Effect of Glutaraldehyde on Cell Viability, Triphenyltetrazolium Reduction, Oxygen Uptake, and β-Galactosidase Activity in Escherichia coli

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Acid (pH 5) and alkaline (pH 8.5) glutaraldehyde solutions were compared for their effects on cell viability, oxygen uptake, and β-galactosidase activities in Escherichia coli. The action of glutaraldehyde at pH 7 on dehydrogenase activity was also studied. Dehydrogenase activity was inhibited at aldehyde concentrations which had little effect on cell viability. In contrast, oxygen uptake and β-galactosidase activity took place in cells killed by acid or alkaline glutaraldehyde. The effect of glutaraldehyde on dehydrogenase activity and β-galactosidase activity of disrupted suspensions was also investigated. The dialdehyde was considerably less inhibitory to these enzyme systems than to those of whole cells, and it is thus feasible that the results with whole cells are a consequence of its interaction with, and strengthening of, the outer cell surface, thereby preventing ready access of substrate to enzyme.

Glutaraldehyde, especially at alkaline pH, is bactericidal to Escherichia coli (1,7), stabilizes penicillin-induced spheroplasts so that they do not readily lyse in media of low osmotic pressure (8,9), and fixes the configurational state of mitochondria (2). Oxygen uptake is stimulated at low glutaraldehyde concentrations, possibly because the dialdehyde is metabolized. The dual features of preservation of structural integrity and persistence of enzyme activity prompted us to consider the effects of glutaraldehyde on certain enzyme activities of intact cells and of disrupted cell preparations of E. coli in relation to the bactericidal activity of the dialdehyde. For simplicity, dehydrogenase activity, oxygen uptake, and β-galactosidase activity were selected.

MATERIALS AND METHODS

Organisms. E. coli NCTC 86 was used for the β-galactosidase experiments, and details of its growth are provided under that heading. E. coli NCTC 9001, used in the dehydrogenase and oxygen uptake experiments, was grown for 24 h on the surface of nutrient agar (Oxoid Ltd., London). The growth was washed off with sterile water and the culture was washed twice with sterile water and adjusted to the required density.

Dehydrogenase activity (triphenyltetrazolium reduction). A series of test tubes containing increasing concentrations of glutaraldehyde, obtained from a 25% aqueous solution (Kodak Ltd., Kirkby, England) ±0.3% (wt/vol) sodium bicarbonate, was set up as follows: a washed suspension of E. coli NCTC 9001 (3.2 mg, dry weight/ml), 0.25 ml; phosphate buffer (0.05 M, pH 7), 1 ml; glucose (0.01 M), 1 ml; triphenyltetrazolium chloride (TTC) (British Drug Houses Ltd., London) 0.1% (wt/vol), 1 ml; and glutaraldehyde or distilled water to 5 ml. After incubation for 30 min at 37°C, the tubes were transferred to an ice bath to terminate metabolic activity. After 5 ml of butanol was added, the bacteria were removed by centrifugation, the upper butanol layer was pipetted off, and the absorbance was measured in a Unicam SP600 spectrophotometer at 525 nm. Controls were made without glutaraldehyde and without either aldehyde or bacteria. The latter control was used as the reference cell for absorption measurements. Preliminary experiments showed that glutaraldehyde did not react with TTC.

Results are expressed as an absorbance ratio, which is the absorbance of the supernatant fluid from glutaraldehyde-treated cells per the absorbance of the supernatant fluid from control cells without glutaraldehyde. Thus, maximum dehydrogenase activity is 1.

Oxygen uptake. Oxygen uptake of E. coli NCTC 9001 at 37°C was determined in a series of Warburg flasks which contained: E. coli suspension (approximately 2 x 10^8 viable cells per ml) in the side arm,
0.25 ml; glucose (0.06 M), 1 ml; 0.66 M phosphate buffer (pH 5) or sodium bicarbonate at 3% (wt/vol), 0.3 ml; and glutaraldehyde to 3 ml. The final sodium bicarbonate concentration (when present) was thus 0.3% (wt/vol). Final pH values were 5 when phosphate buffer was present, or 8.5 when sodium bicarbonate was included. The center well contained 0.2 ml of 10% (wt/vol) potassium hydroxide.

β-Galactosidase activity. E. coli NCTC 86, in which the β-galactosidase enzyme is inducible, was grown on agar (Oxoid) plus 1% (wt/vol) lactose plus 1% (wt/vol) yeast extract (Difco) at 37°C for 18 h. The cells were harvested, and a washed suspension was prepared as for E. coli 9001. E. coli suspensions (0.8 mg, dry weight/ml) were incubated with glutaraldehyde ±0.3% (wt/vol) sodium bicarbonate, final volume 3 ml, for 80 min at 37°C. The final pH values were 5 or 8.5. The suspensions were centrifuged, and the pellet was washed with water and suspended in 5 ml of 0.066 M phosphate buffer (pH 7) containing 0.08% (wt/vol) o-nitrophenyl-β-D-galactoside (ONPG). The tubes were incubated for 1 h at 37°C. The suspensions were centrifuged, and 1 ml of the supernatant fluid was removed and added to 9 ml of sodium carbonate solution (final concentration 0.4 M) to terminate the color reaction. The absorbance of the resulting mixture was measured at 420 nm, the \( \lambda_{\text{max}} \) of o-nitrophenol (ONP), and the amount of ONP produced was calculated from a previously prepared standard curve.

Viable counts. To ensure a valid comparison between the effects of glutaraldehyde on enzyme activity and viability, containers of identical composition to those in the enzyme tests were set up; at intervals, samples were removed and serially diluted in sterile water, and survivor levels were determined by the pour-plate method by using nutrient agar (Oxoid) as the recovery medium. Sodium bisulfite was not used as an inactivating agent because it is toxic to E. coli (7). Colonies were counted after 48 h at 37°C. Viable counts thus were carried out under the exact conditions of the tests.

Cell-free enzyme systems. By using a Braun tissue disintegrator at 4°C for 5 min, 25 ml of washed suspensions of E. coli 9001 (obtained from growth on agar, dry weight 8.8 mg/ml) and of E. coli 86 (obtained from growth on yeast extract-lactose-agar, dry weight 8 mg/ml) were disintegrated. The debris was removed and the cell-free enzyme system was used in experiments for TTC reduction (E. coli 9001) and β-galactosidase activity (E. coli 86).

For TTC reduction, test tubes were set up as described for whole cell suspensions, with 0.25 ml of the disrupted cell preparations replacing an equal volume of whole cell suspension. Incubation was for 30 min at 37°C, and an absorbance ratio was calculated as described above.

For β-galactosidase activity, a series of tubes was set up as follows: E. coli disrupted preparation, 0.25 ml; 1% glutaraldehyde, as required; 6% sodium bicarbonate, absent or 0.25 ml; 0.16% ONPG in 0.066 M phosphate buffer, 2.5 ml; and water to 5 ml. Final aldehyde concentrations (µg/ml) were 100, 200, 500, 800, and 1,000. The tubes were incubated at 37°C for 1 h, and the amount of ONP produced was calculated as described above.

**RESULTS**

Effect of glutaraldehyde on dehydrogenase activity of whole cells. Figure 1 compares the effects of glutaraldehyde after 30 min at 37°C on the viability of, and TTC reduction by, E. coli 9001 in phosphate buffer, pH 7. Preliminary experiments showed that this enzyme system was markedly inhibited at both pH 5 and 8.5 (glutaraldehyde absent), and thus only one pH value was used. This dehydrogenase system at pH 7 is extremely sensitive to the action of the dialdehyde; the viable counts indicate that, at glutaraldehyde concentrations of 200 µg/ml or less, there is no kill after 30 min and only a 20 to 30% kill with the higher concentrations used, although an appreciable decrease in enzyme activity is apparent.

Effect of glutaraldehyde on oxygen uptake of whole cells. A comparison of the effects of different concentrations of alkaline or acid glutaraldehyde on viability of E. coli after 90 min at 37°C with the amount of oxygen uptake after this period is given in Fig. 2 and 3. Alkaline glutaraldehyde (30 µg/ml) for a period (90 min) in which there was a 90% kill did not significantly affect the oxygen uptake; acid glutaral-
dehydrate (40 μg/ml), which similarly rendered 90% of the cells nonviable, reduced the oxygen uptake. This uptake with acid glutaraldehyde-treated cells tends to decrease gradually with increasing aldehyde concentration, whereas with alkaline glutaraldehyde, an abrupt "cut-off" point appears at approximately 35 μg/ml.

Effect of glutaraldehyde on β-galactosidase activity of whole cells. In Fig. 4 and 5, the inhibition of β-galactosidase activity by pretreatment of E. coli 86 with glutaraldehyde at pH 8.5 or 5 for 90 min at 37 C is expressed as the percentage of reduction of ONP released from ONPG compared with control (untreated) bacteria. These figures also show the percentage of cells rendered nonviable in identical aldehyde suspensions. There is little difference between alkaline and acid glutaraldehyde in their ability to prevent β-galactosidase activity detected by this method. Also, it is apparent that there is substantial enzyme activity after the cells have been killed by aldehyde treatment. The viable counts indicate that the relative susceptibility of E. coli 86 to alkaline and acid glutaraldehyde is similar to E. coli 9001 and is not strain specific.

Effect of glutaraldehyde on disrupted cell preparations. The effects of glutaraldehyde on TTC reduction (Table 1) by a disrupted cell preparation of E. coli 9001 indicates that there was no reduction of dehydrogenase activity. Glutaraldehyde did inhibit to some extent the β-galactosidase activity of a disrupted cell preparation of E. coli 86 (Table 2), but even here at least 69% of the activity of the control preparation (glutaraldehyde absent) remained.

DISCUSSION
Glutaraldehyde will combine with several amino acids (7), with bacteriological peptone and with casein (unpublished data), and with other proteins (5), and its interaction with enzyme has been reported (4, 10, 11). Frequently, cross-linked forms of the enzymes, which are stable and retain a reduced enzymatic activity, are produced. Ellar, Munoz, and Salton (3)
have recently shown that glutaraldehyde prevents the selective release of certain enzymes from the cytoplasmic membrane of Micrococcus lysodeikticus, which normally occurs by controlled washing with buffers. In this context, another aldehyde (formaldehyde) has been found to retard the release of $^{55}$Mn after suspension of radioactive cells of E. coli K-12 in a nonradioactive medium (12) and to "seal" the membrane surface of E. coli (6). Similar studies with glutaraldehyde would be valuable.

A "sealing" of the outer cell surface could provide one explanation for our results. Most of the enzymatic activity responsible for oxygen uptake occurs at or within the cytoplasmic membrane. Thus, any glutaraldehyde interaction with the outer cell layers to give a nonviable cell need not affect this uptake. The point at which there is a decreased uptake may represent prevention of glucose access by cross-linking of the outer layer of the cell or the point at which alkaline glutaraldehyde penetrates to the cytoplasmic membrane, to act directly with the enzymes. Glutaraldehyde at acid pH does not react immediately with the outer cell layers or to the same extent as alkaline glutaraldehyde (7). Thus, more acid glutaraldehyde might be available for diffusion to the membrane, where it could act directly, but gradually, on the enzyme system.

Direct interaction of glutaraldehyde with an enzyme system could also explain the inhibitory effect of this antimicrobial agent on oxygen uptake, $\beta$-galactosidase activity, and dehydrogenase activity of whole cells. However, the facts that (i) TTC reduction is relatively unaffected and (ii) $\beta$-galactosidase activity is reduced by more than 30%, in disrupted cell preparations, do not support this contention. It has recently been shown (7, 8, 9) that glutaraldehyde binds strongly to the surface layers of E. coli, and thus it is likely that this interaction, involving a cross-linking process, effectively prevents access of substrate to enzyme.

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