The Lysis of Cholera and El Tor Vibrios

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SUMMARY

Vibrio cholerae and V. eltor underwent considerable lysis after treatment with tris + EDTA, tris + EDTA + lysozyme or sodium lauryl sulphate. Lysis induced by tris + EDTA or tris + EDTA + lysozyme was completely inhibited by 0.3 M sucrose, 0.15 M NaCl or 0.01 M MgCl₂. The extent of death after treatment with tris + EDTA depended on EDTA concentration: 10 min. with 10 µg EDTA/ml left 43 to 50% of V. eltor viable and 71 to 75% of V. cholerae. Labelled organisms exhibited maximum leakage of ³²P compounds when exposed to 71% (v/v) ethanol for V. cholerae and 50% (v/v) ethanol for V. eltor; leakage in 100% ethanol was about 77% of maximum for V. cholerae and 66% for V. eltor. Both ³²P labelled and unlabelled vibrios released considerable amounts of nucleic acid, phospholipid, protein and carbohydrate and practically all their acid soluble phosphates with EDTA.

INTRODUCTION

Studies on the sensitivity of different bacteria to agents such as ethylene diaminetetra-acetic acid (EDTA), lysozyme, sodium lauryl sulphate, etc., are likely to throw light on the physico-chemical structure of their surface layers. According to Wilkinson (1967), sensitivity of an organism to EDTA depends on the wall structure and may have some taxonomic value. Similarly the nature of release of the ³²P compound by bacteria in ethanol of different concentrations can be broadly correlated with their response to Gram-staining and hence to wall structure (Salton, 1963).

This communication records our study of the sensitivity of cholera and el tor vibrios to di-sodium EDTA, di-sodium EDTA + lysozyme and sodium lauryl sulphate and also of the mode of release of ³²P compounds by the vibrios in ethanol.

METHODS

Organisms. Vibrio cholerae, Ogawa 154; V. cholerae, Inaba 66/64; V. eltor, OE/27; V. eltor, E 249/67; V. eltor, E 252/67; and V. eltor, E 256/67 were obtained from Dr K. N. Neogy, Department of Bacteriology of this institute.

Test procedure. Vibrios harvested after 18 hr at 37° on peptone agar or from the logarithmic phase of growth in alkaline peptone water were washed twice in tris buffer (100 µM, pH 8.0) and finally obtained as a thick suspension in the buffer. 1 ml. portions were added to 9 ml. each of five test solutions: (a) 100 µM-tris buffer, (b) EDTA (10, 50, 150 or 266 µg/ml.) in 100 µM-tris buffer (Repaske, 1958) with or without 0.15 M NaCl, 0.3 M sucrose or 0.01 M MgCl₂, (c) lysozyme (50 µg/ml.) in 100 µM-tris buffer, (d) EDTA (266 µg/ml.) and lysozyme (50 µg/ml.) in 100 µM-tris buffer
with or without 0.15 M- NaCl, 0.3 M-sucrose or 0.01 M-MgCl and (e) 0.5% (w/v) sodium lauryl sulphate in 0.15 M-saline (in this case the washing fluid was 0.15 M-saline); pH value in all tests was adjusted to 8.0, initial optical density to about 0.8. Changes in optical density relative to the test solutions were then measured in a photoelectric colorimeter at 650 μμ at room temperature (22 to 25°).

**Viability determination.** Bacteria were treated with EDTA in 100 μμ-tris buffer for 10 min. and their viability relative to a control without EDTA was determined after serial dilution in 0.15 M-saline; 0.1 ml. volumes of suitable dilutions were spread on peptone agar plates and colonies were counted after incubation at 37° for 18 to 24 hr.

**Electron microscopy.** Vibrios treated with (a) EDTA (50 μg./ml.) in tris buffer (100 μμ) for 10 min. (b) EDTA (50 μg./ml.) + lysozyme (50 μg./ml.) in tris buffer for 10 min. and (c) sodium lauryl sulphate, 0.5% (w/v) in 0.15 M-saline for 80 min. were immediately fixed in formaldehyde (4%, w/v) for 2 hr, washed, resuspended in distilled water and prepared for electron microscopy (Das & Chatterjee, 1966).

**Release of 32P.** The distribution of 32P among different chemical fractions of *Vibrio cholerae* and *V. eltor* was determined using the membrane filter technique of Britten, Roberts & French (1955) as modified by Roodyn & Mandel (1966). The membrane filters were the BAC-T-FLEX type B-6 of Schleicher and Schull Co., Keene, New Hampshire, U.S.A. Release of 32P from the labelled vibrios treated with EDTA (50 μg./ml.) in tris buffer (100 μμ) was studied similarly. Release of 32P in ethanol was studied by Salton's (1963) method. In all cases, vibrios labelled during the logarithmic phase of growth were used.

32P activity on the membrane filters was measured by a thin window G.M. Counter and corrected for background and 32P decay; activity released was expressed as % intact organism activity.

EDTA (50 μg./ml.) extracts of the labelled vibrios were subjected to one dimensional ascending chromatography on Whatman No. 1 paper in the following solvents: ammonium sulphate (sat.), 160 ml. + sodium acetate (8.2 g./100 ml.), 36 ml. + isopropanol, 4 ml. (Smith, 1960). The paper was dried at room temperature, cut into 1 cm. strips perpendicular to the direction of the run and their 32P activities were measured.

**Chemical tests.** The O.D. values of filtrates of vibrios treated with EDTA in tris buffer were measured with a Backman spectrophotometer DB in the range 200 to 300 μμ. Protein in the filtrate was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline serum albumin as a standard. Carbohydrate in the filtrate was determined by the method of Dubois *et al.* (1956) using glucose as a standard. For comparison, equal amounts of bacteria were heated at 100° for 20 min., filtered and the protein and carbohydrate in the filtrates were also determined.

**RESULTS**

**Decrease in turbidity.** Vibrio suspensions of all strains, incubated with 266 μg. EDTA/ml. in 100 μμ-tris buffer decreased 50% in O.D. in 5 min.; with 50 μg. lysozyme/ml. in addition 72% lysis resulted in the same time. In tris and lysozyme without EDTA, lysis was only 10%. No significant decrease in O.D. was noted when vibrios were incubated for 80 min. in (i) 0.15 M-NaCl; (ii) 100 μμ-tris buffer; (iii) 0.05 M-sodium phosphate buffer; (iv) phosphate buffer + EDTA (266 μg/ml); (v) NaCl
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(0.15 M) + EDTA (266 µg/ml.) or (vi) phosphate buffer (0.05 M) + EDTA (266 µg/ml.) + lysozyme (50 µg/ml.). The lytic action of tris (100 µM) + EDTA (266 µg/ml.) or tris (100 µM) + EDTA (266 µg/ml.) + lysozyme (50 µg/ml.) was completely inhibited when NaCl (0.15 M), sucrose (0.3 M) or MgCl₂ (0.01 M) was added. Decrease in O.D. of the vibrios without these protective additives depended on the EDTA concentration, was maximum with 50 µg/ml. The O.D. in 0.15 M-NaCl containing sodium lauryl sulphate (0.5 %, w/v) decreased by 80% after 70 min. at 37°C.

Loss of viability. Plate counts of Vibrio cholerae and V. eltor showed differences in inactivation at low EDTA concentration. After 10 min. exposure to 10 µg. EDTA/ml. at room temperature, viability of V. cholerae was 71 to 75 % and V. eltor 43 to 50 %. After exposure for 10 min. to 50, 150 and 266 µg. EDTA/ml. both species had viabilities of 8 to 11, 15 to 20 and 36 to 40 % respectively. Controls without EDTA remained 100 % viable.

Electron microscopy. Vibrio cholerae and V. eltor organisms lost most of their electron opacity after EDTA treatment, but retained their original shape (Pl. 1, fig. 1). EDTA + lysozyme left only round structures of different sizes, but of much less electron opacity (Pl. 1, fig. 2). Electron microscopy of the vibrios heated to 100° for 40 to 50 sec. and then treated with sodium lauryl sulphate revealed vibrio shaped ghosts only (Pl. 1, fig. 3).

³²P release in ethanol. Labelled vibrios suspended in 90 % ethanol leaked ³²P compounds rapidly, attaining the maximum value within 5 min. Figure 1 shows that leakage differed according to species and ethanol concentration; 100 % ethanol released about 77 % of the maximum from vibrio cholerae and about 66 % of maximum from V. eltor.

Release of intracellular material. Chemical fractionation revealed that, after EDTA treatment, 40 % of the lipid, 50 % of the nucleic acid and practically the whole of acid-soluble phosphate fraction had been released from the vibrios. 5'-mono- and tri-
phosphates of adenosine, guanosine, thymidine, cytidine, and uridine were identified by chromatography. The ultraviolet absorption spectrum had the expected maximum at 260 m$, ratio $A_{280}:A_{260}$ being 0.71. Carbohydrate in the EDTA extract was almost double that released by vibrios heated at 100° for 20 min. while the protein was almost the same. No significant chemical differences between the extracts of two vibrio biotypes were observed.

**DISCUSSION**

The initial effect of tris+EDTA is probably at the cell surface (Goldschmidt & Wyss, 1967; Gray & Wilkinson, 1965). Sensitive organisms possess surface layers which are easily altered by EDTA, which allow the chelating agent to enter the cell readily and to damage sensitive intracellular targets so as to produce inactivation of the cell (Kaufman & McDonald, 1957; Martell & Calvin, 1953). The present study reveals a significant difference in the sensitivity of cholera and el tor vibrios to EDTA at concentrations round about 10 $\mu$g./ml. Thus there may be a small difference in the physicochemical structures of the walls of the two vibrio biotypes. The release of $^{32}$P compounds in ethanol of different concentrations also indicates some difference in the physicochemical structures of their walls and recalls the two species of *Pseudomonas* which exhibited different degrees of release of $^{32}$P compounds in ethanol (Salton, 1965).

Electron micrographs of ultrathin sections of *Vibrio cholerae* and *V. eltor* have revealed no differentiating feature in wall structure (unpublished observation). Fimbriae were detected on *V. eltor* (Barua & Chatterjee, 1964; Tweedy, Park & Hodgkiss, 1968) and on one strain of *V. cholerae* (Tweedy et al. 1968). But only the el tor strains examined by ourselves possessed fimbriae (unpublished observation). These fimbriae may have some relation with the greater susceptibility of the *V. eltor* strains to EDTA toxicity. However, there may exist other differentiating features in the physico-chemical structures of their surface layers, such as presence of metallic cations, the nature of their binding with the cell wall components, chemical composition of the cell wall, etc. contributing to the difference in their sensitivities to EDTA and ethanol; a difference in the sensitivity of the cell wall of cholera and el tor vibrios to polymyxin has recently been reported (Takeya, Koike & Iida, 1967).

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**REFERENCES**


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EXPLANATION OF PLATE

Fig. 1. Electron micrograph of Vibrio cholerae treated for 10 min. with tris (100 μM)+EDTA (50 μg./ml.) x 6000.

Fig. 2. Vibrio cholerae treated for 10 min. with tris (100 μM)+EDTA (50 μg./ml.)+lysozyme (50 μg./ml.) x 11,400.

Fig. 3. Vibrio cholerae treated for 80 min. with sodium lauryl sulphate (0.5 %, w/v) in 0.15 M-NaCl. The vibrios were preheated at 100° for 40 to 50 sec. x 9000.