SHORT COMMUNICATIONS

Effect of Glutaraldehyde on Protoplasts of *Bacillus megaterium*

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Glutaraldehyde is a 5-carbon dialdehyde with a wide antimicrobial spectrum; its effectiveness is markedly increased by buffering, usually with sodium bicarbonate, between pH 7.5 and 8.5. Its properties as an antimicrobial agent and chemosterilizer have been reviewed by Rubbo, Gardner & Webb (1967), and it is used extensively as a fixative in electron microscopy. We suggested (Munton & Russell, 1970) that glutaraldehyde acted on the wall of *Escherichia coli*, although other sites of action were also possible. We report here the effects of the dialdehyde on the stabilization of wall-less forms (protoplasts) with particular emphasis on their ability to withstand osmotic shock.

METHODS

Organism and method of culture. *Bacillus megaterium* NCTC6005 was grown for 18 h. at 37° in 100 ml. of nutrient broth (Oxoid) in a shaking incubator operating at 70 oscillations/min. The culture was centrifuged, and the resulting pellet washed twice with sterile glass-distilled water and finally resuspended in sterile water to give the required bacterial density.

Chemicals. Aqueous solutions of glutaraldehyde, with or without added sodium bicarbonate (0.3 %, w/v), were prepared from a 25 % solution (Kodak Ltd, Kirby, Liverpool). Other chemicals were of analytical reagent grade. Lysozyme was purchased from British Drug Houses Ltd, London.

Measurement of turbidity changes. Optical density (O.D.) was measured with the Unicam SP 600 spectrophotometer using 1 cm. cells. The reference cell contained distilled water.

Protoplast formation. Protoplasts of *Bacillus megaterium* were formed by suspending the bacteria (0.6 mg. dry wt./ml.) in a medium containing sucrose (0.5 M), lysozyme (200 µg./ml.) and phosphate buffer (0.013 M, pH 7.2) at 0°. Formation of protoplasts was complete after 30 to 60 min. Magnesium sulphate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, was added to give a final concentration of 5×10^{-3} M. The protoplasts were deposited by centrifugation at 500 g for 30 min. and resuspended in the same stabilized buffer (phosphate + sucrose + Mg^{2+}) to the required O.D. value.

Stability of glutaraldehyde-treated protoplasts. This was investigated in two ways: (a) 1.5 ml. of protoplast suspension was added to 8.5 ml. volumes of aqueous glutaraldehyde solutions, water, or stabilized buffer; (b) protoplast suspensions (9 ml.) were

pretreated with 1 ml. glutaraldehyde for 5, 30 or 60 min., after which 1.5 ml. was added to 8.5 ml. of water, and O.D. measured at intervals. Untreated protoplasts were similarly diluted in stabilized buffer as control. Results are expressed as % of the O.D. of the control in buffer at 0 min. Samples were also examined under a phase-contrast microscope ($\times 400$).

RESULTS

Effect of glutaraldehyde on O.D. of protoplast suspensions. Low glutaraldehyde concentrations slightly reduced the O.D. of protoplast suspensions, whereas high concentrations give a significant increase. The reduction in O.D. was not associated with any decrease in protoplast numbers. Optical density readings at 450 and 660 nm. showed no significant difference and yellow colour production (Munton & Russell, 1970) is thus not involved in O.D. changes. The effect of glutaraldehyde was unchanged by the addition of sodium bicarbonate (0.3 %, w/v), although bicarbonate alone reduced the O.D. of protoplast suspensions.

Table 1. *Lysis of protoplasts of Bacillus megaterium in aqueous or alkaline glutaraldehyde (method (a))*

Readings are expressed as % increases (+) or decreases (–) of O.D. at 450 nm. of control in stabilizing buffer at 0 min.

	Time (min.)					
	0	5	30	60	90	120
Stabilized buffer	0	0	0	0	–2.1	–2.4
Water	0	–56.8	–56.8	–56.8	–57.0	–57.0
Protoplast suspension diluted with glutaraldehyde (% w/v)						
0.01	0	–49.6	–48.3	–48.3	–47.7	–47.2
0.1	0	–45.4	–43.8	–43.5	–43.5	–42.8
0.2	0	–38.3	–35.7	–35.2	–35.7	–35.7
0.6	0	+11.1	+4.5	–1.3	–12.4	–19.8
1.0	0	+21.7	+16.8	+7.2	–0.7	–0.9
Protoplast suspension diluted with 0.3 % (w/v) sodium bicarbonate and glutaraldehyde (% w/v)						
0.0*	0	–57.0	–57.0	–57.0	–56.5	–56.5
0.01	0	–56.9	–54.8	–52.0	–49.8	–49.5
0.1	0	–52.0	–50.1	–49.5	–49.5	–49.5
0.2	0	–52.0	–52.0	–49.5	–49.5	–49.5
0.6	0	–49.7	–48.4	–48.0	–48.0	–48.0
1.0	0	–47.0	–47.0	–46.5	–46.5	–46.5

* Protoplasts in 0.3 % (w/v) sodium bicarbonate only.

Stability of glutaraldehyde-treated protoplasts. More protoplast stabilization occurred in aqueous than in alkaline glutaraldehyde (Table 1, method (a)) and was complete at high aldehyde concentrations. The O.D. with high concentrations of aqueous glutaraldehyde was higher than that of the control, because of the increase in protoplast density, as outlined above. The subsequent reduction in O.D. (Table 1, with 0.6 % and 1.0 % aqueous glutaraldehyde) results from aggregation of the stabilized protoplasts in acid medium, as observed microscopically.

Alkaline glutaraldehyde (method (a)) slightly stabilized protoplasts only at high concentrations, e.g. 0.6 and 1.0% (Table 1). Negligible lysis of protoplasts occurred when they were diluted with stabilizing buffer (Table 1).

Pretreatment with aqueous glutaraldehyde before dilution in water (method (b)) stabilized the protoplast suspensions; 30 min. treatment with an aldehyde concentration as low as 0.01% (w/v) conferred some stabilization of protoplasts. The O.D. of suspensions of untreated protoplasts diluted in water fell by 65%, whereas that of suspensions treated with 0.01% (w/v) glutaraldehyde for 30 and 60 min. fell by 40% and 20% respectively. Protoplasts treated with 0.01% (w/v) glutaraldehyde were observed to swell in water. This was reflected in a two-stage decrease in O.D. When pretreated with 0.2% (w/v) glutaraldehyde there was no subsequent swelling of protoplasts in water and no lysis.

DISCUSSION

Lysis of protoplasts in media of low osmotic pressure is due to water uptake and subsequent bursting of the membrane, although mechanical factors may also be involved (Weibull, 1958; Edebo, 1961; Razin & Argaman, 1963; Marquis, 1967; Corner & Marquis, 1969). Marquis (1965) and Marquis & Corner (1967) have concluded that the protoplast membrane is perforated with small aqueous channels through which lipophobic molecules could diffuse. Protoplasts are stabilized by formaldehyde, osmium tetroxide (Fitz-James, 1958), acid pH (Edebo, 1961) and ethanol or ethanol-acetic acid fixation which permanently immobilizes protoplasts (Fitz-James, 1958).

Glutaraldehyde-treated protoplasts do not lyse in water, possibly because aqueous glutaraldehyde is acid (pH 3.4 to 5) and will precipitate protoplasmic constituents. However, even at pH 8 (with bicarbonate) glutaraldehyde stabilizes protoplasts to some extent, suggesting an inherent stabilization (fixation) property of the molecule.

Slight swelling of protoplasts has been observed at low aldehyde concentrations and short contact times. In such conditions, the membrane seems partially stabilized, allowing some water to enter, and in some cases burst the protoplast or produce a swollen form. High concentrations of alkaline glutaraldehyde do not allow this swelling, water access is prevented probably by blocking off the aqueous channels, and the membrane is strengthened to resist lysis.

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