

# CHARACTERISATION AND NEUTRALISATION OF *AEROMONAS HYDROPHILA* ENTEROTOXIN IN THE RABBIT ILEAL-LOOP MODEL

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## PLATE XXII

*AEROMONAS HYDROPHILA* strains have been shown to be enteropathogenic in experiments with the rabbit ileal-loop model (Sanyal, Singh and Sen, 1975; Annapurna and Sanyal, 1975, 1977; Wadström, Ljungh and Wretling, 1976; Gurwith *et al.*, 1977), and Annapurna and Sanyal (1977) showed that *A. hydrophila* produced a protein enterotoxin that caused the accumulation of fluid in the ileal loops. Enterotoxins have been identified from many diarrhoeagenic organisms such as *Vibrio cholerae* (Finkelstein, 1969 and 1973), enteropathogenic *Escherichia coli* (Dupont *et al.*, 1971; Gyles *et al.*, 1974; Finkelstein *et al.*, 1976), *Pseudomonas aeruginosa* (Kubota and Liu, 1971), *Klebsiella pneumoniae* (Klipstein and Engert, 1975), *Clostridium perfringens* (Duncan and Strong, 1969), *Shigella dysenteriae* type I (Keusch, Mata and Grady, 1970), and *V. parahaemolyticus* (Sakazaki *et al.*, 1974). The measurement of enterotoxic activity of the cell-free toxin of *V. cholerae* and titration of the toxin and antitoxin in the rabbit ileal loop were described by Burrows and Musteikis (1966) and Kasai and Burrows (1966). The present study of the effects of sterile culture filtrates and partially purified (crude) enterotoxin preparations from six isolates of *A. hydrophila* was undertaken: (i) to determine whether they all cause accumulation of fluid in the rabbit ileal loop; (ii) to study the effects of temperature and pH on the activity of the preparations; (iii) to measure the toxicity of the crude enterotoxin; and (iv) to determine the effect of antitoxin on the enterotoxin in neutralisation experiments.

## MATERIALS AND METHODS

**Organisms.** The six strains of *A. hydrophila* used in the study were isolated in this laboratory; three were from the stools of children and one adult with diarrhoea and one each from a healthy individual, a calf and the water from a hand pump. They were identified by the method of Hugh (1970) and maintained in peptone-agar stab cultures; they did not undergo more than three subcultures. A toxigenic *V. cholerae* strain 569B was a positive control.

**Preparation of culture filtrates.** Volumes (50 ml) of Brain Heart Infusion Broth (Difco) in

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250-ml conical flasks were each seeded with 2 ml of a 4–5 h peptone-water culture of the test strain. The flasks were incubated at 37°C for 18 h with constant shaking at 120 oscillations/min. and the cultures were then centrifuged at 22 000 *g* for 30 min. at 4°C. The supernatants were filtrates and crude enterotoxin preparations were used as inocula; the animals were killed after 6 h and the reactions in the loops were noted. The results were expressed as the volume of fluid

*Preparation of crude enterotoxin.* A modification of the method of Annapurna and Sanyal (1977) was used; 100-ml volumes of basal medium (Finkelstein and Lankford, quoted by Craig, 1966) in ten 500-ml conical flasks were each seeded with 5–6 colonies from pure growths of the strains on nutrient-agar plates. The flasks were incubated at 37°C for 18 h with constant shaking at 120 oscillations/min. and the cultures were then centrifuged at 22 000 *g* for 30 min. at 4°C. The supernatants were pooled and saturated with recrystallised ammonium sulphate at 4°C; the pH was maintained at 7.2 with ammonia solution. The suspension was held overnight at 4°C and centrifuged at 22 000 *g* for 30 min. at 4°C. The precipitate was dissolved in 10 ml of 0.4M phosphate-buffered saline (PBS) at pH 7.2 and dialysed against the same buffer at 4°C with 5–6 changes of buffer. The dialysate was passed through a membrane filter (Millipore) of 0.45  $\mu$ m average pore diameter and stored at –20°C. This filtrate was the partially purified (crude) enterotoxin preparation. The protein content was estimated by the method of Lowry *et al.* (1951).

*Enterotoxicity tests* were performed in ileal loops of adult albino rabbits weighing 1.5–2.0 kg by the method of De and Chatterje (1953) and Sanyal *et al.* (1975). One-ml portions of culture filtrates and crude enterotoxin preparations were used as inocula; the animals were killed after 6 h and the reactions in the loops were noted. The results were expressed as the volume of fluid accumulated per cm of ileal length. Each experiment was repeated 3–6 times.

*Effect of temperature on enterotoxin activity.* Culture filtrates and crude enterotoxin preparations were held at 56°C for 10, 20 and 30 min. and at 60°C for 10 and 20 min. in a water bath, and the heated preparations were tested for enterotoxicity in the rabbit ileal loop. Each experiment was done in triplicate.

*Effect of pH on enterotoxin activity.* Culture filtrate and crude enterotoxin preparations from *A. hydrophila* strain 7888 were adjusted to fixed pH values from 3.0 to 10.0 in citrate phosphate buffers (pH 3.0–8.0) or ammonia solution (pH 8.0–10.0). The preparations were held at 4°C for 12 h and then tested for enterotoxicity at pH 7.2. Each test was done in triplicate.

*Preparation of antitoxin.* Rabbits weighing 2.5 kg (Belgian strain) were immunised with crude enterotoxin prepared from *A. hydrophila* strain 20571 that contained protein 1.0 mg/ml. The first dose was given intracutaneously and contained 2 mg of protein in Freund's incomplete adjuvant; this was followed by six doses each of 500  $\mu$ g of protein, without adjuvant, at 5-day intervals by the same route. The animals were bled 7 days after the last injection. The crude serum was used as antitoxin. Normal serum collected before immunisation was the control.

*Assay of toxin.* The method of Burrows and Musteikis (1966) was used. The inocula were graded doses of crude enterotoxin prepared from *A. hydrophila* strain 20571 and seven or eight ileal loops of 10 cm length with 5-cm intervals were tied in each rabbit. The animals were killed after 6 h and the volumes of fluid accumulated and the lengths of loops were measured. The experiments were done in triplicate with each dose and the results were expressed as the average volume of fluid accumulated per cm of ileal length. The amount of toxin required to give a 50% reaction, equivalent to one unit of toxin, was calculated by interpolation of the dose-response curve of crude enterotoxin plotted as the volume of fluid accumulated per cm of ileum against the log<sub>10</sub> toxin concentration. Culture filtrate and PBS in 1-ml volumes served as positive and negative controls respectively.

*Neutralisation of toxin by antitoxin.* The method of Kasai and Burrows (1966) was used. Neutralising antibody was assayed with toxin prepared from *A. hydrophila* strain 20571; 0.5-ml volumes of toxin solution that contained 32.5  $\mu$ g (1.38 units) of toxin were mixed with 0.5-ml volumes of doubling dilutions of antitoxin from 1 in 2 to 1 in 512 and inoculated into the loops. The animals were killed after 6 h and the volumes of fluid accumulated and the lengths of ileal loops were measured. The neutralisation coefficients for all the dilutions of antitoxin were calculated by the method of Kasai and Burrows (1966). The volume of serum that contained one unit of antitoxin, i.e., the amount of antitoxin required to neutralise one unit of toxin, was

calculated by interpolation of the 50% point in a logarithmic plot of neutralisation coefficient versus volume of serum. Crude enterotoxin 0.5 ml mixed with 0.5 ml of normal rabbit serum and 1 ml of PBS served as positive and negative controls respectively. Similar experiments were done with toxin preparations from the other strains and the same antitoxin.

## RESULTS

### *Accumulation of fluid in ileal loops infected with culture filtrates and crude enterotoxins*

Culture filtrates and crude enterotoxin preparations from the six test strains of *A. hydrophila* caused the accumulation of fluid in ileal loops (fig. 1).

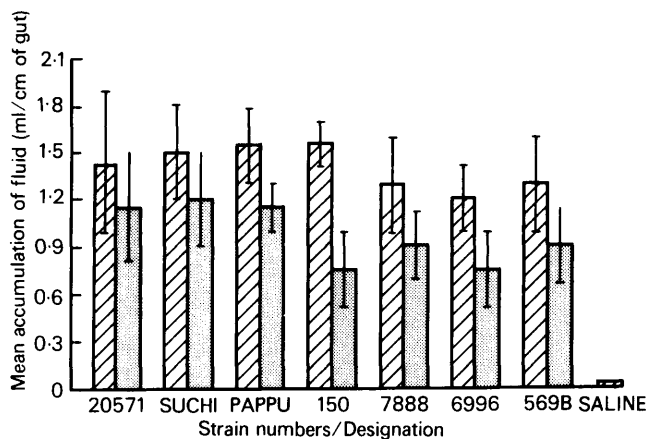


FIG. 1.—Volume of fluid accumulated in ileal loop after inoculation of culture filtrates (▨) and crude enterotoxin preparation (▤) of six strains of *Aeromonas hydrophila* and *Vibrio cholerae* strain 569B. I = Range of results in 3–6 tests.

The volume of fluid varied from strain to strain but was comparable with that produced by *V. cholerae* strain 569B. Culture filtrates caused the accumulation of 1.0–1.9 ml of fluid per cm of ileum whereas the crude enterotoxin preparation produced 0.5–1.5 ml per cm. Culture filtrates and the crude enterotoxin prepared from the toxigenic *V. cholerae* strain 569B under similar experimental conditions caused the accumulation of 1.1–1.7 and 0.7–1.2 ml of fluid per cm of ileum respectively.

### *The effect of temperature on culture filtrates and crude enterotoxin preparations*

Culture filtrates held at 60°C for 20 min. before testing failed to cause any accumulation of fluid in the ileal loop, whereas no significant change was detected after the filtrates were heated at 56°C for 10, 20 or 30 min. or at 60°C for 10 min. (fig. 2). There was no accumulation of fluid after inoculation of any crude enterotoxin preparation that had been heated at 56°C for 30 min. or 60°C for 20 min.

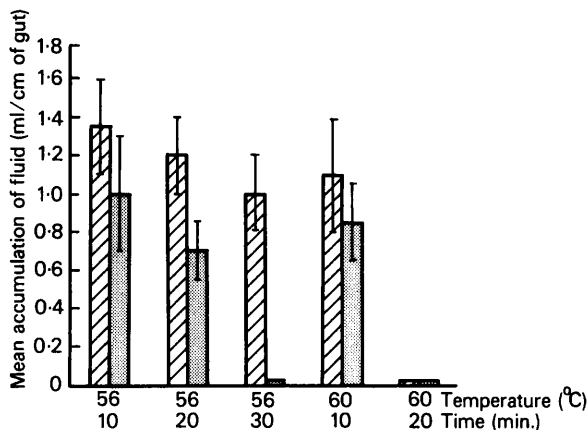


FIG. 2.—Volume of fluid accumulated in ileal loop after inoculation of culture filtrates (▨) and crude enterotoxin preparations (■) held at 56°C and 60°C. The data represent the average of six strains tested. I = Range of results in 3 tests.

### *The effect of pH on culture filtrates and crude enterotoxin preparations*

The culture filtrates and crude enterotoxin preparations showed maximum activity when held at pH 8.0–10.0 and a gradual diminution with decreasing pH (fig. 3). There was no accumulation of fluid when the culture filtrate was held at pH 3.0 or the crude enterotoxin preparation at pH 4.0.

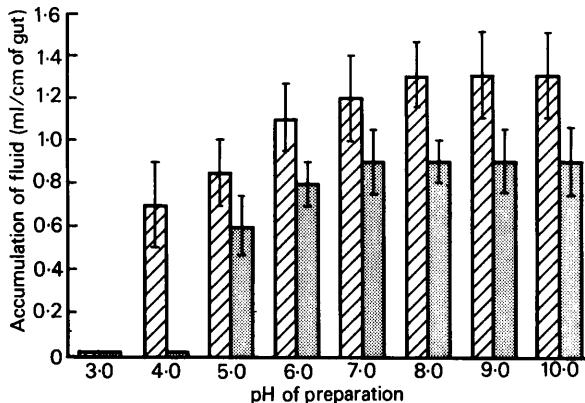


FIG. 3.—Volume of fluid accumulated in ileal loop after inoculation of culture filtrates (▨) and crude enterotoxin preparation (■) of *A. hydrophila* strain 7888 held at different pH values. I = Range of results in 3 tests.

### *Titration of enterotoxic activity*

The titration of the crude enterotoxin preparation from *A. hydrophila* strain 20571 showed that it contained 325 µg of protein per ml; 32.5 µg gave a maximal reaction and caused the accumulation of 1.5 ml of fluid per cm of ileum (fig. 4). The toxin-titration curve (fig. 5) shows that the upper asymptote was 1.4 ml per cm of ileum and the ED<sub>50</sub> was 0.71 ml/cm corresponding to

## AEROMONAS HYDROPHILA ENTEROTOXIN

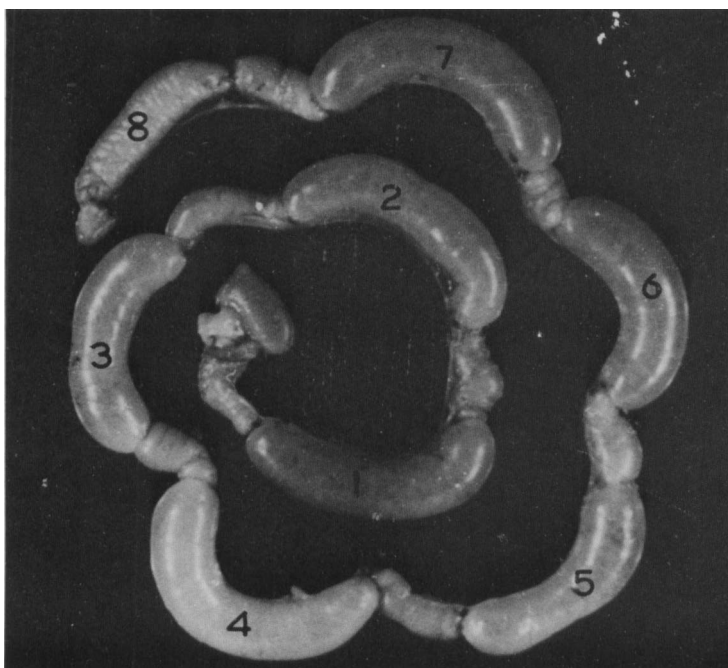


FIG. 4.—Results of the ileal-loop tests for the titration of *A. hydrophila* enterotoxin. Loop 1 (positive control) contained 1 ml of culture filtrate; loops 2–7 contained 32·5, 81·25, 162·5, 243·75, 325 and 487·5  $\mu$ g of protein respectively; loop 8 contained PBS (negative control).

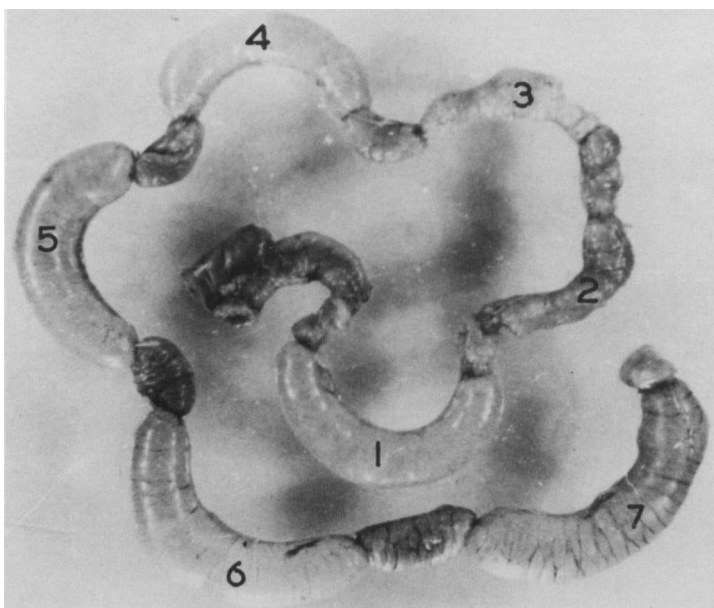


FIG. 6.—Results of the ileal-loop tests for the neutralisation of enterotoxin with antitoxin. Loop 1 (positive control) contained 0·5 ml of crude enterotoxin mixed with 0·5 ml of normal rabbit serum; loops 2–7 contained 0·5 ml of 1 in 32, 1 in 64, 1 in 128, 1 in 256, 1 in 512 and 1 in 1024 dilutions of antitoxin respectively plus 0·5 ml enterotoxin preparation that contained 32·5  $\mu$ g of protein.

[facing page 350.]

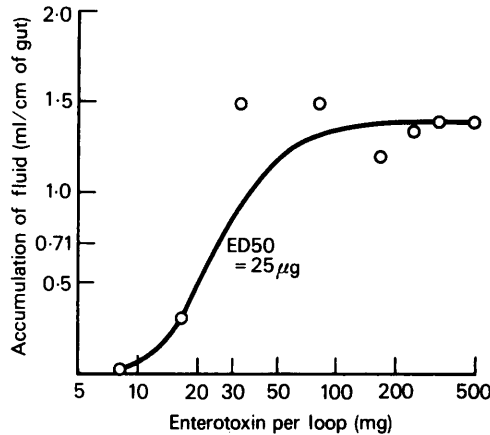


FIG. 5.—Dose-response curve of the crude enterotoxin preparation of *A. hydrophila* strain 20571.

1 unit of toxin activity. One unit was contained in 25  $\mu$ g dry weight of protein and the crude enterotoxin preparation contained 13.8 units of toxin per ml.

#### *Neutralisation of toxin by antitoxin*

There was complete neutralisation of toxin by antitoxin in dilutions up to 1 in 32 (fig. 6) and no neutralisation with 0.5 ml of toxin mixed with normal serum. Proportionally decreasing neutralisation was observed at higher dilutions of antitoxin. By interpolation of the 50% point in the logarithmic plot of neutralisation coefficient versus volume of antiserum (fig. 7), it was found that  $42 \times 10^{-4}$  ml of antiserum contained one unit of antitoxin, i.e., 238 units of antitoxin per ml of antiserum. The points on the graph fitted a slightly curved

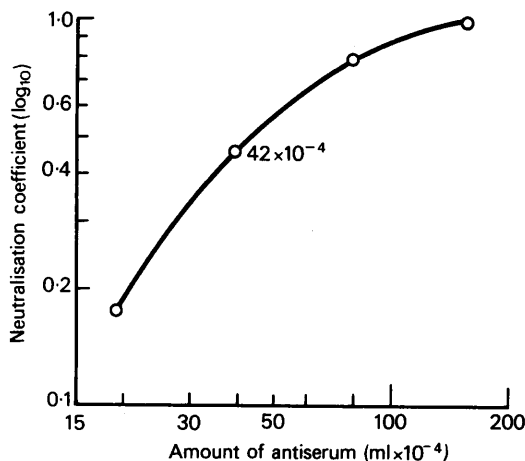


FIG. 7.—Graph of the neutralisation coefficient versus volume of antiserum, for interpolation of 50% neutralisation point (see text).

line when  $\log_{10}$  neutralisation coefficients were plotted against  $\log_{10}$  antitoxin concentrations over the range of neutralisation coefficients 0.178–1.0 and the 50% neutralisation volume was calculated by interpolation. Fig. 7 shows the titration of a single toxin preparation that contained 325  $\mu\text{g}$  of protein per ml against a single pool of hyperimmune rabbit antiserum. Similar neutralisation patterns were obtained in experiments with toxin preparations from the other strains.

## DISCUSSION

The results of this study show the enterotoxic properties of *A. hydrophila* strains isolated from a variety of sources. Culture filtrates and crude enterotoxin preparations contain a non-dialysable, heat- and acid-labile, antigenic protein(s) that causes the accumulation of fluid in rabbit ileal loops similar to that caused by a toxigenic strain of *V. cholerae*. The volume of fluid accumulated differed from strain to strain and the enterotoxin preparations from strains grown in brain-heart infusion broth and the basal medium varied in potency. Furthermore, the enterotoxic factor was not precipitated entirely by ammonium sulphate (unpublished data).

The enterotoxic factor was more temperature- and acid-stable in culture filtrates than in crude enterotoxin preparations. This might indicate that the enterotoxin is more stable in a complex medium than in a synthetic medium. The enterotoxic activity of culture filtrates and the crude enterotoxin preparations was lost when they were held at 60°C for 20 min. and the activity of crude enterotoxin preparation was lost when held at 56°C for 30 min. Furthermore the activity of culture filtrates and crude enterotoxin preparations was lost when they were held for 12 h at pH 3.0 and pH 4.0 respectively; the maximum activity was found in preparations tested at pH 8.0–10.0.

The present series of experiments have shown that *A. hydrophila* strains produce an enterotoxin that is probably implicated in the pathogenesis of diarrhoea in humans infected with *A. hydrophila*. It can be assayed in the rabbit ileal loop and shares several properties with cholera enterotoxin (Kasai and Burrows, 1966). The toxin is antigenic and can be neutralised by antitoxin, which suggests that local antitoxin production *in vivo* may confer immunity to *A. hydrophila* diarrhoea as has been shown for cholera. This hypothesis requires further investigation.

## SUMMARY

Cell-free culture filtrates and crude enterotoxin preparations from six strains of *Aeromonas hydrophila* caused the accumulation of fluid in rabbit ileal loops. This activity was due to a non-dialysable, heat and acid-labile antigenic protein and was lost when culture filtrates and crude enterotoxin preparations were heated at 60°C for 20 min. or 56°C for 30 min. respectively. Maximum activity was observed at pH 8.0–10.0; there was a gradual loss at lower pH and

activity was abolished in culture filtrates held at pH 3.0 and crude enterotoxin preparations held at pH 4.0. Titration of the crude enterotoxin preparations in rabbit ileal loops showed that the ED<sub>50</sub> (equivalent to 1 unit of toxin) was contained in 25 µg of protein; a logarithmic plot of the neutralisation coefficients against antiserum concentrations showed that one unit of antitoxin was contained in  $42 \times 10^{-4}$  ml of the antiserum.

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