DIALYSIS AND ULTRAFILTRATION OF A HEAT-STABLE ENTEROTOXIN FROM ESCHERICHIA COLI

R. J. Bywater

Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, University of Edinburgh

Smith and Halls (1967) described a heat-stable enterotoxin produced by certain strains of Escherichia coli that caused dilatation of ligated loops of intestine in pigs and calves. An enterotoxin produced by one of these strains caused net secretion of fluid and electrolyte in Thiry-Vella loops in calves (Bywater, 1970).

In their first report of the properties of heat-stable enterotoxin, Smith and Halls found that its dilating ability was retained on dialysis in Visking tubing against tap-water for 2 days. However, Kohler (1968) used a different test preparation, the production of diarrhoea in piglets after intragastric inoculation of enterotoxic material, and found that enterotoxic activity was present in dialysate, and that most but not all the activity in the dialysand was lost on prolonged dialysis. Later, Smith and Gyles (1970) found that dialysis produced a definite loss of heat-stable enterotoxin.

It seemed desirable to repeat the dialysis experiments, using the calf Thiry-Vella loop as a test preparation, and to define the molecular size of the enterotoxin more precisely by ultrafiltration.

MATERIALS AND METHODS

The organism, E. coli strain B44 (untypable), which was enteropathogenic for calves, was supplied by Dr H. Williams Smith, of the Houghton Poultry Research Station, Hunts.

Enterotoxic culture filtrate (240 ml) was prepared by culturing the E. coli on soft agar and separating the culture fluid as described previously (Bywater). The method is essentially that of Smith and Halls and involves heating the culture filtrate for 10 min. at 65°C. This killed any remaining organisms and destroyed heat-labile enterotoxin (Gyles and Barnum, 1969). Acetone (2040 ml) was added to the filtrate and the precipitate was allowed to settle overnight at −30°C. The supernatant acetone was decanted; the precipitate was dried and redissolved in 20 ml of sterile distilled water. The redissolved extract was spun at 4000 r.p.m. for 10 min. to remove undissolved material.

Dialysis for 48 hr. For each of the four tests, 5 ml of the concentrated extract was placed in Visking dialysis tubing and was dialysed against 200 ml of distilled water at 4°C for 48 hr. The dialysates and dialysands were then freeze-dried. Uninoculated culture medium was similarly treated for use as a control.

The test-preparations were Thiry-Vella loops previously prepared from the distal ileum of calves. The loops were 30 cm long, and retained intact blood and nerve supplies. The ends were exteriorised and attached to the abdominal wall; the remaining intestine was joined to restore continuity. During experiments, the stomata were sealed by modified Foley catheters. Loops remained responsive to the effects of enterotoxin and useful as test preparations for several months.

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Enterotoxic activity was tested by comparing absorption of fluid and electrolytes from the Thiry-Vella loops during consecutive periods of 30 min. During the first period, 250 mg of the control dialysate dissolved in an electrolyte solution was placed in the loop; absorption from this solution was compared with absorption from a solution containing 250 mg of enterotoxic dialysate during the next 30 min. Fluid movement was assessed with polyethylene glycol 4000 as a non-absorbable marker. Details of the method and solutions used have been described (Bywater). Osmolalities of control and test solutions were determined cryoscopically.

The effect of 250 mg of enterotoxic dialysand was tested similarly by comparison with 250 mg of dialysand from an extract of uninoculated medium.

**210 ml Culture filtrate**
- Precipitate with 8 vol. acetone;
- redissolve dry precipitate in 70 ml distilled water;
- centrifuge at 10,000 r.p.m. for 10 min.

- **65 ml Supernatant**
  - Filter through PM-10 membrane at 20 lb. per in.² for 5 hr

- **58 ml Filtrate**
  - Filter through UM-2 membrane
  - Fr. Dr. wt = 0.89 g (0.84 g)

- **8 ml Residue**
  - Fr. Dr. wt = 0.72 g (0.58 g)

**FIG. 1.—Preparative procedure for ultrafiltrate fractions of culture fluid.** Figures in parentheses refer to weights of freeze-dried material obtained from equal volumes of uninoculated medium (control). Fr. Dr. wt = Freeze-dried weight.

Dialysis for 7 days. To examine the proportion of activity that was dialysable over a longer period, 5 ml amounts of concentrated enterotoxic extract were prepared as above. The sacs were dialysed against 200 ml volumes of distilled water at 4°C with daily changes of water for 7 days. At the end of this time, the dialysand was freeze-dried and 250 mg amounts were compared with the same weight of material obtained from uninoculated culture medium treated in the same way.

**Ultrafiltration experiments.** From the B44 strain of *E. coli* 210 ml of culture filtrate was prepared and precipitated by eight times its volume of acetone and the precipitate was treated as shown in fig. 1. Uninoculated culture medium was similarly treated to provide control material.

The membranes used were Amicon PM-10 and UM-2 (Amicon Ltd, High Wycombe, Bucks) with pore sizes such that the cut-off was at molecular weights of about 10,000 and 1000 respectively. Ultrafiltration was carried out under nitrogen at 20–40 lb. per in.² (1-41–2.8 x 10⁴ kg per m²) in a cell fitted with a magnetic stirrer to prevent clogging of the membrane. The residue from the PM-10 membrane (fraction D), the residue from the UM-2 membrane (fraction E), and the filtrate from the UM-2 membrane (fraction F) were all freeze-dried.

Samples from the three fractions were added to the test solution and absorption from enterotoxic fluid was compared during consecutive absorption periods with that from fluid containing equal weights of corresponding material from uninoculated culture medium. The weights of material tested were: fraction D 120 mg, fraction E 100 mg, fraction F 50 mg.
RESULTS

Dialysis for 48 hr. Figure 2 shows the net absorption of fluid, sodium, bicarbonate and chloride from solutions containing 250 mg of freeze-dried dialysate of control and enterotoxic material respectively. Figure 3 shows similarly the effect of 250 mg of dialysand. In both cases, the effect of dialysed extract on all four parameters was significant. Differences in osmolality between test and control solutions were negligible.

![Graph showing net absorption of fluid and electrolytes](image)

**FIG. 2.**—The effect of 250 mg of freeze-dried dialysate (48 hr dialysis) on net absorption of fluid and electrolytes from a Thiry-Vella loop. Control material was 250 mg of freeze-dried dialysate of uninoculated medium. Each column represents the mean of five observations. Vertical bars show standard errors. Positive signs indicate net absorption, negative signs indicate net secretion. Differences were tested by paired t-test.

Dialysis for 7 days. The table shows the effect on fluid absorption of 250 mg of dialysand after 7 days' dialysis against seven changes of distilled water. Most of the enterotoxic activity was lost, and the difference between the fluid absorption during control and test periods was not significant. The effects on electrolyte movement were not tested.

Ultrafiltration experiments. In fig. 4 the stippled histograms show the differences between the mean fluid absorption during control periods and the subsequent control periods when the loops contained fractions from enterotoxic filtrate. The differences were attributed to enterotoxin activity and are referred to as exudate.
The clear histograms, T1, T2 and T3, represent the theoretical response of the loops to the amount of enterotoxin in the three cases: T1—if all the toxic activity is retained by the PM-10 membrane; T2—if all the toxic activity is retained by the UM-2 membrane, but none by the PM-10; and T3—if neither the PM-10 nor the UM-2 membrane retains enterotoxic activity.

**Table**

*Amount of fluid absorbed from Thiry-Vella loops in the presence of 250 mg of enterotoxin 7-day dialysand*

<table>
<thead>
<tr>
<th>Solution in loop</th>
<th>Fluid absorbed from loop (ml per 30 min.)</th>
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<tbody>
<tr>
<td>Control extract</td>
<td>7.1 (±2.2)</td>
</tr>
<tr>
<td>Enterotoxic extract</td>
<td>4.1 (±0.76)</td>
</tr>
</tbody>
</table>

Figures are means (±SE) of five observations. Difference between means is not significant (P>0.05 paired t-test).

These figures were calculated from the means of at least three previous responses of the loops to standard doses of enterotoxin (i.e. that obtained from 30 ml of culture filtrate) given that the total amount of enterotoxic culture filtrate at the start of the experiment was 210 ml (seven standard doses).
Material from each fraction was tested at least twice. Differences in osmolalities between solutions of control and test material were small but when they were found, adjustments were made to correct them.

The results showed that the responses corresponded most closely to the theoretical case T2, indicating that the molecular weight of most of the active material lay in the 1000–10,000 range. However, some activity was found in fraction F (molecular weight less than 1000) and, although the amount of exudate involved was small, this was significant (P<0.05).

**DISCUSSION**

The dialysis experiments showed that enterotoxic material passed through Visking tubing, and could be demonstrated in the dialysate. Prolonged dialysis removed most of the activity from the dialysand. Kohler (1968) obtained similar results using a heat-stable enterotoxin from *E. coli* of porcine origin. His test method involved intragastric inoculation of piglets, which may be subject to problems such as coincidental diarrhoea. Smith and Gyles (1970), also found that heat-stable enterotoxin was lost on dialysis, but this was contrary to the results of Smith and Halls (1967). However, in neither of these two studies was
the dialysate examined, and each involved the use of the ligated loop, a technique that may be less suited to quantitative observation than the Thiry-Vella loop (Bywater, 1970).

The dialysability of the heat-stable enterotoxin may be contrasted with the non-dialysability of the heat-labile enterotoxin described by Gyles and Barnum (1969), although it has been suggested that the heat-labile and heat-stable enterotoxins may be different forms of the same enterotoxin (Smith and Gyles).

The fact that the heat-stable toxin was dialysable suggested that its molecular weight was probably less than 10,000, but a closer approximation was obtained by ultrafiltration. These experiments showed that most of the enterotoxic activity lay in the molecular-weight range, 1000–10,000 because the results corresponded more closely to the theoretical case T2 than to T1 or T3 (fig. 4). However, fractions D and F appeared to possess more activity than would have been expected if T2 was the complete explanation. The greater activity of fraction D than forecast may be a result of the fact that the enterotoxin batch from which the fractions were obtained differed from that on which the calculation for T1, T2, and T3 were based. In general, batches of culture filtrate appeared to differ little in their enterotoxin content, but in this case it may be that the fractionated batch (210 ml) contained more than seven times the standard dose.

The significant activity of fraction F could not be explained simply in terms of T2, because whatever the potency of the original preparation, no activity would pass the UM-2 membrane if T2 were correct. This suggested that some of the material had a molecular weight of less than 1000. This formed only a small proportion of the total activity.

The molecular size appeared to be quite different from that of cholera enterotoxin, which has been estimated to be 61,000 (Finkelstein and Lospalluto, 1969). However, the present experiments were carried out with acetone-precipitated material, and the acetone precipitation may have affected the molecular weight of the precipitate, though the results are in general agreement with dialysis experiments which did not involve acetone precipitation (Kohler).

The properties described suggest that ultrafiltration may be a useful method of removing large and small molecular-weight contaminants as an initial step in purification of heat-stable enterotoxin.

**SUMMARY**

A heat-stable enterotoxin from a strain of *Escherichia coli* enteropathogenic for calves was subjected to dialysis and ultrafiltration, and the resulting fractions were tested in Thiry-Vella loops in calves.

The active material was dialysable, and could be demonstrated in the dialysate. After 7 days' dialysis, the activity remaining in the dialysand was not significant.

Ultrafiltration suggested that the molecular weight of most of the active material was between 1000 and 10,000, although a small amount appeared to have a molecular weight less than 1000.
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REFERENCES


