The rapid loss of viability of *Azotobacter* in aqueous solutions

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When a low number of *Azotobacter vinelandii* 12837 log phase vegetative cells (2 × 10^3 cells/ml) were removed from the culture liquid to water of the same temperature, a rapid loss of viability occurred depending on the procedure of washing and suspending. Death was not accompanied by visible lysis and the rate of loss of viability was less at lower temperatures, and in the presence of salts or cell-free filtrates from heavy cell suspensions in water. The die-off was erratic at increased cell concentrations and was accelerated by utilizable energy sources. Cells standing in a favorable ionic solution (0.1 % NaCl) do not lose their viability while cells washed by a series of centrifugations with the same ionic solution show a progressive loss of viability with each washing. Phospholipids were found to leach from the cells into the aqueous solutions. Such cell death suggests instability of the cell membrane and the loss of osmotic or ionic control in the cells.

**INTRODUCTION**

In doing quantitative counts of vegetative cells of *Azotobacter* in our laboratory we have observed a rapid death of the bacteria in the water dilution blanks used for the plate count procedure. In highly diluted cultures where the count was only a few hundred cells per ml of dilution water all cells might die off in a matter of minutes while in the lower dilutions of the same series very little death would occur. This resulted in plates of one dilution showing too many organisms to count while a 10-fold greater dilution would show no organisms at all. This rate of death of *Azotobacter* is far more rapid than that observed by Strange, Dark and Ness (1961), and Postgate and Hunter (1963a, b) using other species. Our first presumption was that the rapid erratic death was due to active chlorine or other volatile toxic substances in the distilled water or to the presence of residual soap on the glassware. When these explanations were proved wrong by carefully controlled studies, we investigated the factors involved in the rapid death. Lysis of the cells was not observed when the cells were concentrated from the water dilutions but following such removal of the dying cells lipid materials
leached from them could be found in the distilled water. Since experience in our laboratory indicates unusual difficulty in quantitative plate counts with Azotobacter and discussions with other scientists working with this genus revealed that they have had similar experiences, publication of these results should be useful.

**MATERIALS AND METHODS**

**Cell preparations.** Azotobacter vinelandii ATCC 12837 was grown on Burk’s nitrogen-free salt medium (Wilson and Knight, 1952) with 0.5% sucrose as the carbon source. The cells were harvested during the exponential phase of growth (Klett 80–90).

**Viability studies.** The cells were collected on Millipore membrane filters and washed on the filters with either a salt solution such as 0.02 M NaCl and 0.02 M potassium phosphate buffer (pH 7.5) or cold distilled water. The cells were resuspended in water or experimental solution to a concentration of 2 x 10^8 cells per ml. The temperature of the water or solutions was 33 C unless otherwise stated. For some experiments, the cells were washed by a series of centrifugations. The cells were then plated directly on Burk’s sucrose agar overlain on 0.1% casein hydrolysate medium since our experience indicated that the casein hydrolysate eliminated irregularities in growth due to trace impurities contaminating the glassware. The average results of at least 3 experiments were used to determine each curve in this report.

**Phospholipid studies.** Phospholipids were extracted by the Folch procedure (Folch, Lees and Sloane Stanley, 1957) using chloroform–methanol 2:1 (v/v). The extract was washed free of non-lipid impurities using 0.88% KCl. The upper solvent used to wash the interface contained 0.37% KCl. The lipid solution was flash-evaporated and dried by successive treatments with benzene–absolute ethanol 4:1 (v/v) and once with chloroform. The final residue was resuspended in a small volume of chloroform. Polar phase chromatography on commercially made Whatman silica-gel-impregnated paper SG 81 (made in England by W & R Balston, Ltd. and distributed by H. Reeve Angel and Co., Inc., Clifton, N. J.) was performed as described by Marinetti (1965). In other experiments cells were labeled with P³² and extracted. The lipid extract was placed in a silicic acid column and the lipids were eluted according to their polarity. The solvents employed were chloroform, chloroform: acetone 1:1, acetone, a discontinuous methanol in chloroform gradient, and methanol. The fractions were concentrated and placed in vials containing Bray’s solution (Bray, 1960), and the P³² activity was counted with a Ansitron scintillation counter.

Water used in this study was distilled from a commercial metal still, deionized