions, C_2H_2 immediately precipitated as the carbides of the respective metals. C_2H_2 could be regenerated in toto by the addition of HCl to these precipitates. On a milligram basis, effectiveness of these salts was in the following descending order: Ag > Cu > Hg. The minimum concentrations of silver nitrate, cuprous chloride, and mercuric acetate required to quantitatively precipitate all the C_2H_2 were 3, 5, 17, and 20 mg/ml, respectively (Fig. 1). However, cuprous chloride was unstable, being susceptible to oxidation, and was therefore not tested further. The times required by silver nitrate and mercuric acetate to precipitate all the C_2H_2 quantitatively were found to be 2 and 10 min, respectively (Fig. 2).

Considerable reduction in the time for analysis was obtained when the above procedure was used for the routine C_2H_2 reduction assay for measuring cyanobacterial nitrogenase activity (Fig. 3). Under the operating conditions (mentioned in the legend to Fig. 3) the retention times for C_2H_4 and C_2H_2 were 30 and 46 s, respectively. Because of the large quantity of C_2H_2 (0.1 atm) normally used in such assays, its complete elution is considerably delayed. For instance, the time required for analysis of one cyanobacterial sample was found to be 6 min (Fig. 3A). Treatment with ammoniacal silver nitrate reduced this time to less than 1 min (Fig. 3B). Similar results were obtained with suitable concentrations of ammoniacal cuprous chloride and mercuric acetate. Significantly, treatment for 3 h with any of these salts did not interfere with the C_2H_4 peak which could be accurately reproduced even at the low concentration of 0.25 nmol.

Treatment of *Anabaena* with the salt solutions resulted in immediate cell lysis (as revealed by microscopy), and further incubation in light did not result in increased C_2H_4 production. Injection of 1 ml of ammoniacal silver nitrate (10 mg) to vials containing excised peanut nodules also terminated the C_2H_2 reduction activity immediately. The salt solutions can therefore be conveniently used as agents to terminate the assay, thus replacing the trichloroacetic acid used commonly. The samples can also be analyzed conveniently, and there is no need to transfer the gas phase.

The time required for analysis of a sample in the C_2H_2 reduction assay has been reported to be 3 to 6 min (7). With the present procedure,

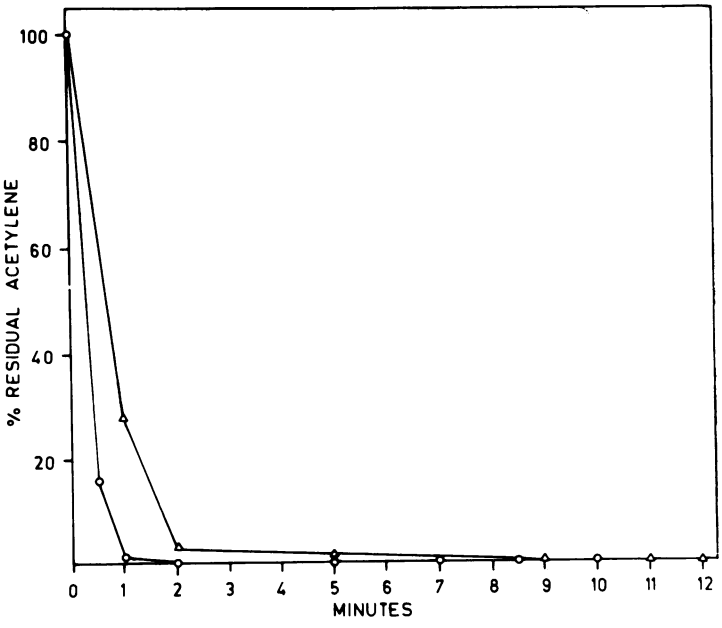

FIG. 2. *Time course of acetylene precipitation with ammoniacal silver nitrate (final concentration 10 mg·ml^{-1} (○) or ammoniacal mercuric acetate (final concentration, 40 mg·ml^{-1}) (△). After injecting the ammoniacal solutions into the vials, the gas phase was transferred to preevacuated vials at the indicated time intervals. Other details as in legend to Fig. 1.*

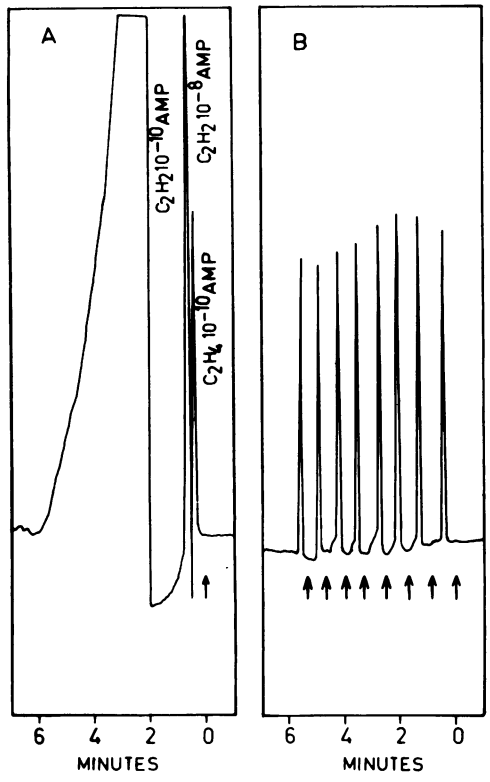

FIG. 3. *Gas chromatograph tracing of acetylene reduction assays performed with Anabaena L-31. Algal suspensions (2 ml) were incubated with 0.1 atm of acetylene (in air) in 5-ml sealed vials on a rotary wheel (11 rpm) at 5,000 lux and 25°C. After 30 min, ammoniacal silver nitrate solution was added to one set of vials (B) to give a final concentration of 10 mg·ml^{-1}. An equal volume of distilled water was added to the control vials (A). After mixing the contents for 5 min, portions of the gas phase from each vial were withdrawn and analyzed with a gas chromatograph. Alternatively, the entire gas phase from each vial was transferred to another preevacuated vial for later gas chromatographic analysis. The algal samples could then be examined immediately for cell lysis. Gas samples in the silver nitrate-treated vials (B) show only peaks for ethylene formed (approximately 0.25 nmol). Arrows indicate time of injection of samples. In (A) ethylene peak was measured with electrometer amplifier set at 10^{-10} A. The acetylene peak was measured at 10^{-8} A for the first 2 min and then at 10^{-10} A.*

analysis can be done at the rate of one sample in less than 1 min. The method would be of great advantage, especially when large numbers of samples have to be analyzed. When dry, metal carbides are known to be unstable and may explode by heat or shock (6). We did not find this property hampering the analysis in any way since the precipitated carbides were in extremely small quantities and were always in liquid suspension. No special precaution was found necessary. Nevertheless, it may be desirable to treat the vials with dilute mineral acid (thus releasing C_2H_2) before disposal.

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