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Quantification and Removal of Some Contaminating Gases from Acetylene Used to Study Gas-Utilizing Enzymes and Microorganisms

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Acetylene generated from various grades of calcium carbide and obtained from commercial- and purified-grade acetylene cylinders was shown to contain high concentrations of various contaminants. Dependent on the source of acetylene, these included, at maximal values, H_2 (0.023%), O_2 (0.779%), N_2 (3.78%), PH_3 (0.06%), CH_4 (0.073%), and acetone (1 to 10%). The concentration of the contaminants in cylinder acetylene was highly dependent on the extent of cylinder discharge. Several conventional methods used to partially purify cylinder acetylene were compared. A small-scale method for extensively purifying acetylene is described. An effect of acetylene quality on acetylene reduction assays conducted with purified nitrogenase from *Azotobacter vinelandii* was demonstrated.

The effects of acetylene on microbial enzymes involved in gas metabolism are numerous and highly diverse in their modes of action. These effects have made the use of acetylene a versatile tool in studies of microbial physiology, ecology, and enzyme mechanisms.

The most common biological application of acetylene is as an alternative substrate for nitrogenase. This reaction forms the basis of the acetylene reduction assay for nitrogenase (6). The simplicity, high sensitivity, cost effectiveness, and general applicability of the acetylene reduction assay to highly diverse nitrogen-fixing systems have contributed to make this assay a standard means of estimating apparent rates of biological nitrogen fixation worldwide. Also of considerable importance is the increasing use of acetylene as an inhibitor of nitrous oxide reductase. This inhibition forms the basis of the acetylene blockage assay used to estimate denitrifying activity. This assay has been successfully applied to a wide variety of denitrifying systems (9, 16, 20, 23). In addition to the well-characterized inhibitions of N_2 and N_2O reduction, acetylene also inhibits a variety of other microbial gas-utilizing processes. Acetylene acts as a suicide substrate of both ammonia monooxygenase in the nitrifying bacterium *Nitrosomonas europaea* (7) and the methane monooxygenase of the methanotroph *Methylococcus capsulatus* (Bath) (14). Acetylene irreversibly inhibits methanogenesis (11) in addition to reversibly inhibiting the growth of hydrogen-utilizing sulfate-reducing *Desulfovibrio* species (13).

The results presented in this paper arose from attempts to clarify the confusion which exists concerning acetylene as an inhibitor of the hydrogenase from aerobic nitrogen-fixing microorganisms. The development of a purification process and a detailed analysis of acetylene and its associated contaminants was prompted when it was found that acetylene from various sources was often contaminated with hydrogen. The results of this investigation showed that contamination in acetylene was a far greater problem than was originally suspected and might therefore be of significance in other experiments where acetylene is used as a reagent.

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MATERIALS AND METHODS

H_2, O_2, N_2, and CO were detected with a dual column 8A gas chromatograph fitted with a thermal conductivity detector (Shimadzu, Kyoto, Japan). A 16-ft by ⅛-in. (ca. 4.88-m by 3.1-mm) stainless steel column containing molecular sieve 5A was used at 100°C. The detector was operated at 130°C with a current of 70 mA. Argon was used as carrier gas at a flow rate of 40 ml/min.

CO_2, C_2H_6, PH_3, and H_2S were also quantified with the chromatograph described above. For these gases, a Teflon-coated stainless steel column (6 ft [ca. 1.83 m] by ⅛ in.) containing Porapak T (80-100 mesh; Waters and Associates, Inc., Milford, Mass.) was operated at 50°C. The detector was operated at 120°C with a current of 160 mA. Helium was used as carrier gas at a flow rate of 35 ml/min.

CH_4, C_2H_4, C_2H_2, and C_3H_8 were detected with a Varian Aerograph 1400 gas chromatograph (Varian, Palo Alto, Calif.) fitted with a flame ionization detector. A stainless steel column (6 ft by ⅛ in.) containing Porapak N (80-100 mesh) was used at 75°C. The injection port and detector temperatures were 200 and 230°C, respectively. Nitrogen was used as carrier gas at a flow rate of 40 ml/min.

Acetone, propyne, and ethanol were also detected with the flame ionization gas chromatograph and the conditions described above, except that a column temperature of 150°C and a nitrogen flow rate of 50 ml/min were used.

Each gas was identified by cochromatography with a sample of pure gas. The gas chromatographs were calibrated for each gas with standards generated by making additions of the pure gas to stoppered 160-ml vials which contained a diluent gas. For H_2, O_2, N_2, CO, and CO_2 the diluent gas was argon (99.998%); for CH_4, C_2H_4, PH_3, and acetone the diluent gas was air; and for H_2S, ethanol, and propyne the diluent gas was highly purified cylinder acetylene. The acetylene was purified as described in Results. All gases used for calibrations were the highest quality available from either Liquid Carbonics (Chicago, Ill.) or Matheson Scien-

TABLE 1. Concentration of contaminants in acetylene generated from calcium carbide[a]

Calcium carbide source (wt [g])	Gas concn in gas phase (μl/liter)						
	H_2	O_2	N_2	CH_4	C_2H_4	PH_3	Propyne
Baker (1)	214	7,792	15,089	6.9	38.3	600	60.1
(2.5)	176	1,340	4,034	22.1	27.7	425	13.1
(5)	189	151	514	10.7	28.2	391	12
Aldrich (1)	95.5	5,758	20,218	11.4	6.6	461	ND
(2.5)	60.6	648	2,137	21.0	5.8	384	ND
(5)	108	119	419	24.8	5.8	370	ND
Fisher (1)	149	6,758	28,859	13.2	25.4	535	29.6
(2.5)	197	3,332	6,456	20.0	20.8	447	22.7
(5)	137	924	2,588	36.2	20.0	353	5.6

[a] Calcium carbide was added to the lower chamber of a 250-ml gas-generating bottle completely filled with tapwater. After a few seconds, the chamber was sealed with a serum stopper; samples were removed 2 min later. The following gases were not found in acetylene generated in a gas-generating bottle above these limits of detection (microliters per liter): CO_2, 25; H_2S, 35; CO, 15; C_2H_6, 4; C_3H_8, 1; ethanol, 1.5; acetone, 2.5. Other limits of detection (microliters per liter) were as follows: H_2, 2.5; O_2, 10; N_2, 10; CH_4, 1; C_2H_4, 1; PH_3, 10; propyne, 1.5. ND, Not detectable.

tific Inc. (East Rutherford, N.J.). Acetone standards were generated by adding appropriate volumes of acetone-saturated air at atmospheric pressure to stoppered and evacuated 160-ml vials. The pressure in the vial was returned to atmospheric pressure by puncturing the stopper with a needle for a few seconds. The vapor pressure of acetone at 26°C was taken as 260 mm of Hg (24).

All gases were quantified by peak area measurements made with a Hewlett-Packard 3390A integrator (Hewlett-Packard Co., Fullerton, Calif.). Gas sample injections were 0.5 ml and were made with a 1-ml gas-tight syringe (Glenco).

Nitrogenase assays. All reactions were carried out at 30°C in stoppered 13-ml serum vials. The reaction mixture (0.5 ml) contained the following components: creatine phosphate (25 mM), creatine kinase (0.1 mg/ml), $MgCl_2 \cdot 6H_2O$ (10 mM), ATP (5 mM), and sodium dithionite (20 mM). *N*-2-Hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid–NaOH (50 mM), pH 7.2 (at 30°C), was used as buffer. The gas phase consisted of 10% acetylene added as an overpressure in the nitrogen-filled vials. Assays were initiated by adding samples of MoFe protein 30 s after the addition of the Fe protein. After 5, 10, and 15 min, 0.1-ml samples were removed, and the ethylene content was measured by gas chromatography as described above. Purified *Azotobacter vinelandii* nitrogenase components were a gift from B. Burgess, Department of Molecular Biology and Biochemistry, University of California, Irvine.

The sources of calcium carbide were J. T. Baker Chemical Co., Phillipsburg, N.J. (Baker grade; lot no. 802335); Fisher Scientific Co., Fair Lawn, N.J. (granular, screened, approximately ¼ to ½ in. [ca. 6.2 to 12.5 mm]; lot no. 721241); and Aldrich Chemical Co., Milwaukee, Wis. (lump technical grade, approximately 80%; lot no. 03108CM).

Purified (99.6%) and commercial grade acetylene cylinders (175 ft^3 [ca. 4.95 m^3]) were obtained from Liquid Carbonics.

RESULTS

Contaminants present in acetylene generated from calcium carbide. The simplest laboratory method for generating acetylene in situ is the contained reaction of calcium carbide added to excess water. Burris described a simplified version of a Kipps apparatus (3) which is now commonly used for this purpose. Throughout this paper, we refer to this apparatus simply as a "gas-generating bottle." Table 1 shows the levels of various contaminants present in this gas-generating bottle after the addition of various amounts of calcium carbide obtained from three major commercial suppliers. The acetylene generated from all weights and grades of calcium carbide contained significant amounts of hydrogen, oxygen, nitrogen, and phosphine and lower concentrations of hydrocarbons. Increasing the weight of calcium carbide used led to a decrease in the concentration of oxygen and nitrogen. This effect is probably due to an increased displacement of trapped and dissolved air with an increasing quantity of acetylene. Apart from oxygen and nitrogen, the remaining gases listed in Table 1 are all products of the water-carbide reaction. In these cases, the relation between the weight of calcium carbide added and resulting contamination level is less clear. The concentration of methane typically increased with increasing amounts of calcium carbide, whereas the concentrations of propyne, ethylene, and phosphine decreased. Note that the concentrations of phosphine present in the gas-generating bottle were the highest of all the non-air contaminants.

Time course experiments showed that the levels of the contaminants listed in Table 1 remained effectively constant for at least 1 h, with the exception of methane. The concentration of methane increased with time, and its rate of formation was directly related to the weight of calcium carbide added. The concentration of methane in day-old acetylene can exceed 100 μl per liter.

Static contaminant levels in cylinder acetylene. An alterna-

TABLE 2. Concentrations of contaminants in commercial and purified (99.6%) cylinder acetylene[a]

Cylinder type	Component concn in gas phase (μl/liter)											
	H_2	O_2	N_2	CO	CH_4	C_2H_4	C_2H_6	C_3H_8	PH_3	Acetone	Propyne	Ethanol
Commercial grade	2,087	65	7,971	ND	732	49	68	222	420	11,377	ND	ND
Purified	2,346	109	37,832	131	65	73	7.4	ND	ND	12,784	3.3	34.2

[a] The first 10 liters of gas was discharged from each cylinder at a rate of 5 liters per min into a stoppered 160-ml vial fitted with a second needle to allow excess gas to escape. After 2 min, the vial was removed, and samples (0.5 ml) were removed for analysis. ND, Not detectable.

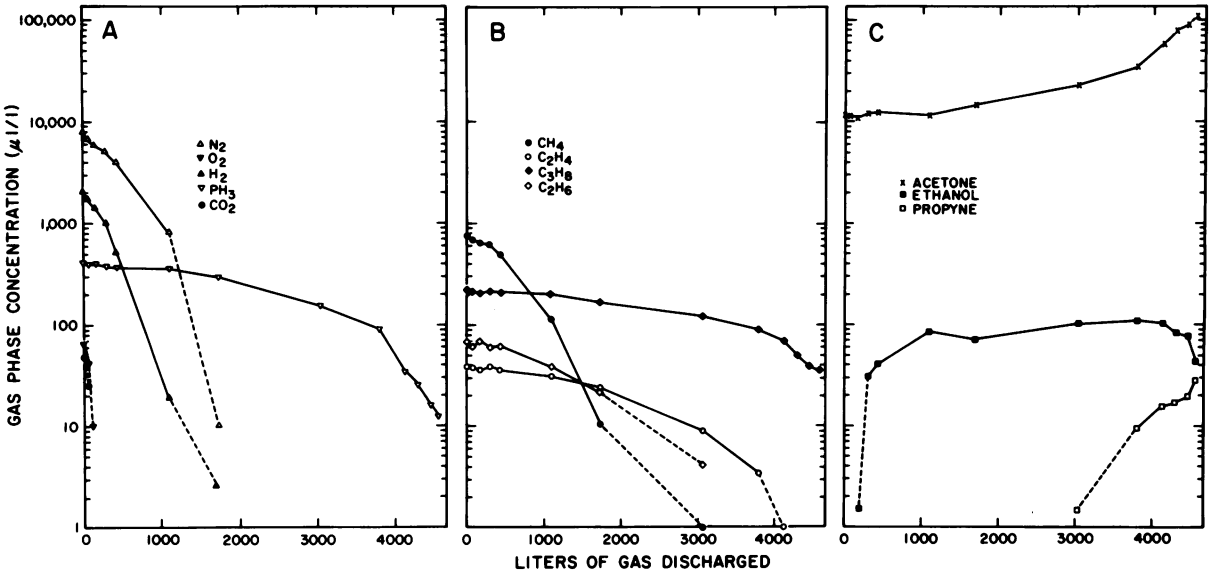

FIG. 1. Changes in the contaminant levels in commercial-grade cylinder acetylene as a function of total discharge volume. Commercial-grade cylinder acetylene was discharged at 5 liters per min for the first 450 liters. After this, the cylinder was discharged at an initial rate of 20 liters/min until 4,600 l had been discharged from the cylinder. Sample vials were filled as described for Table 2. Shown are (A) the inorganic gases, (B) the main hydrocarbon gases, and (C) acetone and the minor hydrocarbons. Dashed lines represent extrapolated results when a given gas concentration fell below detection limits between sampling times.

tive source of acetylene to that generated in the laboratory is commercially manufactured and bottled gas. Commercially produced acetylene is derived either from calcium carbide or from the thermal and electrical cracking of hydrocarbon gases such as methane (10). In both cases the acetylene is usually sold in low-pressure cylinders in the form of a solution in acetone. Table 2 shows the level of several contaminants present after the first 10 liters of gas had been discharged from two randomly selected cylinders of acetylene, one of commercial-grade gas and the other purified (99.6%). The essential differences between the two grades of acetylene were that the commercial-grade gas contained high levels of phosphine in addition to higher levels of the hydrocarbon gases. Both cylinders contained much higher concentrations of hydrogen than the carbide-generated acetylene described in Table 1. Notably, the purified acetylene contained readily detectable levels of carbon monoxide.

Dynamics of contaminant levels in cylinder acetylene. The data presented in Table 2 only provide a static analysis of the initial differences between contaminants present in two differing grades of cylinder acetylene. The laboratory use of a cylinder gas is a dynamic process. For this reason, it is to be expected that the composition of the gas released from the cylinder will change in relation to the total volume of gas released. Figure 1 shows how the composition of the cylinder of commercial grade acetylene described in Table 2 changed with the total volume of gas discharged. Inorganic gases (H_2, O_2, N_2, CO_2) were released most rapidly (Fig. 1A). Hydrocarbon gases (Fig. 1B) were released at a slower rate than the inorganic gases, methane was released most rapidly, and propane was released the least rapidly. Surprisingly, phosphine (Fig. 1A) was released at a similar rate to propane and was still present at readily detectable levels after 4,600 liters (162 ft^3) of gas had been discharged from the cylinder. Not all of the contaminants decreased with increasing discharge volume (Fig. 1C). The ethanol concentration rose sharply over the first 500 liters, slowly increased with further discharge, and then declined again in later samples. Conversely, the propyne concentration increased sharply in later samples, although only to low concentrations. The most dramatic increase occurred with acetone; its concentration started at approximately 1% of the gas phase and rapidly increased in later samples to a level in excess of 10%. Higher concentrations of acetone are known to be released from exhausted cylinders and may reach as high as 18% under certain circumstances (10). Obviously, the results presented in Fig. 1 can be taken only as indicative of the trends to be expected with other cylinders. The variables that may influence the discharge pattern include the purity of the acetylene charged into the cylinder, the initial acetone concentration at that time, the extent of cylinder use, and the temperature at which the cylinder is maintained and used.

Purification of cylinder acetylene. In the past, several authors have described purification schemes designed to remove mainly acetone and phosphine from cylinder acetylene gas streams (3, 6, 22). However, in most cases these reports fail to quantify the efficiency of the methods proposed. Table 3 shows the effects of various common methods employed to remove acetone from acetylene gas streams. The effects of these methods were compared for a cylinder of commercial grade acetylene at an early and late stage of discharge which exhibited a low and high level of acetone contamination, respectively. The sulfuric acid trap was the most efficient means of removing acetone at high or low concentrations (Table 3).

The only other gas removed by the sulfuric acid trap was phosphine (Table 4). In some instances, ethanol and propyne were removed also by this acid treatment, but the results were dependent on the starting concentration of these contaminants. Although not considered in this study, ammonia, another common contaminant in acetylene, would presumably be removed by this treatment (10).

Figure 2 shows the purifying apparatus we use to remove some of the contaminants still present after scrubbing with

TABLE 3. Acetone removal from cylinder acetylene gas streams[a]

Treatment	Acetone concn in gas phase (μl/l)	
	Low acetone	High acetone
No treatment	9,501	50,712
Acetone-dry ice	1,959	22,876
Acetone-dry ice + water trap	321	1,879
Water trap	298	1,751
Sulfuric acid trap	ND	40.1

[a] Commercial-grade cylinder acetylene was discharged at a rate of 3 liters per min through various traps. The acetone-dry ice trap consisted of a glass U tube with a 20-ml collection bulb at the base. The water trap consisted of two 270-ml Milligan wash bottles. The sulfuric acid trap was a 500-ml Dreschel bottle containing 300 ml of concentrated acid. The gas vented from each trap was collected in stoppered, 160-ml vials (see footnote *a* to Table 2). ND, Not detectable.

TABLE 4. Purification of commercial-grade cylinder acetylene by sulfuric acid and cryogenic methods[a]

Gas	Gas concn in gas phase (μl/liter)		
	No treatment	After acid scrubbing	After cryogenic purification
H_2	1,077	1,071	ND
O_2	258	263	ND
N_2	5,401	5,411	ND
CH_4	599	610	12.8
C_2H_4	33.7	34.7	32.4
C_2H_6	67.1	69.4	66.1
C_3H_8	199	206	244
PH_3	370	ND	ND
Acetone	13,676	ND	ND

[a] Commercial-grade cylinder acetylene was discharged at 3 liters per min through the apparatus shown in Fig. 2. ND, Not detectable.

sulfuric acid. After the acid scrubbing, the acetylene is passed through a second trap containing 5 M NaOH in water. This trap removes any acid spray, any sulfur dioxide released from the acid, and any hydrogen sulfide present in the acetylene (10). A replaceable cartridge containing Drierite and soda lime (4-8 mesh) is then used to remove moisture and carbon dioxide. In the final step, the gas is solidified in the purifier, which is partially immersed in liquid nitrogen. During the solidification process, stopcocks 1, 3, and 4 are left open. After the system is operated for 5 min with an acetylene flow rate of 3 liters per min, the gas supply is disconnected by closing stopcock 4. Stopcock 1 is switched from the gas exhaust position to the vacuum pump, and stopcock 2 is opened to a stoppered 160-ml vial through a needle vent. (Any suitable gas collection vessel, including a manifold, can be used; the only requirement is that the apparatus be able to withstand and maintain a vacuum.) The entire apparatus is then evacuated for 5 min. This step serves to remove the noncondensed gases (H_2, O_2, N_2, CO, CH_4). After evacuation, stopcock 1 is closed, and the purifier is removed from the liquid nitrogen to allow the solidified acetylene to sublime. At all stages, the pressure in the system is monitored by the manometer. Overpressures are released through the manometer. Once the system has reached atmospheric pressure, stopcock 2 is closed, and the gas-filled vial can be removed. The sublimation process can be halted by reimmersing the purifier into the liquid nitrogen bath.

A typical purification run under these conditions can generate in excess of 5 liters of purified acetylene; the whole process requires less than 30 min. The results of a typical purification are shown in Table 4. The cryogenic purification step was effective at removing hydrogen, oxygen, nitrogen, and methane. However, it was ineffective at removing ethane and ethylene and, in the case of propane, slightly increased the level of contamination. The increase in propane concentration is due to the similar vapor pressure-versus-temperature profiles for propane and acetylene. For the same reason, it is necessary to remove carbon dioxide, hydrogen sulfide, and phosphine from acetylene before condensing the gas at liquid nitrogen temperatures; otherwise they could be concentrated by the cryogenic purification step.

Effect of acetylene grade on nitrogenase activity. The results presented in Fig. 3 plot the ethylene production by purified *Azotobacter vinelandii* nitrogenase in the presence of 10% acetylene of various qualities. Surprisingly, we observed that acetylene generated from calcium carbide gave consistently higher rates of ethylene production (1,162 nmol/min per mg of MoFe protein) than purified cylinder acetylene (99.6%), whether untreated or cryogenically purified as described above (907 and 850 nmol/min per mg of MoFe protein, respectively). These results are reproducible, and subsequent experiments have enabled us to discount obvious factors which might account for this effect. These include differential effects of acetylene grade on assay pH, absolute acetylene concentration, and differential hydrogen production or inhibition. Also, the apparently selective

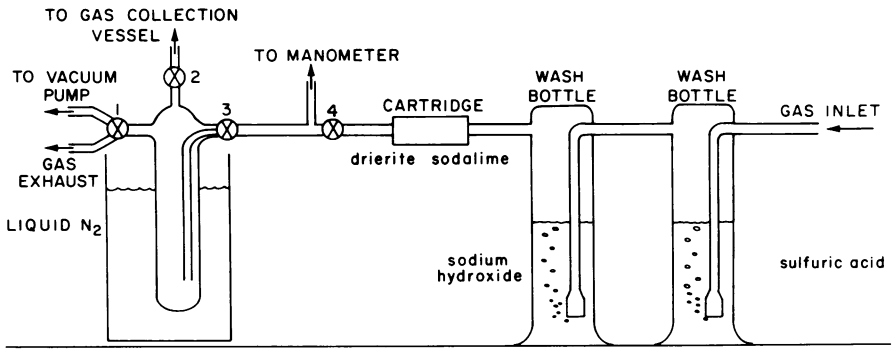

FIG. 2. Cryogenic purification scheme for cylinder acetylene. The train used to purify cylinder acetylene is shown in diagrammatic form. The roles and contents of each component are described in Results.

removal of acetone and phosphine from commercial grade cylinder acetylene by acid scrubbing resulted in lower rates of ethylene production compared with the same gas in the untreated form (data not shown).

DISCUSSION

It is apparent from the results presented in this paper that acetylene from a variety of sources can be heavily contaminated with various gases. Within the field of nitrogen fixation, several authors have stressed the need to use high-purity acetylene in the acetylene reduction assay (6, 22). However, despite the steadily increasing use of acetylene for both the acetylene reduction assay and as an inhibitor of other microbial processes, little attention seems to have been given to the quality of the acetylene used for these purposes. To our knowledge, there is only one report which considers and demonstrates an effect of acetylene quality on an acetylene reduction assay (21). In this work, we also report an effect of acetylene quality on the measured rate of nitrogenase activity (Fig. 3). In contrast to the previous report (21), our acetylene reduction rates were actually higher with the lower-quality acetylene generated from calcium carbide. Although the reason for the different rates is not known, these results demonstrate the need to be aware of the source and quality of acetylene used in acetylene reduction assays of nitrogenase activity.

Given the wide number of effects acetylene is now known to have on microbiological systems, it is impossible to discuss here all the potential ramifications of the impurities shown to be present in acetylene in this study. The effects of a particular contaminant need to be determined for each system and application. It is also to be expected that variations in calcium carbide quality, methods of gas generation, and cylinder gas compositions will be large. For example, hydrogen sulfide and carbon monoxide, both well known as respiratory inhibitors, are two other gases which have been suggested to be present in acetylene generated from calcium carbide (10, 22). We failed to detect either of these gases in any acetylene generated with our gas-generating bottle. However, these gases may be produced in alternative generating systems such as those which operate by the addition of small amounts of water to calcium carbide. In these systems, high localized temperatures and the varied ratio of water to air present may significantly alter the resulting composition of the acetylene produced (10). It is also important to note that the list of contaminants and the sources of acetylene discussed in this paper are not exhaustive. However, two particular contaminants warrant further discussion.

First, the quantity of phosphine found in carbide-generated and commercial-grade cylinder acetylene is a matter for concern for two reasons. The Occupational Safety and Health Administration total weekly allowance for this gas for a 40-h week is a maximal level of 0.3 μl/liter (Material safety data sheet, 074; Matheson Gas Products). Given the levels of phosphine we measured (Tables 1 and 2), care is warranted in the production and use of acetylene. We have found that Kitagawa gas detector tubes (Matheson) are a very effective semiquantitative means of estimating phosphine levels in gas samples and the general laboratory environment. On the other hand, phosphine is an effective metal ligand and can reduce heavy metal salts to the free metal (18). As such, it is possible that some of the effects of acetylene on metalloenzymes presently ascribed to acetylene may in part be due to contaminating phosphine.

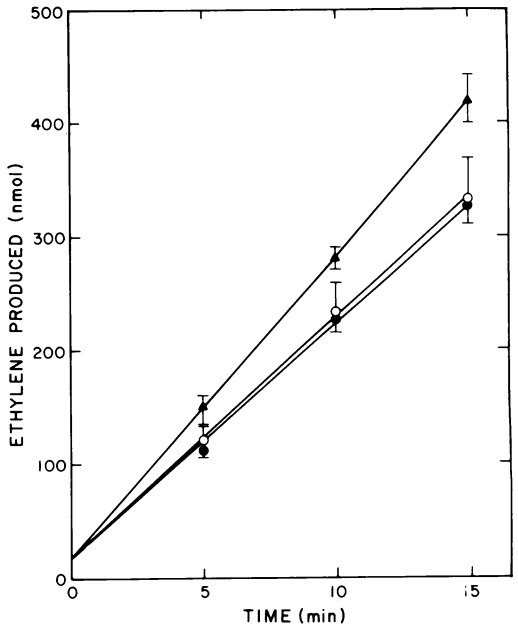

FIG. 3. Ethylene production by purified nitrogenase from *A. vinelandii*. Ethylene production by purified nitrogenase from *A. vinelandii* in the presence of three grades of acetylene is shown. Acetylene reduction assays were conducted in triplicate. A MoFe/Fe protein ratio of 1:10 was used with 23 and 68 μg of each protein present, respectively. Symbols: ▲, acetylene generated from 1 g of calcium carbide (Fisher); ○, nontreated, purified cylinder acetylene (99.6%); ●, purified cylinder acetylene (99.6%) after cryogenic purification. The plotted points are the averages of the three determinations. The error bars indicate the ranges of ethylene concentrations detected for each plotted point.

The second contaminant worthy of consideration is hydrogen. The role of hydrogen as an inhibitor and product of and as a substrate for microbial gas-utilizing enzymes is also beyond the scope of this discussion. However, it has been suggested by Payne (12) that the apparent susceptibility of hydrogenases to inhibition by acetylene may account for the as yet uncharacterized effects of acetylene on various hydrogenase-containing microorganisms. If this is the case, then the presence of hydrogen in acetylene is clearly of considerable importance. Furthermore, the presence of hydrogen in acetylene may account for some of the confusion which exists over the role of acetylene as an inhibitor of hydrogenases (1, 2, 5, 15, 19). Another example of where this contamination appears to have been overlooked is the report that hydrogen is unnecessary for the expression of hydrogenase in *Rhizobium japonicum* (5). In this case, 2% acetylene was used to inhibit H_2 production from nitrogenase. The need for authors to clearly state the source and quality of the acetylene used in their experiments is evident.

A final point relating to contaminants in general is the use of acetylene as a growth-supporting substrate for bacteria. Several reports have been made concerning newly isolated bacteria which can grow on acetylene and related unsaturated compounds (4, 8, 17). The possible presence of growth-supporting substrates in acetylene, such as methane and H_2, makes the use of highly purified acetylene a necessity in the study and isolation of these organisms.

The purification schemes described in this paper demonstrate that cylinder acetylene can be purified to a great extent by using a simple, small-scale laboratory process. Such

stringent purification may not be necessary for all applications which use acetylene. However, the increasing use of acetylene as a common laboratory reagent, especially in ecological and kinetic studies, clearly requires that workers be made aware of the possible complications arising from the use of this gas.

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