SEPARATION AND PURIFICATION OF ENTEROTOXINS FROM A STRAIN *ESCHERICHIA COLI* PATHOGENIC FOR PIGS

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INVESTIGATIONS on the pathogenic role of *Escherichia coli* enterotoxin (Kohler, 1968, 1971*a*; Smith and Gyles, 1970*b*; Kohler and Cross, 1971) and mode of action (Al-Awqati, Wallace and Greenough, 1972; Evans *et al.*, 1972) have been performed with very impure material (Jacks *et al.*, 1973). Enterotoxins that are more accurately defined and of greater purity are needed so that (1) results obtained by workers in different laboratories can be properly compared (2) the importance of enterotoxin in scouring can be further elucidated, and (3) the development of a method of assay more reliable than that made in ligated segments of intestine may be facilitated.

Attempts to concentrate and purify enterotoxins produced in culture supernates by pathogenic *E. coli* have given contradictory results (Kohler, 1968, 1971*b*; Smith and Gyles, 1970*a*; Larivière, 1971; Bywater, 1972; Jacks *et al.*, 1973). This may be because different enterotoxins are produced by different strains (Truszczyński and Ciosek, 1972) or because a single strain may produce enterotoxins with a variety of molecular weights; a further possibility is that the method of culture influences which of the various enterotoxic components predominates.

We have examined, by ultrafiltration and column chromatography, enterotoxins produced by pathogenic *E. coli* in defined medium (Mitchell, Tame and Kenworthy, 1974) thereby avoiding some of the complexities introduced by media containing much ultraviolet-absorbent material.

MATERIALS AND METHODS

Bacterial strain. *E. coli* strain P16 (serotype O9; K9) was obtained from Dr H. W. Smith.

Defined medium and culture technique. These have been described previously (Mitchell *et al.*, 1974).

Assay method for enterotoxin. The ligated intestinal-segment method (De and Chatterje, 1953; Moon, Sorensen and Sautter, 1966; Smith and Gyles, 1970*b*) was used as by Mitchell *et al.* (1974).

Concentration methods. Enterotoxic supernates were prepared from defined-medium cultures of *E. coli* by centrifugation for 20 min. at 75,000 *g*. These preparations were then either (a) freeze-dried and redissolved in distilled water to give 10- to 25-fold concentration, or (b) reverse-dialysed against 30% w/v polyethylene glycol for 24 hours to give between 7- and 15-fold concentration; the cellulose membrane used had a molecular-weight exclusion of between 10^4 and 2×10^4 daltons.

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Ultrafiltration. Diaflo apparatus was used at 138–276 kN per m² with XM.100, PM.30, PM.10 or UM.2 membranes having, respectively, molecular-weight exclusion values of 10⁵, 3 × 10⁴, 10⁴ and 10³. Freeze-dried concentrates were too viscous for ultrafiltration, and ultrafiltrations of reverse-dialysed material was inconveniently slow; most of the studies were therefore performed directly on broth supernates.

In an enterotoxigenic broth, the proportion of the total enterotoxigenic activity caused by the unfilterable toxins and the proportion due to the filterable toxins could be calculated from the filtration data by means of formulae derived as follows: let T = enterotoxigenic activity per ml in the filtrate; X = activity per ml in the retentate; A = activity per ml due to unfilterable toxin; a = activity per ml due to filterable toxin; v = volume of retentate in ml; V = total volume in ml; P = activity per ml due to unfilterable toxin if the retentate was reconstituted to the original volume V ; H = the percentage of unfilterable toxin before filtration; L = the percentage of lower molecular weight (MW) filterable toxin before filtration. If it is assumed that any loss of activity occurs equally in the filterable and unfilterable toxin and that there is complete separation, then

$$\begin{aligned} a &= T \text{ (no } A \text{ passes through the filter)} \\ X &= A + a \text{ (by definition)} \end{aligned} \quad (1)$$

$$\therefore A = X - a \quad (2)$$

Substituting for a from (1) in (2)

$$A = X - T \quad (3)$$

$$\text{and } P = A \frac{v}{V} \text{ (by definition)} \quad (4)$$

Substituting A from (3) in (4)

$$P = (X - T) \frac{v}{V} \quad (5)$$

$$\text{and } H = \frac{P}{P + a} \times 100 \text{ (by definition)} \quad (6)$$

Substituting P and a in (6) from equations (5) and (1)

$$H = \frac{(X - T) \frac{v}{V}}{(X - T) \frac{v}{V} + T} \times 100 \quad (7)$$

$$\text{and } L = 100 - H \text{ (by definition)} \quad (8)$$

Separation of enterotoxins on Sepharose and Sephadex. Concentrates were applied in volumes of 10 to 20 ml to columns of Sepharose 4B, Sephadex G50 or Sephadex G10. Eluants consisted of 0.85% NaCl adjusted to pH 7.4 by the addition of alkali, or phosphate buffer (pH 7) made up of 0.7% K₂HPO₄ and 0.3% NaH₂PO₄. The following substances were used for the preparation of standard curves for MW calculations: 12 crystals of dextran blue, MW 2 × 10⁷, used to find the void volume; 10 ml impure porcine IgM, MW 9 × 10⁵; 50 mg dextran sulphate, MW 5 × 10⁵; 50 mg porcine IgG, MW 1.5 × 10⁵; 25 mg lysozyme, MW 1.44 × 10⁴; 4 ml standard (40 units per ml) insulin, MW 5.8 × 10³; 50 mg streptomycin sulphate, MW 1358, assuming monodissociation; 25 mg ATP, MW 623; 0.5 mg tetracycline, MW 444; 0.5 mg riboflavin, MW 376; 25 mg adenosine, MW 267; 12.5 mg 2-amino-purine, MW 198; 12.5 mg 5-bromo-uracil, MW 191; 12.5 mg adenine, MW 135. The IgM and IgG samples were obtained from Dr P. Porter, of this laboratory, and the rest were commercially available. Fractions were collected in 4.75- or 9.5-ml portions and the effluent from the columns was continuously monitored for ultraviolet-light transmission at 280 nm. All the standard substances absorbed in the ultraviolet region except streptomycin, which was assayed by measurement of the zone of inhibition on a lawn of sensitive *E. coli*

strain G1253 (O147; K89; K88ac; H19). The streptomycin could not be used with phosphate buffer. Enterotoxigenic activity was assayed by the ligated-intestine method. For comparative purposes, all results are expressed in KAV units, defined as follows:

$$\text{KAV} = \frac{\text{elution volume} - \text{void volume}}{\text{bed volume} - \text{void volume}}$$

Standard curves for MW determinations were drawn by plotting KAV against the reciprocal of the MW.

RESULTS

Concentration of enterotoxin

Initial attempts at concentration by means of solvent and salt precipitations were unsuccessful, giving at best less than 10% recovery of enterotoxigenic activity.

TABLE I

Ultrafiltration of enterotoxigenic culture supernates from Escherichia coli strain P16

Starting material	Number of separate filtrations	Filter used	Fraction tested	Volume reduction	Degree of concentration of activity*	Percentage of unfilterable activity†	Percentage of activity lost in filtration
CS	1	XM.100	Retentate Filtrate	$\times 10$ $\times 1$	$\times 3.5$ $\times 1.0$	20	0
CS	3	PM.30	Retentate Filtrate	$\times 15$ $\times 1$	$\times 2.8$ $\times 0.8$	14	7
CS	2	PM.10	Retentate Filtrate	$\times 8$ $\times 1$	$\times 2.9$ $\times 0.4$	44	29
PM.30 filtrate of CS	2	UM.2	Retentate Filtrate	$\times 12$ $\times 1$	$\times 4.3$ $\times 0.4$	45	27

* Derived from ligated-loop assay.

† Calculated by equation (7) derived under *Materials and methods*.
CS = Culture supernate.

Reverse-dialysis gave about 50% recovery whilst freeze-drying gave 80 to 100%. Unfortunately the viscosity of freeze-dried concentrates rendered them difficult to apply to columns and almost impossible to ultrafilter.

Ultrafiltration

The slime in culture supernates tended to block ultrafilters as filtration progressed. Thus the time taken for the procedure became excessively long, either where the material was fairly concentrated or where filters with small pore size were used. The data in table I indicate that activity tended to be lost in filtrations of long duration.

The data in tables I and II show that the enterotoxigenic activity in the supernates of strain P16 could be split into four fractions according to MW, as follows: $>10^5$, 3×10^4 – 10^4 , 10^4 – 10^3 , $<10^3$.

XM.100 filtration. This indicated that 14 to 20% of the enterotoxigenic activity was associated with MW values exceeding 10^5 .

PM.30 filtration. An insignificant amount of the activity that passed through an XM.100 filter was retained by a PM.30 filter (table II) showing that no significant activity was associated with MW values in the range 10^5 to 3×10^4 .

PM.10 filtration. The results in table I suggest that 44% of the activity of the enterotoxin supernates was contained in fractions having a MW exceeding 10^4 . The data obtained with XM.100 and PM.30 membranes showed that 14–20% of the total toxin had a MW exceeding 3×10^4 ; therefore 24–30% must have had a MW between 3×10^4 and 10^4 .

UM.2 filtration. Because of their slime content, it was impracticable to filter the enterotoxin culture supernates directly through a UM.2 membrane. Therefore, all UM.2 filtrations were performed on PM.30 filtrates, which lacked 14–20% of the total activity (tables I and II). From the data in table I, it can be calculated that about 44–47% of the total enterotoxin activity was associated with a MW of less than 10^3 , and 33–42% with a MW of more than 10^3 but less

TABLE II

Sequential ultrafiltration of enterotoxin concentrate prepared by reverse dialysis of culture supernates of E. coli strain P16

Filter used	Fraction tested	Volume reduction	Degree of concentration of activity*	Percentage of unfilterable activity†
XM.100	Retentate	$\times 10$	$\times 2.4$	14
	Filtrate	$\times 1$	$\times 0.9$	
PM.30	Retentate	$\times 10$	$\times 1.1$	2
	Filtrate	$\times 1$	$\times 0.9$	
UM.2	Retentate	$\times 6$	$\times 3.4$	24
	Filtrate	$\times 1$	$\times 1.2$	

Footnotes as in table I.

No activity was lost in filtration.

than 3×10^4 . As already indicated, 24–30% had a MW between 3×10^4 and 10^4 ; therefore 3–18% of the total activity was associated with MW values in the range 10^4 – 10^3 .

Comparison of the data for UM.2 filtrations in tables I and II shows that a much higher percentage of low-MW toxin was obtained after sequential filtration. This contradicts the theoretical expectation, because the toxin used in sequential filtrations was first concentrated by reverse dialysis and should have lost much of the material of MW less than 10^3 . This apparent anomaly can best be explained by assuming that one or more of the higher MW toxins fragmented during filtration.

Gel-filtration

The standard curves for MW calculation are shown in fig. 1.

High-MW toxin ($>10^5$). Because the 25-fold concentrate produced by freeze-drying was very slimy and viscous, the ultraviolet absorption peak, obtained from Sepharose-4B gel filtration, was retarded and excessively broad (fig. 2a). However, when the fractions responsible for this peak were re-concentrated 10-fold by reverse dialysis and run again on Sepharose 4B (fig. 2b)

a much sharper peak was obtained. Of the total activity added to the column in the first run (fig. 2a) 17% was recovered between KAV 0.2 and 0.7 in the

FIG. 1.—Standard curves for determining MW values from gel-filtration results. The curves were obtained with (a) Sepharose 4B, (b) Sephadex G50, and (c) Sephadex G10.

second run (fig. 2b), i.e., it lay between $MW 5.2 \times 10^5$ and 9.9×10^5 as calculated from the standard curve (fig. 1a). If the apex of the ultraviolet absorption peak were coincident with maximum enterotoxic activity, the MW would be 5.9×10^5 .

FIG. 2.—Separation of high-MW enterotoxin on Sepharose 4B. The column measured 48×3.5 cm and the eluant was saline. (a) The toxin consisted of 15 ml of a 25-fold concentrate prepared by freeze-drying. (b) The toxin consisted of 22 ml of a 10-fold concentrate prepared by reverse dialysis of fractions between KAV 0.5 and 1.1 from the run shown in (a).

Medium-MW toxin (3×10^4 – 10^3). This was separated on Sephadex G50 with either 0.85% NaCl or phosphate-buffer eluant. When a 0.1% NaCl

FIG. 3.—Separation of medium-MW enterotoxin on Sephadex G50. ○—○ = Ultraviolet transmission. ■ = Enterotoxigenic activity. (a) The column measured 90 × 3.5 cm and the eluant was phosphate buffer. The toxin consisted of 20 ml of a 15-fold concentrate prepared by freeze-drying the exclusion fractions from a G10 column. (b) The column measured 52 × 3.5 cm and the eluant was saline. The toxin consisted of 15 ml of an XM. 100-membrane filtrate from a 10-fold concentrate prepared by reverse dialysis. (c) The column and eluant were as in (b), and the toxin consisted of 20 ml of a PM. 30-membrane filtrate from a 10-fold concentrate prepared by reverse dialysis.

eluant was used the peaks spread and little activity was recovered, indicating instability in eluants of low ionic strength.

The results (fig. 3) show that the medium-MW toxins were found between KAV 0.3 and 0.8 i.e., between MW 1.7×10^4 and 5.6×10^3 (fig. 1). If a line of closest fit were drawn through the histograms in figs. 3b and 3c there would be a double peak of enterotoxin activity exactly coinciding with the peaks of ultra-violet absorbence. This strongly suggests that the ultraviolet absorbence was at least partly measuring enterotoxin and indicates that there were two toxins, one of MW 9.5×10^3 and the other of MW 6.7×10^3 . It is interesting that the second peak of ultraviolet absorbence was lost on UM.2 filtration (fig. 4) whilst the first peak remained stable.

FIG. 4.—Gel filtration of UM.2-membrane retentate on Sephadex G50. The column measured 52×3.5 cm and the eluant was saline. Toxin, concentrated 10-fold by reverse dialysis, was filtered through a PM.30 membrane. The filtrate was used as follows: $\circ \cdots \circ$ = 20 ml unconcentrated filtrate; $\times \cdots \times$ = 20 ml filtrate, concentrated 2.5 times by UM.2 filtration; $\square \cdots \square$ = 12.5 ml filtrate, concentrated 6 times by UM.2 filtration.

All the enterotoxin activity in each reverse-dialysed ultrafiltered preparation applied to the G50 Sephadex columns was recovered between KAV 0.3 and 0.8 whereas only 60% of the total activity in a freeze-dried preparation was recovered in this region.

Low-MW toxin ($>10^3$). To avoid loss of low-MW material, freeze-dried concentrates were applied directly to a G10 column. Because preliminary ultrafiltration was impracticable we tried, with only partial success, to remove some of the slime and residue by centrifugation for 1 hour at 75,000 g. Even so the exclusion peak spread further than was desirable (fig. 5). As expected, most activity was found in the exclusion peak but 17% occurred outside this region, between KAV 1.0 and 2.0, giving MW limits of 500–280, as calculated from the standard curve (fig. 1c). A line of closest fit drawn through the histogram in fig. 5 would give a MW of 380, but this does not coincide with any peak of ultra-violet absorbence; the nearest one would be at KAV 1.65, giving a MW of 333.

DISCUSSION

The results from ultrafiltration suggest that enterotoxins occurred in four MW classes. If mean results are taken, supernates from cultures of strain P16 appear to have contained about 17% of toxin with $MW > 10^5$, 27% with $MW 3 \times 10^4$ – 10^4 , 10% with $MW 10^4$ – 10^4 and 46% with $MW < 10^4$. However, it seems likely that some of the higher-MW toxins fragmented to yield units of $MW < 10^4$; the results from G50 Sephadex-gel filtration (fig. 4) suggest that the fraction which broke down was the toxin of $MW 6.7 \times 10^4$.

FIG. 5.—Separation of low-MW enterotoxin on Sephadex G10. The column measured 38×2.5 cm and the eluant was phosphate buffer. The toxin consisted of 10 ml of a 20-fold concentrate prepared by freeze-drying. Symbols as in fig. 3.

When freeze-dried concentrates were used in gel filtrations, 17% of the total enterotoxin activity was recovered between KAV 0.2 and 0.7 on Sepharose 4B, 60% between KAV 0.3 and 0.8 on Sephadex G50 and 17% between KAV 1 and 2 on Sephadex G10. These results thus accounted for 94% of the total activity in freeze-dried concentrates and probably included all the major enterotoxin components in culture supernates of strain P16.

In general, the results from gel filtration and ultrafiltration agreed well. Both the Sepharose 4B and XM.100 filtrations indicated that about 17% of the total enterotoxin activity had a MW exceeding 10^5 . The Sephadex G50- and PM.10-filtration data both suggested that there were two toxins with a MW between 3×10^4 and 10^4 , but it must be assumed that the toxin having a MW of 9.5×10^3 did not pass through the PM.10 membrane, perhaps because of some steric factor. The UM.2 filtration data (table I) indicated a MW of $< 10^3$ for

46% of the total activity and a MW within the range 10^3 – 3×10^4 for 38%. The Sephadex G10 and G50 data suggested, on the other hand, corresponding percentage values of 17 and 60. This discrepancy may have resulted from the fragmentation which, as has already been explained, probably affected the toxin of MW 6.7×10^3 during ultrafiltration; if so, it is probable that the gel-filtration results reflected more accurately the original composition of enterotoxic supernates than did the ultrafiltration figures. Reassessing the UM.2 and PM.10-filtration data in the light of this observation, we suggest that 27% of the total activity represented stable toxin of MW 9.5×10^3 , 33% represented unstable toxin of MW 6.7×10^3 and 17% represented toxin of MW $< 10^3$.

To summarise our results with reference to those of other laboratories, we found that a strain of *E. coli* (serotype 09; K9) produced enterotoxins having MW values consistent with those found by others. Some investigators (Larivière, 1971; Jacks *et al.*, 1973) have suggested that most enterotoxic activity is associated with a MW of about 10^6 , whilst others (Kohler, 1968, 1971*b*; Smith and Gyles, 1970*a*) have stated that only low-MW toxin is found in culture supernates; Kohler (1971*b*) has presented data that suggest that the MW is just below that of sucrose. Bywater (1972), using a pathogenic strain of *E. coli* from a calf, suggested that there was no high-MW toxin but that most of the activity was associated with MW values within the range 10^4 – 10^3 , and only a little with values of $< 10^3$. However, his results showed some increase in activity in the PM.10 retentate which might have been caused by high-MW toxin; furthermore, some high-MW activity might have been lost during precipitation by acetone, a solvent which in our experience (unpublished observation) has resulted in poor recovery of activity. The high-MW toxin produced in our studies had a MW slightly lower than that found by Larivière (1971) and Jacks *et al.* (1973) whilst our low-MW toxin was very similar to that found by Kohler (1971), especially if the weight of 333 suggested by ultraviolet absorbence was correct. Likewise our medium-MW toxins had weights within the range stated by Bywater (1972), *viz.*, 10^4 – 10^3 . One of our toxins had a MW of 9.5×10^3 yet would not pass a PM.10 membrane, but it should be pointed out that the MW was only just below that excluded by this type of filter.

Thus it appears that the differences between our results and those of others are quantitative rather than qualitative and might well be related to the use of different strains of bacteria and of different methods of culture and preparation. We have noted (unpublished observation) that prolonged incubation of cultures increases the proportion of low-MW toxin and that another strain of *E. coli*, serotype O141; K85*ab*; K88*ab*; H4, seems to produce negligible amounts of high-MW toxin. This agrees well with other observations on strain variability (Truscynski and Ciosek, 1972). One of the toxins with a MW in the medium range apparently fragmented during ultrafiltration whilst the other remained stable, indicating that their molecular structures may have been quite different and that the interrelationship of enterotoxins may not be quite as simple as suggested by Smith and Gyles (1970*a*); moreover, it would seem that, in molecular terms, *E. coli* enterotoxin may not be directly comparable with *Vibrio cholerae* toxin (Finkelstein, Peterson and Lospalluto, 1971; Lospalluto and

Finkelstein, 1972). The MW of the low-MW toxin is very close to that of cyclic 3',5'-adenosine monophosphate (c-AMP; MW 347) which has been implicated in *E. coli* enteritis (Al-Awqati *et al.*, 1972). This coupled with the probable fragmentation of one of the medium-MW toxins to give low-MW toxin leads to the speculation that a polymer might be capable of breaking down to yield mono-nucleotides that interfere with a system normally regulated by c-AMP.

SUMMARY

Enterotoxin was produced by growing *Escherichia coli* strain P16 (serotype O9; K9) in a defined medium. Ultrafiltration and gel-filtration studies suggested that toxic activity was produced by enterotoxins, 17, 27, 33 and 17% of which had, respectively, MW values of 5.9×10^5 , 9.5×10^3 , 6.7×10^3 and 380. Inactivation accounted for the remaining 6%. There was some evidence that toxin of MW 6.7×10^3 fragmented to produce toxin of MW 380.

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