Ussing chambers were mounted with stripped rabbit ileal mucosa and either crude or pure toxin A added to the mucosal side of the chamber to final concentrations of 800 and 30 µg/ml respectively; increases in short-circuit currents of the order of 25 µA were observed in both preparations over a period of 90 min, at which point the tissue was responsive to theophylline but not glucose. Histology showed that damage to villus tips had occurred by 90 min in contrast to Hughes et al. (1983) who claimed no damage was induced in similar experiments. Unidirectional flux experiments have not yet been carried out and may be of evaluative value in analysing perturbations in active transport systems until and unless effective means are found for controlling the effects due to cell damage.


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**Clostridium perfringens enterotoxin: a brief review**

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The enterotoxin produced by *Clostridium perfringens* is responsible for one of the most common types of food poisoning in this country. During the period 1970–1981, there were 11603, reported cases of food poisoning in England and Wales: *Salmonella* accounted for 91953 (79%) cases and *C. perfringens* was responsible for 19888 (17%) cases. Illness often follows the preparation of meat or poultry dishes to be served to large numbers of people. The food is frequently pre-cooked the day before serving and then inadequately stored, so that spores surviving the cooking process may germinate and multiply to hazardous levels. Ingestion of large numbers of vegetative cells of *C. perfringens* in food leads to multiplication and sporulation in the intestine. When the mature spores are released from the sporangia there is an accompanying release of enterotoxin, leading rise to the symptoms of diarrhoea and abdominal cramps. Between 1970 and 1981, 438 (71%) of the 617 outbreaks caused by *C. perfringens* occurred in mass-catering situations. Fatal cases are relatively rare, most people recovering within 12–15 hr. Spores surviving the cooking process are abnormal in that they are not able to sporulate, nor do they produce enterotoxin. Thus the lethal potential must be recognized, especially since such a high proportion of outbreaks occurs in hospitals and old people's homes.

Duncan et al. (1972) isolated mutants of *C. perfringens* with altered abilities to sporulate, and used them to show that enterotoxin was a sporulation-specific gene product. About 3 h after inoculation of vegetative cells into sporulation medium, heat-resistant spores develop, followed closely by the accumulation of intracellular enterotoxin. Maximum numbers of spores are obtained after 10 h, and at this time free spores can be detected, which reach a maximum after 14–15 h. With the liberation of mature spores enterotoxin is released and hence intracellular toxin decreases whilst extracellular toxin increases in parallel with the increase in numbers of free spores (Duncan, 1973).

The role of enterotoxin at sporulation is still not clear, but it is possible that its accumulation may result from over-production of a protein synthesized for some specific function.

Abbreiviation used: ELISA, enzyme-linked immunosorbet assay.


Frieben & Duncan (1973) extracted a component of the spore coat protein which was identical in biological and serological activity to enterotoxin. It was also observed that some toxigenic strains develop large paracrystalline inclusions at the same stage that deposition of fragments of spore coat material occurs (Duncan et al., 1973). Studies with mutants suggested that a single gene mutation was responsible for lack of spores, enterotoxin and paracrystalline inclusion. Although enterotoxin is only produced by sporulating organisms, not all strains which sporulate produce the toxin, and even amongst toxigenic strains there is not always a direct relationship between good sporulaters and good toxin producers.

Various sporulation media have been developed in attempts to obtain maximum toxin production in *vitro* but there is apparently no single medium which is suitable for all strains (Skjelkvale et al., 1979). Heat-resistant strains (i.e. those which withstand heating at 100°C for 60 min) produce more spores and toxin when raffinose is a component of the medium, whereas heat-sensitive strains prefer raffinose (Table 1). Equal in importance to the composition of the medium are the culture conditions employed. For example, heating the cultures at 75°C for 20 min before transferring to sporulation medium greatly increases the yield of spores and toxin (Tsai & Riemann, 1974). The mechanism of this heat activation is unknown but it has been suggested that heat could induce dormant spores to germinate or that it could select for those cells with an increased inherent ability to sporulate.

Various procedures have been developed for the purification of enterotoxin from sonicated cell extracts, including affinity chromatography (Scott & Duncan, 1975; Barnhart et al., 1976) and preparative polyacrylamide-gel electrophoresis (Enders & Duncan, 1977). Hauschild & Hilsehimer (1971) and Stark & Duncan (1972) employed procedures involving several chromatographic steps. In 1973, Sakaguchi and co-workers published details of a simplified method involving ammonium sulphate precipitation, differential solubilization and gel filtration on Sephadex G-200 (Sakaguchi et al., 1973). This method was improved (Granum & Whitaker, 1980) by replacing the differential solubilization with a second ammonium sulphate precipitation step. This procedure works well providing that the enterotoxin in the sonicated cell extract represents at least 8–10% of the total protein (Bartholomew & Stringer, 1983). Thus great care must be taken in choosing the correct strain and sporulation conditions to provide maximum toxin production.
The enterotoxin is a protein with a single polypeptide chain with a relative mass of 34000–35000 and an isoelectric point of 4.3. It contains a single residue of cysteine with no cystine and has C-terminal glycine. Initial studies suggested that the enterotoxin was resistant to trypsin treatment (Hauschild & Hilsheimer, 1971). However, a recent report (Granum et al., 1981) described how trypsin treatment of the enterotoxin resulted in a 2.5-fold increase in biological activity as measured using the guinea-pig skin test.

Extensive studies have been performed in attempts to elaborate the mode of action of the toxin. Early investigations demonstrated that the toxin could induce fluid accumulation in ileal loops of rabbits and lambs, and erythema and increased capillary permeability in the skin of guinea-pigs (Duncan & Strong, 1969; Hauschild, 1970). Niilo (1971) described the systemic effects of the enterotoxin when injected intravenously into lambs, rabbits and guinea-pigs. Lambs developed transitory diarrhoea, lachrymation, salivation, lassitude and dyspnoea within 1–5h of inoculation. Animals that died had hyperaemic small intestinal mucosa, and congestion in the liver, lungs, spleen and kidneys.

McDonel (1974) established a rat model for studying transport alterations due to the action of the enterotoxin. He reported that in the ileum a reversal of transport, from absorption to secretion of fluid and sodium and chloride ions, was induced. Glucose absorption was inhibited whilst transport of potassium and bicarbonate was unaffected. Histological studies showed that desquamation of intestinal epithelial cells was occurring at villus tips. Using the scanning electron microscope the toxin was found to cause the appearance of spherical structures on the surface of the villus tips. These were apparently epithelial cells degenerating and extruding into the lumen. Ultra-thin sections viewed under the transmission electron microscope revealed that the brush-borders had lost their characteristic folded configuration and that blebs were forming in the apical membrane of epithelial cells (McDonel et al., 1978). The evidence from this study pointed towards the microvillus membrane of the villus tip epithelial cells being the primary site of action of the enterotoxin.

Metabolic studies (McDonel & Duncan, 1975) with rats everted ileal sacs demonstrated that toxin-treated sacs consumed considerably less oxygen than untreated ones. However, glucose uptake and lactate production were unaffected. It was concluded that oxidative metabolism is

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Table 1. Relationship between sporulation of heat-resistant and heat-sensitive strains of C. perfringens isolated from different sources and the presence of starch or raffinose in the sporulation medium

<table>
<thead>
<tr>
<th>Source of strain</th>
<th>Number of HR and HS strains</th>
<th>Number of strains producing maximum numbers of spores in each sporulation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outbreaks of food poisoning</td>
<td>DS 35 DR 30</td>
<td>TR 10</td>
</tr>
<tr>
<td>Various clinical infections</td>
<td>TS 27</td>
<td>TR 4</td>
</tr>
<tr>
<td>Faeces of hospital patients</td>
<td>TS 52</td>
<td>TR 13</td>
</tr>
</tbody>
</table>

HR, heat-resistant (i.e. spores survive 100°C for 60min); HS, heat-sensitive. Vegetative cells of the various strains were transferred after heat activation to each of the following sporulation media: DS, Duncan & Strong medium (Duncan, 1973) with 0.4% (w/v) starch; DR, Duncan & Strong medium with 0.4% (w/v) raffinose; TS, Tsai, Torres-Anjel & Riemann medium (Tsai & Riemann, 1974) with 0.4% (w/v) starch; TR, Tsai, Torres-Anjel & Riemann medium with 0.4% (w/v) raffinose.

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Table 2. Sensitivity of biological and serological methods for the detection and assay of C. perfringens enterotoxin

<table>
<thead>
<tr>
<th>Method</th>
<th>Enterotoxin (µg)</th>
<th>Dose volume (ml)</th>
<th>Extrapolated minimal concentration of enterotoxin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythema activity</td>
<td>0.06–0.125</td>
<td>0.05</td>
<td>1.25–2.5</td>
</tr>
<tr>
<td>Mouse lethality (LD₅₀)</td>
<td>3.5</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Rabbit ileal loop (standard)</td>
<td>29.0</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Rabbit ileal loop (90min)</td>
<td>6.25</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Chicken ileal loop</td>
<td>20.0–30.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf ileal loop</td>
<td>42.0–80.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep ileal loop</td>
<td>26.0–80.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single-gel diffusion</td>
<td>0.3</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Double-gel diffusion</td>
<td>0.013</td>
<td>0.025</td>
<td>0.5</td>
</tr>
<tr>
<td>Electro-immunodiffusion</td>
<td>0.01</td>
<td>0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>Counterimmunoelectrophoresis</td>
<td>0.002</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>Reversed passive haemagglutination</td>
<td>0.00005</td>
<td>0.05</td>
<td>0.001</td>
</tr>
<tr>
<td>Radiimmunoassay</td>
<td>0.001</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>ELISA</td>
<td>0.00005</td>
<td>0.05</td>
<td>0.001</td>
</tr>
</tbody>
</table>

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Extensive studies have been performed in attempts to elaborate the mode of action of the toxin. Early investigations demonstrated that the toxin could induce fluid accumulation in ileal loops of rabbits and lambs, and erythema and increased capillary permeability in the skin of guinea-pigs (Duncan & Strong, 1969; Hauschild, 1970). Niilo (1971) described the systemic effects of the enterotoxin when injected intravenously into lambs, rabbits and guinea-pigs. Lambs developed transitory diarrhoea, lachrymation, salivation, lassitude and dyspnoea within 1–5h of inoculation. Animals that died had hyperaemic small intestinal mucosa, and congestion in the liver, lungs, spleen and kidneys.
hibited by the toxin whereas glycolysis remains unaffected. Further studies (McDonel & Duncan, 1977) using isolated rat liver mitochondria demonstrated a 30% inhibition of oxygen consumption.

Cultured Vero cells have proved an excellent model system for studying the mode of action of the toxin (McClane & McDonald, 1979). In this system, the toxin causes rapid inhibition of macromolecular synthesis, loss of viability and morphological alterations. It was proposed that these effects in Vero cells result from binding of toxin to the cytoplasmic membrane causing structural damage with subsequent loss of essential substances. This leads to a failure of macromolecular synthesis, gross morphological damage and eventual cell death.

Methods for assay of enterotoxin may be divided into two groups: those which measure the biological effects of the molecule, and those which assay the toxin by immunological means (Table 2). In addition, a sensitive assay based on the ability of the toxin to inhibit the plating efficiency of Vero cells grown in culture has been described (McDonel & McClane, 1981, Giugliano et al., 1983).

The immunochromatography trials which have been developed include double-gel diffusion (Stringer et al., 1982), electroimmunoaffinity (Duncan & Somers, 1972), counterimmunoelectrophoresis (Naik & Duncan, 1977) and reverse passive haemagglutination (Uemura et al., 1973). These have the advantages of greater sensitivity, specificity and reproducibility. Recently, an ELISA for the enterotoxin has been reported using the four-layer sandwich technique (Olsvik et al., 1982). However, we have developed a double-antibody sandwich method which has proved very satisfactory for rapid assay of faecal enterotoxin. Detection of faecal enterotoxin is one of the criteria we use to confirm C. perfringens outbreaks and our initial results with this ELISA method are encouraging (Table 3).

Scott, V. N. & Duncan, C. L. (1975) Infect. Immun. 12, 536–543