Colloid Titration for Determining the Surface Charge of Bacterial Spores

By K. WATANABE AND S. TAKESUE

*Institute of Microbiology, Faculty of Pharmaceutical Sciences,
Fukuoka University, Fukuoka, Japan*

(Received 3 March 1976; revised 10 May 1976)

INTRODUCTION

Colloid titration was originally devised by Terayama (1948) for studying the colloidal polyelectrolytes. However, the potential of this technique was not fully exploited until Senju (1969) introduced titration reagents of higher molecular weight and adequate purity, and confirmed its usefulness. It has now become a valuable analytical technique for many colloidal polyelectrolytes such as agar, lignin, pectin and some proteins. Recently, colloid titration was used to determine the surface charge of some yeasts (Imahara, Iwai & Nakahama, 1971) and of *Micrococcus lysodeikticus* (Katayama *et al.*, 1975).

To learn more about the mechanism of germination of bacterial spores, we have investigated the surface structure of two types of *Bacillus megaterium* spores that differ in their response to germinants and inhibitors. We found that the spores of QM-B1551 strain (designated GN spores), which respond germinatively to glucose and KNO₃, have more ionizable groups than those of ATCC19213 strain (AL spores), which respond to L-alanine and inosine; and that the buffer action of GN spores against a change in hydrogen-ion concentration is substantially stronger than that of AL spores (Watanabe & Takesue, 1970). Since the surface charge of spores may affect the initiation of germination, we examined whether colloid titration could be used to determine the surface charge of bacterial spores, and then determined the surface charge in terms of the colloid titration value of the two types of *Bacillus megaterium* spores.

METHODS

Bacillus megaterium ATCC19213 (designated AL strain in this paper) and QM-B1551 (GN strain) were provided by Professor L. J. Rode, University of Texas. Spores were produced on an agar medium as described by Rode & Foster (1960). After three days incubation at 30 °C, the spores were harvested in deionized water and washed eight times with water by centrifugation in the cold. Concentrated suspensions of cleaned spores, confirmed by phase-contrast microscopy, were lyophilized.

Colloid titration is based on the phenomenon that positively and negatively-charged colloidal particles combine stoichiometrically with each other and the end point of a titration can be detected with a suitable indicator. Protamine sulphate (PS) was used as a positive colloidal reagent and potassium polyvinyl sulphate (PVS-K; polymerization $n = 1500$, esterification 98.4 %, certificated by Wako Jun-Yaku Co. Japan) as a negative reagent. For titrating negatively-charged colloidal particles such as bacterial spores, an excess (electrically) of 2.5 mM-PS (5 ml) was added to an appropriate concentration of spores (e.g. 5 ml at 2 mg ml⁻¹) in deionized water or sometimes in 143 mM-veronal acetate buffer. The unneutralized PS was back-titrated with 1.0 mM-PVS-K using toluidine blue as indicator.

Fig. 1

Fig. 2

Fig. 1. Relationship between spore concentration and the colloid titration value. Graded amounts of spores (1.25 to 20.0 mg) were suspended in water (5 ml) and the colloid titration was conducted at pH 6.0 with 2.5 mM-PS (5 ml) and 1.0 mM-PVS-K. Spores: ●, GN; ○, AL.

Fig. 2. pH-colloidal charge curve of spores. An aqueous suspension of spores (10 mg in 5 ml) was adjusted to various pH values with HCl and NaOH, and the colloid titration was conducted with 2.5 mM-PS (5 ml) and 1.0 mM-PVS-K. Spores: ●, GN; ○, AL; ■, control (no spores).

The end point was indicated both by metachromasy (the colour change of toluidine blue from blue to red-violet) and by aggregation of the spores. The value of the colloidal charge was expressed as milliequivalents per gramme of spores (m equiv./g spores), using standardized PVS-K as a criterion for titration.

RESULTS AND DISCUSSION

To test whether colloid titration is applicable for the quantitative determination of the surface charge of bacterial spores, various amounts of spores suspended in 5 ml deionized water were titrated by the technique described in Methods. The titration value depended on the spore concentration (Fig. 1). Both the GN and AL spores were found to be charged negatively and the magnitude of their negative charge increased almost linearly with spore concentration to 4 mg ml⁻¹ under the conditions used. This indicated that colloid titration by this method could be used for bacterial spores. In the following experiments, a spore suspension at a final concentration of 2 mg ml⁻¹ was used. Of the positive colloidal commercially available reagents tested, protamine sulphate was the most useful; others such as glycol chitosan and methyl glycol chitosan were unsuitable because they did not give a sharp colour change at the end point.

The titration value of GN spores was about three times that of AL spores (Fig. 1). This indicated that about three times as many negatively charged groups were located on the surface of GN spores as on AL spores, although the contribution of cationic groups, such as the amino group, to the surface charge was not known. According to Kondo & Nishihara (1970), the outer layer of the coat of GN spores is composed mainly of galactosamine-containing phosphomucoprotein, and its phosphorous content is about four times that of AL spores. The high phosphorous content of the outer layer in GN spores may be related to the fact that the surface is more negatively charged and the buffer action against a change in pH is stronger than in AL spores.

The degree of dissociation of weakly dissociable groups changes with the concentration of

hydrogen ions in the environment, and, since colloid titration is concerned with weakly dissociated groups on the surface of colloidal particles, the titration value must be influenced by the pH of the surroundings. Therefore, colloid titration of the spores suspended in water and adjusted to different pH values with HCl and NaOH was conducted to determine the optimum conditions. The isoelectric points of the spores were also determined. The highest titration values were obtained at pH values above 6 with both types of spores (Fig. 2). No appreciable change in the titration value was observed between pH 6 and 9 (the plateau region). When 143 mM-veronal acetate buffer was used instead of HCl and NaOH to adjust the pH, the pH-colloidal charge curve was almost the same. For GN spores, the colloidal charge at pH values in the plateau region was about -0.6 m equiv./g spores. At pH values below 5, the negative charge decreased rapidly. In the pH range about 1 or 2, it was slightly positive, showing that the isoelectric point of GN spores lies in that region. Accurate values for the isoelectric point could not be obtained, because the charge of the control (protamine sulphate solution without spores) did not remain constant in the tested pH range (Fig. 2), and, especially at the lower pH values, it tended to reverse and varied significantly between experiments. The general shape of the curve for AL spores was similar to that for GN spores, but both the negative charge at optimum pH (about -0.2 m equiv/g spores) and the isoelectric point (pH 3 to 4) were different.

Since GN spores were more heat-resistant than AL spores (Rode, 1968) and the outer layer of the coat of GN spores was thicker than that of AL spores (Kawasaki, Nishihara & Kondo, 1970), it seemed that there might be a difference in resistance to mechanical shock between the two types of spores. However, subjecting the spores of either type to several cycles of freezing and thawing did not cause any change in their colloidal charge. The colloidal charges of spores suspended in water were not altered by storage for 6 months in a refrigerator, indicating that their surface structures were stable over long periods.

The authors wish to thank Professor H. Imahara, Kyoto College of Pharmacy, for his helpful advice on this work. They acknowledge the assistance of the following undergraduate students of Fukuoka University with some of the experiments: M. Fukudome, K. Kanamasa, H. Obata, K. Sekiya, Y. Wada, and M. Yokoyama.

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