Interference by Oxygen in the Acetylene-reduction Test for Aerobic Nitrogen-fixing Bacteria

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The reduction of acetylene (Dilworth, 1966; Schöllhorn & Burris, 1967) with the detection of ethylene by gas-liquid chromatography is a widely used method of assaying nitrogen fixation (Stewart, Fitzgerald & Burris, 1967; Hardy, Holsten, Jackson & Burns, 1968). Nitrogen fixation by the Azotobacteriaceae, which are important aerobic nitrogen-fixing bacteria in nature, can be inhibited by oxygen, particularly in carbon- or phosphate-limiting conditions (Dalton & Postgate, 1969*a*). We now report that the oxygen sensitivity of Azotobacteriaceae can lead to false assessments of nitrogenase activity by the acetylene-reduction test.

Batch cultures of *Azotobacter chroococcum* (NCIB 8003), *A. vinelandii* (NCIB 8660) and *A. macrocytogenes* (NCIB 8700) were grown in Burk's sucrose medium (Newton, Wilson & Burris, 1953) and 2 ml. samples of growing cultures were shaken at 75 strokes/min. at 30° in 25 ml. flasks under atmospheres of $A + O_2 + C_2H_2$; the latter always at 0.03 atm. Maximum rates of acetylene reduction usually occurred at pO_2 values well below atmospheric (Fig. 1*A*). Comparable bell-shaped curves relating acetylene reduction to pO_2 in the aerobic nitrogen-fixing bacteria *A. chroococcum* (Dalton & Postgate, 1969*b*) and *Mycobacterium flavum* (Biggins & Postgate, 1969) and the blue-green alga *Anabaena flos-aquae* (Stewart, 1969) have been reported earlier. The optimal pO_2 for each species depended on the population density; only with very dense cultures (e.g. *A. vinelandii* at 2 mg. dry wt./ml.) was the optimal pO_2 for acetylene reduction at or above the atmospheric value.

Shaking samples of the cultures in air on a Griffin flask shaker (Griffin & George, Wembley, England) at 250 vib./min. for 2 to 4 min. before testing normally lowered their maximum acetylene-reducing activity by 25 to 75%; only dense cultures with optimum pO_2 values at or above 0.2 atm. were unaffected by such shaking. The 'switching off' of acetylene-reducing activity in sensitive cultures could be reversed: gentle shaking (75 strokes/min.) at pO_2 0.2 atm. for 10 min. restored the original activity tested at the optimum pO_2 . However, prolonged shaking in air (up to 1 hr) caused irreversible loss of up to 60% of the original activity.

It is possible to 'adapt' populations to high oxygen tension (Fig. 1*B*). Nitrogen-fixing continuous cultures of *Azotobacter chroococcum* were set up at 30° as described by Dalton & Postgate (1969*b*), and 'adapted' to 0.09 and 0.55 atm. O_2 at $D = 0.2 \text{ hr}^{-1}$; oxygen electrode measurements showed the ambient oxygen concentration in both cultures to be about 12 μM and the population densities were the same. The respiratory activities of such populations were related directly to pO_2 : at pO_2 0.55 the QO_2 was about 2700 $\mu\text{l./mg. dry wt./hr.}$; at pO_2 0.09 it was 1400. The low-oxygen population, like a batch culture, was readily 'switched off' by vigorous shaking in air.

The high pO_2 population was not; it even tolerated shaking in pure O_2 , a treatment which caused irreversible suppression of acetylene-reducing activity of the low pO_2 population.

The populations so far discussed were all 'N₂-limited' in the sense used by Dalton & Postgate (1969*b*). Carbon-limited populations are very sensitive to oxygen inhibition of growth; so, to test organisms of a different nutritional status, a carbon-limited continuous culture of *Azotobacter chroococcum* growing in air was studied. Its pO_2

Fig. 1. (A). Effect of pO_2 on acetylene reduction by azotobacters. 2 ml. samples of batch cultures of: ●—●, *A. chroococcum*; ▲—▲, *A. macrocytogenes*; ■—■, *A. vinelandii*; tested at 75 strokes/min, 30° under argon+oxygen+acetylene, the latter always at 0.03 atm. (B). Effect of pO_2 during growth, and respiratory activity, on the optimum pO_2 for acetylene reduction by *A. chroococcum*. 1 ml. samples containing 0.5 mg. dry wt organisms from continuous culture at $D = 0.2 \text{ hr}^{-1}$ tested at 150 strokes/min. ●—●, culture grown at $pO_2 = 0.55 \text{ atm}$, $QO_2 = 2700 \mu\text{l./mg. dry wt/hr}$; ■—■, culture grown at $pO_2 = 0.09 \text{ atm}$, $QO_2 = 1400 \mu\text{l./mg. dry wt/hr}$.

optimum for acetylene reduction was 0.025 atm. and no activity at all was detected at 0.2 atm. Shaking populations comparable to the N_2 -limited ones on a vortex mixer in air for 1 min. lowered activity at the optimum pO_2 by 50%.

The present work shows three points relevant to the routine use of the acetylene-reduction test. (i) Aerobic bacteria rarely show their optimal activity at the atmospheric pO_2 value; (ii) aerobic bacteria can undergo, partially or completely, a reversible 'switching off' process when vigorously aerated by shaking; (iii) the sensitivity to 'switching off' depends on population density and nutritional status.

Field samples are usually tested at the atmospheric pO_2 of 0.2, which is reasonable because, even if the oxygen inhibits some of the bacteria present, this condition fairly represents their natural state. Soil and water organisms are likely to be growing under carbon-limited conditions and adapted to sub-atmospheric pO_2 values; conditions which may be expected to maximize oxygen damage on handling. Therefore, if natural samples are vigorously shaken in air or otherwise unduly oxygenated, it is likely that aerobic nitrogen-fixing bacteria will partly or wholly 'switch off' their nitrogenase systems and the test will give a falsely low estimate of the original nitrogenase activity. The nature of the 'switch-off' and inhibition processes will be discussed in more detail elsewhere; we present the above data because of their relevance to the general use of the acetylene-reduction test.

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