Purification and Physico-Chemical Analysis
of Fractions from the Culture Supernatant of Escherichia coli O78K80: Free Endotoxin and a Non-Toxic Fraction

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SUMMARY

The extracellular production of 'free endotoxin' from Escherichia coli serotype O78K80 is discussed. Isolation of purified toxic and non-toxic fractions from crude extracellular material and the physico-chemical properties of these fractions are described. Purified free endotoxin possessed similar properties to endotoxins extracted from the bacteria by conventional procedures; a purified non-toxic extracellular fraction corresponded to the so-called 'native hapten'. When dissolved in buffers containing sodium dodecylsulphate, the non-toxic fraction was not disaggregated, but the endotoxin produced sub-units of a smaller particle size than the non-toxic material. From these experiments it was concluded that the non-toxic material did not constitute simple sub-units of endotoxin.

INTRODUCTION

The term 'endotoxin' describes certain lipopolysaccharides of high molecular weight found in Gram-negative bacterial cell walls. Although Shear and his associates (e.g. Shear & Turner, 1943) have isolated tumour-necrotizing substances (endotoxins) from supernatant fluids of cultures of Gram-negative bacteria, most other workers have preferred to extract endotoxins from the bacteria themselves. An exception has been Work and her collaborators (Bishop & Work, 1965, Taylor, Knox & Work, 1966), who found endotoxic lipopolysaccharides in culture supernatant fluids of lysine-requiring mutants of Escherichia coli grown under lysine-limited conditions.

The release of large quantities of free endotoxin into the culture supernatant fluids of Gram-negative bacteria, under favourable growth conditions, has recently been described (Crutchley, Marsh & Cameron, 1967a). The present paper will deal with the fractionation of supernatant fluids of cultures of Escherichia coli serotype O78K80 and the subsequent physical and chemical characterization of the fractions; the accompanying paper (Crutchley et al. 1967b) will discuss the biological properties of these fractions.

Origin of toxicity. The published discussions of two symposia concerning endotoxins (led by Westphal et al. 1957 and Ribi et al. 1964) show that there is still a wide divergence of opinion about the chemical composition and physical properties of the moieties believed to be responsible for endotoxic activity. The German school of Westphal and his collaborators (Westphal, 1960; Westphal & Lüderitz, 1954; Westphal et al. 1957) concluded that lipid, possibly in conjunction with a polysac-
The minimal molecular size of the complex required for full endotoxic activity. Most workers seem to favour a unit possessing a sedimentation coefficient of about 10S (cf. Ribi et al. 1964). Mild acidic hydrolysis (e.g. with 0.1 M-acetic acid for 90 min. at 100°C) destroyed virtually all the toxicity of Ribi's endotoxin and produced a parallel decrease in the average sedimentation coefficient of the solute, although the 'acid hapten' obtained by this process (s = 1.4S) retained certain antigenic characteristics of the parent endotoxin (Ribi et al. 1962). By using non-hydrolytic conditions of disaggregation, in solutions containing sodium dodecylsulphate (SDS), two groups of workers reached opposite conclusions: Oroszlan & Mora (1963) found that the endotoxic complex of Serratia marcescens dissociated into particles of about 3S, with significant concomitant decrease in the biological activity responsible for tumour damage; on the other hand, Beer, Staehelin, Douglas & Braude (1965) found that an endotoxic preparation from Escherichia coli could be treated with SDS without measurable loss of toxicity.

The above findings, and many other conflicting reports about endotoxins, may have arisen partly from the use of different bacterial species and serotypes, but probably mainly from the widely different experimental procedures used, particularly as regards extraction and purification. It is uncertain whether the extraction procedures commonly used to isolate endotoxins from bacteria (trichloracetic acid at 4°C, Boivin & Mesro-beanu, 1933; phenol+water at 65°C, Westphal et al. 1952; aqueous ether at 6-12°C Ribi et al. 1961a) yield native endotoxins or degraded or aggregated derivatives of the parent materials. We have therefore developed a series of very mild extraction and purification procedures whereby endotoxic and other bacterial products may be isolated in large amounts from bacterial culture fluids without any significant change in the properties of the native substances.

**METHODS**

Acid-hydrolysed casein was obtained from Oxoid Ltd., ammonium sulphate from I.C.I., protein stains from G. T. Gurr (London), 'Ionagar for Electrophoresis' from Difco (Detroit, U.S.A.), Sephadex G 100 and Blue Dextran 2000 from Pharmacia (Uppsala, Sweden) and DEAE-cellulose DE 11 (1 m-equiv./g.) from Whatman (Balston, England). Yeast extract was prepared at the Wellcome Research Laboratories. All other chemicals were of Reagent or AR quality.
Toxic and non-toxic fractions of E. coli

Preparation of crude endotoxin. Escherichia coli serotype O78K80 was grown in tanks for 42 hr at 35° in a vigorously aerated aqueous medium containing acid-hydrolysed casein (5%, w/v), sucrose (3%, w/v) and yeast extract (10%, w/v), adjusted to pH 6.5 (D. C. Edwards, unpublished). Before inoculation, the medium was clarified, heated to 85° and sterilized by Seitz filtration. This medium will be referred to as ‘casein sucrose’.

The bacteria were removed from suspension by using a de Laval centrifugal separator and the supernatant fluid sterilized by Seitz filtration. Crude endotoxin I was precipitated from this supernatant fluid by addition of ammonium sulphate (about 700 g./l.) and allowing it to stand at 4° for 6 days. The brown flocculent precipitate (I) was recovered by centrifugation, resuspended and then dialysed thoroughly against distilled water. A small amount of insoluble material was removed by centrifugation and the endotoxin then reprecipitated at 4° by adding 65 g. AR ammonium sulphate per 100 ml. solution. The reprecipitated material (II) was treated as above to remove salts, clarified by centrifugation and freeze-dried to give a brown powder (Fig. 1).

\[
\begin{align*}
\text{Bacterial suspension (equivalent to 10-7 g. bacteria/l.)} & \\
1. & \text{Centrifugation-filtration} \\
2. & \text{Sterilization} \\
\text{Supernatant fluid (1 l.)} & \\
1. & \text{Sat. (NH}_4\text{)}_2\text{SO}_4, \text{6 days at 4°} \\
2. & \text{Ppt. dialysed against water} \\
3. & \text{Centrifugation} \\
\text{Crude endotoxin I (solution)} & \\
1. & (\text{NH}_4\text{)}_2\text{SO}_4 (65 g./100 ml.), \text{1 day at 4°} \\
2. & \text{Ppt. dialysed against water} \\
3. & \text{Centrifugation} \\
4. & \text{Freeze-drying of supernatant fluid} \\
\text{Crude endotoxin II (120 mg.)} & \\
\end{align*}
\]

Fig. 1. The preparation and purification of endotoxin and non-toxic fractions from Escherichia coli O78K80. Yields of freeze-dried salt-free fractions per litre of sterile culture fluid are given in brackets after the appropriate fractions.

Purification of endotoxin

Recovery of purified fractions. Volatile buffers based on ammonium salts were used for all chromatographic separations in order to eliminate the necessity for extensive dialysis operations. Normally, the pooled chromatographic effluents were dialysed once against a 50- to 100-fold excess of distilled water, and the remaining traces of the volatile salts were removed during subsequent freeze-drying. This method has been used routinely to recover endotoxic fractions from large volumes of solution without loss in biological activity.
Gel filtration. Crude endotoxin II (40 mg.) dissolved in 3 ml. of 0.05 M-NH$_4$HCO$_3$ was applied to a Sephadex G 100 column (95 cm. x 3.28 cm. diameter), equilibrated with this buffer. Figure 2 shows the elution diagram for this fractionation (S I) for extinction readings at 230 m$\mu$. (Endotoxin II showed a spectral maximum at 274-275 m$\mu$. Readings were also taken at 230 m$\mu$ in order to gain a better assessment of the total solids eluted, including carbohydrate.) Endotoxic activity was shown to be associated mainly with the first peak, fraction S I.1 (Crutchley, Marsh & Cameron, 1967b): the bulk of the brown-pigmented material was found in fraction S I.6. Fraction S I.1 was purified further by a second fractionation (S II) on the Sephadex column described above, and the main endotoxic fraction S II.1 was reserved for further purification by recycling chromatography. In subsequent experiments, larger quantities (about 550 mg.) of the crude fraction II were applied to the Sephadex column but, because of the poorer resolution obtained, two further purification stages were required to achieve the same degree of purity as fraction S II.1.

Recycling chromatography. To obtain the highly purified fraction R (Fig. 1), partially purified endotoxin was repeatedly chromatographed on the long Sephadex G 100 column described previously. A solution of fraction S II.1 (125 mg. in 5 ml. 0.2 M-NH$_4$HCO$_3$) was recycled through the column by means of a peristaltic pump, and the effluent was continuously monitored for extinction at 253.7 m$\mu$ using an LKB Uvicord spectrophotometer and recorder. After six cycles through the column, the effluent was collected and its extinction measured at 230 m$\mu$. All contaminating traces of nucleotide and peptide were removed during the course of the recycling process. The proportion of contamination by fraction S I.2 was also substantially decreased by the recycling procedure.

Ion-exchange chromatography. Since it had proved to be very tedious to resolve completely the major components of fractions S I.1 and S I.2 (Fig. 2) by gel filtration alone, chromatographic separation on columns of DEAE-cellulose and DEAE-Sephadex was investigated. Of these two anion-exchangers, DEAE-cellulose proved to be the more suitable in practice.

The first purification, utilizing a long narrow column of DEAE-cellulose, was applied to fraction S I.1. A high molecular weight, predominantly carbohydrate fraction, S I.D1. (identical with fraction D1.S1 prepared by a slightly different method; Fig. 1), was found to be weakly bound to this column in dilute buffers, whereas most other material was strongly adsorbed. For subsequent large-scale separations, it was more convenient to carry out the first stage in the purification of crude endotoxin II by using a wide shallow DEAE-cellulose column, followed by further purification by gel filtration (Fig. 1).

A solution of crude endotoxin II from the salt-precipitation stages (600 mg. in 30 ml. of 0.005 M-NH$_4$HCO$_3$) was applied to a Buchner column (8 cm. x 10 cm diameter) containing DEAE-cellulose equilibrated to pH 8 and suspended in 0.005 M-NH$_4$HCO$_3$. Elution was performed successively with (i) 0.01 M-NH$_4$HCO$_3$ (1250 ml.) and (ii) M-ammonium acetate acetic acid buffer (pH 5.5, 500 ml.). A single peak of weakly ultraviolet-absorbing material (at 230, 260 and 275 m$\mu$) was eluted with buffer (i), this was fraction D1. The bulk of the remaining material was eluted with buffer (ii), this was fraction D2. (In a subsequent experiment 0.2 M-NH$_4$HCO$_3$ was used as an intermediate buffer between (i) and (ii). This probably had the advantage of avoiding possible contamination of fraction D2 with traces of strongly adsorbed
Toxic and non-toxic fractions of E. coli

D1, although the yield of D2 was lower due to its partial elution with the 0.2 M salt.

Fraction D2 proved to be endotoxic and D1 non-endotoxic, but both were protective in mice against a subsequent challenge with Salmonella typhi 24 hr after injection of fractions D1 or D2 (Crutchley et al. 1967). Fractions D1 and D2 were further purified by gel filtration on a Sephadex G 100 column (167.5 cm. × 1.2 cm. diameter); see Figs. 3, 4. Only the first fractions from each chromatographic experiment (D1.S1 and D2.S1, respectively), possessed the biological activities associates with fractions D1 and D2.

**Ultracentrifugal analyses.** Crude and highly purified preparations were examined in a Spinco Model E Ultracentrifuge, using schlieren optics. Phosphate NaCl buffer (pH 7.8; I = 0.1) was used throughout as the solvent, except in certain experiments where sodium dodecylsulphate was added, 0.5 g./100 ml. of this buffer. Crude Escherichia coli 078K80 endotoxins, extracted from the parent bacteria of our culture by the aqueous phenol method of Westphal, Lüderitz, Eichenberger & Keiderling (1952) and by the aqueous ether method of Ribi et al. (1961a), were also examined in the ultracentrifuge for comparative purposes. The aqueous layer of the phenol-water extract was partially purified to the stage where nucleic acid was removed from the endotoxin by organic solvent precipitation. No further purification of the aqueous ether extract was performed beyond the stage of precipitating the crude endotoxin with 68% EtOH-H2O. Sedimentation coefficients for the higher molecular weight solutes in these preparations (Fig. 8) were calculated and corresponding concentrations were determined from traced magnified areas under the schlieren peaks by ‘counting squares’, using schlieren patterns from known concentrations of fraction D1.S1 as the standards. Appropriate corrections were made for differences in the schlieren-
phase plate angle and for radial dilution effects, but no correction was made for the Johnston & Ogston (1946) effect, since this was insignificant in most cases. The aqueous phenol extract, further purified by passage through Sephadex G 100 (Fig. 5), gave an almost identical elution pattern to that shown in Fig. 4 for fraction D2. The ultracentrifugal data obtained for the major fraction, P1, eluted at the void volume of the column, are also presented in Figs. 7 and 8 and in Table 1.

Fig. 3. Elution diagram for a Sephadex G100 fractionation of fraction D1 (20 mg. in 2 ml. of 0-2 m-NH$_4$HCO$_3$—elution with the same solvent).

Fig. 4. Elution diagram for a Sephadex G100 fractionation of fraction D2 (19 mg. in 2 ml. of 0-2 m-NH$_4$HCO$_3$—elution with the same solvent).

Fig. 5. Elution diagram for a Sephadex G100 fractionation of a partially purified aqueous phenol cell extract (20 mg. in 2 ml. of 0-2 m-NH$_4$HCO$_3$—elution with the same solvent).

Table 1. Ultracentrifugal data for measurable schlieren peaks for buffered solutions (pH 7-8, I = 0-1) of aqueous phenol and aqueous ether extracts of Escherichia coli O78K80

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Fig.</th>
<th>Solute concn. (mg./ml.)</th>
<th>Speed (rev./min.)</th>
<th>Schlieren angle (°)</th>
<th>Aqueous phenol extract</th>
<th>Aqueous ether extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$s_0^0$ (sec. $10^{19}$)</td>
<td>$c$ (mg./ml.)</td>
</tr>
<tr>
<td>1</td>
<td>8a</td>
<td>6-0</td>
<td>59,780</td>
<td>45</td>
<td>(a) 11-2</td>
<td>17-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(b) 5-47</td>
<td>5-42</td>
</tr>
<tr>
<td>2</td>
<td>8b, c</td>
<td>30-0</td>
<td>59,780</td>
<td>70</td>
<td>(a)</td>
<td>14-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(b) 2-94, 4-91</td>
<td>3-56, 1-04</td>
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<tr>
<td>3</td>
<td>8d</td>
<td>30-0</td>
<td>29,500</td>
<td>70</td>
<td>(a) 24-8</td>
<td>15-5</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>(b) 2-97, 5-15</td>
<td>2-28, 2-05</td>
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<td>4†</td>
<td>—</td>
<td>2-0</td>
<td>59,780</td>
<td>65</td>
<td>(a) (65)*</td>
<td>—</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(b) 5-82</td>
<td>0-59</td>
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<tr>
<td>5†</td>
<td>8e, f bottom patterns</td>
<td>6-0</td>
<td>39,460</td>
<td>65</td>
<td>(a) 12-9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(b) 4-90</td>
<td>2-09</td>
</tr>
</tbody>
</table>

* Value obtained from a run at lower speed—probably corresponds to nucleic acid.
† Fraction P1 from phenol extract purified by Sephadex G100 chromatography.
All sedimentation coefficients were corrected to water at 20° (s_{20}) and, for the highly purified preparation D1.S1, a graph of s_{20} versus concentration (c) was plotted in order to determine [s_{20}]_{c=0} (Fig. 7).

**Other estimations of purity**

*Gel filtration.* Highly purified materials were chromatographed on a Sephadex G 100 column (182×1.2 cm.), which was in routine use for the molecular-weight estimations of proteins (Andrews, 1964). The void volume of this and other G 100 columns was measured by using a solution of Blue Dextran 2000 (Pharmacia).

*Electrophoresis.* Cellulose acetate strip electrophoresis in barbitone buffer (pH 8.8, Kohn, 1960) and starch-gel electrophoresis at pH 8.5 (Smithies, 1955), in presence or absence of 6 m-urea, were used to investigate the purity of the endotoxic fractions. After electrophoresis at 175 V. for 1 hr, cellulose-acetate strips were stained with trifaligic acid or Ponceau S for proteins and with periodate-Schiff’s reagent for carbohydrates (Kohn, 1960). Lipid was detected on drying the strips before staining, due to differences in the drying characteristics of cellulose acetate with adsorbed lipid. Lipid was not stained by the ozone–Schiff’s method, presumably because of the absence of C=C bonds. Sliced starch gels were stained for protein with amido black 10B.

*Immunodiffusion.* The double diffusion method, in an agar gel supporting medium, was used to investigate the purity of fractions at each stage in the purification (Fig. 1). The preparation of antisera for these experiments is described in the companion paper (Crutchley et al. 1967b).

*Spectrophotometry.* Ultraviolet absorption spectra for aqueous solutions of purified fractions were automatically recorded on a Unicam SP 800 spectrophotometer.

**Chemical analyses**

*Elementary analysis.* Nitrogen was estimated by the micro-Kjeldahl method (Pregl, 1945) and phosphorus by a modified Gomori (1942) procedure with a sulphuric acid + hydrogen peroxide mixture as digesting agent.

*Amino acids.* Purified fractions D1.S1 (1.4 mg.) and D2.S1 (3.06 mg.) were hydrolysed with constant-boiling HCl (5-7-N) in vacuum for 24 hr. Amino acids were estimated by their colour reactions with ninhydrin, by using a Beckman-Spinco (Model 120) automatic analyser, with β-2-thienyl-dL-alanine added as an internal standard (Siegel & Roach, 1961).

*Carbohydrates.* Various classes of monosaccharides in purified fractions were determined by the sulphuric acid + cysteine procedure of Dische (1955). The hexosamine content for material which had been hydrolysed for 5 hr at 100° in 3 N- or 4 N-HCl (see Ribi et al. 1961a) was quantitatively estimated by the indole + HCl procedure (Dische, 1955) with appropriate non-deaminated controls, or by the short column of the Beckman-Spinco Analyser.

*Fatty acids.* Purified fractions D1.S1 and D2.S1 (3 mg.) were hydrolysed for 3 hr at 100° in 2 N-HCl. The fats were extracted directly from these hydrolysates with 3×1.5 ml. light petroleum (b.p. 60–80°). The pooled light petroleum phases were evaporated to dryness, saponified and examined as free fatty acids by gas-phase chromatography (Schmit & Wynne, 1965).
RESULTS

Ultracentrifugal analyses

The sedimentation patterns for crude endotoxin II (Fig. 1) indicated pronounced heterogeneity (Fig. 6a, b), but all preparations possessed a characteristic hypersharp peak ($[\bar{s}]_{\omega=0} = 3.1$ S). Fractions D1.S1 (Fig. 6c) and R (Figs. 6d, e, f) possessed only this peak together with occasional slight traces of higher molecular weight impurities. These two fractions, both non-toxic (Crutchley et al. 1967b), were identical by ultracentrifugal criteria; each exhibited the same variation of $s$ upon c, yielding an extrapolated value of $[\bar{s}]_{\omega=0} = 6.7 \pm 0.2$ S (Fig. 7).
Subsequent analysis of two endotoxic extracts of the present bacteria (aqueous phenol and aqueous ether extracts) showed heterogeneity and differences of composition between the two fractions (Fig. 8). Both extracts, however, possessed an endotoxic fraction of high molecular weight and also a component with similar sedimentation behaviour to that of non-toxic fraction D1.S1 at the same concentration (Table 1; Fig. 7). (In one aqueous phenol extract two hypersharp peaks were observed: Fig. 8b–d. Since no explanation can yet be given for this phenomenon, the data for these peaks has not been included on the graph shown in Fig. 7.)

![Graph showing the variation of $s_0^0$ with $c$ for fractions D1.S1 and R. Calculated $s_0^0$ and $c$ values for analogous material in aqueous phenol and aqueous ether cell extracts were plotted after drawing the curve (Table 1).](image)

Sodium dodecylsulphate disaggregated traces of high molecular weight material in crude endotoxin II (Fig. 6a) and in fraction R, but did not affect the sedimentation characteristics of the major peak of non-toxic material, other than slightly decreasing its sedimentation coefficient (e.g. $\Delta s_0^0|_{c=0} \approx 0.05$ S). This is probably a reflection of small changes in $\nu$ and $\rho$ for the solute and solvent, respectively.

The sedimentation patterns of the endotoxic fraction D2.S1 showed asymmetry and very rapid spreading of the main schlieren boundary (Fig. 9): probably these effects were due mainly to reversible association of endotoxin molecules and of their sub-units. In addition to the main schlieren boundary, a small quantity of material of very high sedimentation coefficient was observed to migrate across the cell as the centrifuge was accelerating up to the running speed. On adding sodium dodecylsulphate (SDS) (0.5 g./100 ml.) to translucent aqueous solutions of the toxic fraction, there was immediate clarification of the solutions, and a concomitant striking decrease
in the average sedimentation coefficient of the solute occurred (Fig. 9). The extent of disaggregation produced by SDS appeared to vary with different concentrations and with different preparations of purified endotoxin D2.S1. SDS also disaggregated the endotoxic component in the purified phenol extract P1, but had no effect on the non-toxic component (Figs. 8e, f).

![Ultracentrifugal schlieren patterns](image)

**Fig. 8.** Ultracentrifugal schlieren patterns of aqueous phenol (top pictures, a-d) and aqueous ether (bottom pictures, a-d) extracts of bacterial cells, and of purified phenol extracts P1 (e,f). All solutions in phosphate-NaCl buffer (pH 7.8; I = 0.1); solutions shown in the top pictures of (e) and (f) also contain 0.5 g: SDS per 100 ml. Other details given in Table 1.

Approximate $[s_{20}^0]_{c=0}$ values for the disaggregated material from fraction D2.S1 were 2.4 S and 4.1 S (Fig. 9b). Owing to the poor solubility and rapid boundary spreading of the main endotoxic peak in solutions without added SDS (Fig. 9a), no estimate of $[s_{20}^0]_{c=0}$ for this peak could be made. However, calculated values of $s_{20}^0$ for 1% and 2% solutions were, respectively, 14.5 S and 13.2 S.
Estimations of purity

Gel filtration. Fractions D1.S1, D2.S1 and purified phenol extract (P1) all gave single elution peaks upon rechromatography on a long Sephadex G 100 column; this indicated absence of any significant quantity of lower molecular weight contaminants. However, since these peaks emerged with the void volume of the column, small proportions of impurities of molecular weight higher than that of the bulk of the material (noticed in certain purified fractions during ultracentrifugation; Figs. 6, 8 and 9) would not have been detected.

Electrophoresis. On cellulose-acetate electrophoresis at pH 8.8, the endotoxic fraction D2.S1 migrated towards the cathode as a single band in which lipid, carbohydrate and peptide were detected: a trace of insoluble material remained at the origin. The purified phenolic extract, P1, was largely retained at the origin (strong carbohydrate and weak protein staining); a lipid moiety of a similar negative mobility to fraction D2.S1 was also detected in P1. Non-toxic fraction D1.S1 gave a single band of very low apparent negative mobility (possibly due to electro-osmotic flow of buffer) in which carbohydrate, lipid and protein were very weakly detectable. Starch gel did not prove to be a suitable medium for electrophoresis of the above samples, but was used for separation of lower molecular weight protein fractions present in crude extracts (e.g. endotoxin II).

Immunodiffusion. A sample of pooled horse antiserum against the whole extract of Escherichia coli O78K80 was of little value in estimating the homogeneity of purified fractions, since only one major and a trace of one minor precipitin line were formed against crude endotoxin II (Crutchley et al. 1967b). When examined against a potent rabbit antiserum to crude endotoxin II, little heterogeneity was observed in purified fractions R, D2.S1 and P1; D1.S1 did not give an immunodiffusion line with this
antiserum. A decrease in the number of precipitin lines was observed after each purification stage.

**Spectrophotometry.** The ultraviolet absorption spectra for aqueous solutions of purified fractions R, D1.S1, D2.S1 and P1 (Fig. 10) indicated chemical differences between the fractions. Fractions R and D1.S1 possessed identical spectra, typical of carbohydrates; but D2.S1 (and crude also endotoxin II) showed an additional small absorption maximum at about 275 m\(\mu\), possibly due to aromatic amino acids. The maximum at 260 m\(\mu\) for purified phenol extract, P1, was almost certainly due to contamination by nucleic acid.

![Ultraviolet absorption curves for aqueous solution of purified fractions D1.S1 (1 mg./ml., solid line), D2.S1 (0.5 mg./ml., dotted line) and P1 (0.5 mg./ml., dashed line).](image)

**Chemical analyses**

Quantitative chemical differences were found between fractions D1.S1 and D2.S1 (Tables 2, 3). In particular, D2.S1 contained a higher proportion of long-chain fatty acids and a much greater proportion and variety of amino acids than did fraction D1.S1. Fraction D1.S1 was, however, much richer in amino sugar than was D2.S1; acid hydrolysates of these fractions showed that this sugar was chromatographically identical with glucosamine (long and short columns of the amino acids analyser used). High voltage paper electrophoresis of the hydrolysates in 0.1 M-borate buffer (pH 10; Michl, 1951; Foster, 1953), followed by staining with ninhydrin or AgNO\(_3\)+NaOH (Trevelyan, Procter & Harrison, 1950), confirmed that glucosamine was the only amino sugar in both the non-toxic fraction D1.S1 and the toxic fraction D2.S1.

The cysteine + sulphuric acid method of Dische (1955) showed some similarities in the spectra of fractions D1.S1 and D2.S1: hexose (and/or hexose derivatives), small quantities of heptose; but no significant quantity of pentose or 2-keto-3-deoxyoctulosonic acid were detectable, from these spectra. More detailed analysis of carbohydrate and lipid components in fractions D1.S1 and D2.S1 is now under investigation.
Toxic and non-toxic fractions of *E. coli*

Table 2. *Chemical analyses of purified fractions from Escherichia coli O78K80*

<table>
<thead>
<tr>
<th>Sample</th>
<th>N (%)</th>
<th>P (%)</th>
<th>Glucosamine (%)</th>
<th>Amino acid (%)</th>
<th>Fatty acid (%)</th>
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<tr>
<td>D1.S1</td>
<td>4.32</td>
<td>8.89</td>
<td>26-1 (27.8)*</td>
<td>1-2-1.4†</td>
<td>0-1</td>
</tr>
<tr>
<td>D2.S1</td>
<td>4.95</td>
<td>10.06</td>
<td>12-3</td>
<td>14-1-17.7</td>
<td>1-9</td>
</tr>
</tbody>
</table>
* Value obtained by the Dische (1955) procedure: other glucosamine contents obtained using the amino acid analyser.
† Limits defining 0–100% amino acid involved in chemical linkage.

Table 3. *Amino acid analyses of fractions D1.S1 and D2.S1*

<table>
<thead>
<tr>
<th></th>
<th>µmole amino acid/mg.</th>
<th>g. amino acid/100 g.</th>
<th>µmole amino acid/mg.</th>
<th>g. amino acid/100 g.</th>
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<tr>
<td>Glucosamine*</td>
<td>0.813</td>
<td>14.55</td>
<td>0.600</td>
<td>10.74</td>
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<tr>
<td>Lys.</td>
<td>ND</td>
<td>ND</td>
<td>0.087</td>
<td>1.27</td>
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<tr>
<td>His.</td>
<td>ND</td>
<td>ND</td>
<td>0.029</td>
<td>0.45</td>
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<td>NH₄</td>
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<td>0.765</td>
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<td>ND</td>
<td>0.065</td>
<td>1.13</td>
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<tr>
<td>Asp.</td>
<td>0.0151</td>
<td>0.20</td>
<td>0.211</td>
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<td>0.086</td>
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<td>ND</td>
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<tr>
<td>Val.</td>
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<td>ND</td>
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<tr>
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<td>Tyr.</td>
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<td>Phe.</td>
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<td>Totals†</td>
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<td>17.5</td>
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* Low results here due to extended period of hydrolysis.
† ND = None detectable (< 0.0005 µmole amino acid).
‡ Excluding glucosamine and ammonia.

**DISCUSSION**

Two essentially homogenous non-toxic fractions, D1.S1 and R, have been isolated from the extracellular material produced by *Escherichia coli* O78K80 by using two slightly different chromatographic procedures (Fig. 1). Apart from possible differences in trace impurities, these fractions were physically and chemically indistinguishable. Since higher yields were obtained by successive chromatography on DEAE-cellulose and Sephadex G 100, this method was chosen to prepare the non-toxic fraction in bulk. DEAE-cellulose offered a convenient tool for separating this weakly charged fraction from the endotoxic lipopolysaccharide-protein complex (D2.S1), which was strongly adsorbed at alkaline pH values, despite its apparent positive charge under these conditions (see electrophoretic experiments). The adsorptive capacity of DEAE-cellulose for the toxic complex may be partly due to a disaggregation of the complex
into negatively charged lipopolysaccharide and positively charged protein. A similar disaggregation of this complex occurred with Westphal's phenol + water extraction procedure (Marsh, unpublished observations.)

Aqueous ether or phenol + water extraction of the parent bacteria yielded components with similar physical characteristics to the non-toxic fraction D1.S1 (Figs. 7, 8). This fraction is almost certainly equivalent to 'native hapten' found in protoplasmic fractions (or whole bacteria) of several serotypes of Escherichia coli by Anacker et al. (1964, 1966). Both fraction D1.S1 and 'native hapten' possess not only similar sedimentation characteristics but also similar chemical and immunological properties, including the failure to stimulate the production of precipitating antibody in rabbits (Crutchley et al. 1967b). Jann's (1965) acidic polysaccharides, isolated from phenol + water extracts of Escherichia coli, also behaved in the ultracentrifuge similarly to non-toxic fraction D1.S1. The difference in toxicity between these two substances is probably due to the presence of endotoxin in 'acidic polysaccharide'; the charge difference between them may arise from a difference in the amounts of bound protein in the two substances.

Extracellular endotoxin (D2.S1) showed similarity in sedimentation behaviour to endotoxic preparations obtained from the parent bacteria by conventional extraction procedures. However, there was some evidence of a greater degree of aggregation in cell-wall endotoxins, particularly with the aqueous ether preparation. The higher peptide content of fraction D2.S1 as compared with other preparations of purified endotoxic lipopolysaccharide (Westphal et al. 1952; Ribi et al. 1964) is due to loosely bound protein which may be removed by phenol + water extraction (Marsh, unpublished observations).

By the mild fractionation procedures described in this paper, highly purified endotoxic, and non-toxic lipopolysaccharide-protein complexes were readily isolated from culture fluids of Escherichia coli O78K80, yielding substances which were essentially the same as those naturally occurring within the bacteria. The conventional procedures for extracting endotoxin from bacteria are more laborious than the method used here for obtaining free endotoxin. The vigorous extraction procedures which have been used previously (particularly those using phenol or trichloroacetic acid) probably lead to degradation of the endotoxic complex.

The relationship between fractions D1.S1 and D2.S1

Both Ribi et al. (1964) and Westphal et al. (1964) considered the toxic lipopolysaccharide unit of endotoxin to be an aggregate of polysaccharide sub-units held together by lipid moieties. This hypothesis is now further substantiated by the demonstration that sodium dodecylsulphate disaggregate both the free endotoxin liberated extracellularly (fraction D2.S1; Fig. 9b) and that extracted from the bacteria (fraction P1; Fig. 8e, f). Similar results were obtained by Oroszlan & Mora (1963) for endotoxin from Serratia marcescens. Disaggregation by sodium dodecylsulphate appears to be the mildest procedure available for obtaining 'haptenic' non-toxic sub-units from endotoxins.

The marked differences between the [s_w]_∞ values for sub-units of endotoxic fraction D2.S1 and of the non-disaggregatable non-toxic fraction D1.S1, suggest that D1.S1 does not consist of simple sub-units of D2.S1. Differences of chemical composition between fractions D1.S1 and D2.S1 also support this conclusion. These
results show that fraction D1.S1 as a whole does not constitute a structural element of endotoxin (D2.S1). Immunological similarity between the two fractions is probably associated with regions of chemical similarity, but this does not necessarily imply that part of non-toxic D1.S1 constitutes a structural element or a precursor of endotoxin (see Anacker et al. 1964). Therefore we find no justification, at present, in designating fraction D1.S1 as a 'native hapten' in the sense considered originally by Anacker and colleagues (1964). We reserve judgement on whether or not the non-toxic and endotoxic fractions are interrelated until more is known about the detailed structure of both substances.

We are particularly pleased to thank Mr J. M. Summerell for help with the ultracentrifugal experiments; also Dr A. W. Phillips, Dr Paley Johnson (Cambridge) and Mr A. W. Pink for making certain ultracentrifugal, amino acid, elemental and G.P.C. analyses, and Mr H. King for technical assistance. We are indebted to Drs M. Sterne and J. Cameron for helpful discussions.

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