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2.1 Organism

Vibrio parahaemolyticus strain 28E/68 and 1213/68 were used in this study. The vibrio strains were isolated from cases of human diarrhoea at Calcutta. They were obtained through the courtesy of Dr. B. D. Chatterjee of the Bacteriology Department of School of Tropical Medicine, Calcutta and were maintained by fortnightly subculture on nutrient agar slopes.

2.2 Growth media

The vibrios were grown at 37°C in either of the following media:

(i) peptone water medium (PW) containing Bactopeptone (Difco), 3.0 gm; NaCl (analytical reagent grade, British Drug House Ltd.), 2.0 gm in 100 ml redistilled water, pH 7.6,

(ii) peptone-agar medium (PA) containing 2% (W/V) Bactoagar (Difco) in peptone water medium, or

(iii) nutrient-agar medium (NA) containing Bactopeptone, 1.0 gm; beef extract (ACAS), 1.0 gm; NaCl, 2.0 gm and Bactoagar, 2.0 gm in 100 ml redistilled water, pH 7.6.

2.3 Treatment of the vibrios with EDTA and lysozyme

After desired period of growth in PW medium, the vibrios were harvested by centrifugation, washed twice in tris (B.D.H.) buffer (33 mM, pH 8.0) and finally suspended in a suitable volume of the buffer. One ml aliquots of this vibrio suspension were added to 9 ml aliquots of the following test solutions whose final concentrations were thereby made as desired:

(a) tris buffer (33 mM, pH 8.0), (b) EDTA (A.R., B.D.H.) (10, 60, 100 or 200 /μg/ml) in 33 mM tris buffer with or without 0.15 M NaCl, 0.01 M MgCl₂ (A.R., B.D.H.) or 0.3 M sucrose (A.R., B.D.H.); (c) lysozyme (A.R., B.D.H.) (50 /μg/ml) in 33 mM tris buffer; (d) EDTA (10, 60, 100 or 200 /μg/ml) and lysozyme (50 /μg/ml) in 33 mM
tris buffer with or without 0.15 M NaCl, 0.01 M MgCl₂ or 0.3 M sucrose. Similar sets of experiments were performed by replacing tris buffer (33 mM) with tris buffer (0.1 mM, pH 8.0), 0.1 M phosphate buffer (0.05 M - KH₂PO₄ + 0.05 M - Na₂HPO₄), pH 8.0 or saline (0.15 M, pH 8.0). In tris + EDTA system, tris buffer (33 mM) of pH 7.6, 8.0 and 9.0 were used, the pH values were adjusted with 0.1 N HCl (A.R., B.D.H.).

2.4 Treatment of the vibrios with sodium lauryl sulphate

For studying the effect of sodium lauryl sulphate, vibrios harvested from the logarithmic phase of growth (4.5 hours static culture in FW medium) were washed twice in saline (0.85% W/V, pH 8.0), added to saline containing sodium lauryl sulphate (B.D.H.) of desired concentrations (0.01, 0.05, 0.1, 0.5 or 1.0% W/V) and incubated at 37°C.

2.5 Treatment of the vibrios with sodium deoxycholate

V. parahaemolyticus cells, harvested from logarithmic phase of growth, were washed in tris buffer (33 mM, pH 8.0) and finally suspended in tris buffer containing sodium deoxycholate (E. Merck) of desired concentrations (0.5% or 1.0% W/V).

2.6 Treatment of the vibrios with camphor

The vibrios were grown in FW medium at 37°C. Camphor (I.P., Aurora Pharmaceuticals, India) was used after 3 times repeated sublimation in laboratory. Stock solution was prepared by dissolving 1 gm of camphor in 2 ml of 100% ethyl alcohol in a sterile test tube. Suitable volumes of this stock solution were added to the growing cultures to give desired concentrations of camphor. Ethanol control was routinely included in each experiment to determine the effect of this solvent on the growth of V. parahaemolyticus.
2.7 Treatment of the vibrios with toluene

For studying the effect of toluene, the vibrios were grown in PW medium at 37°C. After desired period of growth, suitable volumes of toluene (A.R B.D.H.) were added to the growing cultures to give the desired final concentrations (0.2%, 0.5% or 1.5% V/V).

2.8 Treatment of the vibrios with phenethyl alcohol

The vibrios were grown at 37°C in PW medium. After desired period of growth, requisite volumes of phenethyl alcohol (PEA) (Fluka) were added to the growing cultures so as to give the desired final concentrations (0.1%, 0.3% or 0.5% V/V).

2.9 Measurement of optical density

Changes in optical density of the above bacterial suspensions relative to the controls (test solutions) were measured at room temperature with the help of a photoelectric colorimeter at 680 nm at suitable intervals of time.

The colorimeter readings (optical density values) were found proportional with the concentration of vibrio suspensions up to an optical density of 0.4. Above that optical density, the proportionality of the scale was ensured in the following way:

Vibrio cultures or suspensions of different optical density values above 0.4 were diluted with suitable volumes of the respective medium to an optical density less than 0.4. The corrected optical density of the original culture/suspension was obtained by multiplying the measured optical density (after dilution) by the dilution factor. A calibration graph was obtained by plotting corrected optical density values against the observed ones and this was used for all subsequent optical density measurements (Fig. 2.1). (Table 2a).
Fig. 2.1. Calibration graph for colorimeter readings. The graph was drawn with the optical density observed before dilution against the corresponding corrected values.
2.10 **Determination of viability**

The vibrios were grown in PW medium at 37°C. After desired period of growth, they were treated with suitable reagents (EDTA, camphor, toluene or PEA) for requisite period of time. Viability of the vibrios was determined after serial dilution in 0.15 M saline; 0.1 ml volumes of suitable dilutions were spread on peptone-agar (PA) plates which had been previously dried for 2 hours at 50°C. Numbers of colony forming units were counted after overnight incubation of the plates at 37°C.

2.11 **Electron microscopy**

*V. parahaemolyticus* cells suspended in 0.15 M saline (control) or treated with (a) EDTA (60 /μg/ml) in tris buffer (33 mM, pH 8.0) for 20 minutes, (b) EDTA (60 /μg/ml) + lysozyme (50 /μg/ml) in tris buffer for 20 minutes, (c) sodium lauryl sulphate (0.1% W/V in 0.15 M saline) for 60 minutes or (d) camphor (500 /μg/ml) in PW medium for 60 minutes were immediately fixed by adding suitable volumes of formaldehyde in the above suspensions so as to give final aldehyde concentration 4% (W/V). In another experiment, vibrio suspension was kept at 100°C for 45 seconds and then subjected to similar sodium lauryl sulphate treatment and subsequent fixation. After 1-2 hours fixation, the vibrios were harvested by centrifugation, washed twice in distilled water and then resuspended in suitable volume of distilled water to give a slightly turbid suspension. One drop from each of these suspensions was deposited on carbon collodion coated copper wire meshes with the help of capillary pipettes, dried, washed by rinsing in distilled water, again dried and finally shadowed obliquely with chromium at an approximate shadow to height ratio of 1:3 with the help of a Hitachi H500-3 vacuum evaporator.

Electron micrographs were taken with a Siemens Elmiskop 1 electron microscope at instrumental magnifications ranging between 6,000 x and 10,000 x.
2.12 Chemical procedures

(a) Release of intracellular materials from the vibrios

(i) Collection of bacteriafree filtrate

For measuring the release of intracellular materials from V. parahaemolyticus cells after EDTA treatment, thick suspensions of the vibrios in tris buffer (33 mM, pH 8.0) containing EDTA (60 /μg/ml) were kept at room temperature for 20 minutes. The suspensions were then centrifuged at 8000 x g for 15 minutes followed by filtration through membrane filters (27 mm diameter disks of BAC-T-Flex type B-6 of Schleicher and Schull Co., Keene, New Hampshire, U.S.A.). The filters were placed on a perforated metal sheet that covered a hole (24 mm diameter) of a metal cylinder that fitted on a filter flask. Another metal cylinder with axial hole (24 mm diameter) was placed on the filter. This cylinder served to keep the filter in place while maintaining the vacuum and also contained the suspension to be filtered. The filter flask was connected to a vacuum pump through a U-tube filled with fused calcium chloride. The filtrates thus obtained were found bacteriafree when examined under the electron microscope and also tested for viability by the method described earlier. This filtrate was used for measuring the release of intracellular materials (protein, carbohydrate and 260 nm absorbing materials) from the vibrios after treatment with EDTA (60 /μg/ml).

For comparison, equal amounts of bacteria, similarly harvested and suspended in tris buffer (33 mM, pH 8.0) alone, were heated at 100°C for 20 minutes. It was cooled, filtered through the membrane filters and the cell-free filtrates were subjected to similar subsequent measurements.
To study the release of intracellular materials from the vibrios after treatment with camphor (750 μg/ml) for 2 hours, cell free filtrates were collected from treated vibrio cultures as well as from ethanol controls by the above method. The filtrates were used for measuring the release of protein, carbohydrate and 260 nm absorbing materials.

(ii) **Estimation of protein in the filtrate**

The amount of protein in the filtrate was estimated by the method of Lowry et al. (1951) using crystalline 'Bovine serum albumin' as standard.

(iii) **Estimation of carbohydrate in the filtrate**

Carbohydrate material in the filtrate was quantitatively estimated by the method of Dubois et al. (1956) using glucose as standard.

(iv) **Ultraviolet spectrophotometry of the filtrate**

Optical density of the bacteria-free filtrate relative to the test fluid was measured in the wavelength range 200 - 300 nm with the help of a Beckman DU spectrophotometer.

(b) **Chemical fractionation and estimation**

For studying the effect of camphor, toluene or phenethyl alcohol on macromolecular synthesis of *V. parahaemolyticus* cells, the chemical fractionation method adopted by Raychaudhuri et al. (1970) on *V. cholerae* was followed.

Protein and nucleic acid determinations were carried out on aliquots (40 ml) of the vibrio cultures removed at suitable intervals of time and chilled rapidly. The vibrios were harvested by centrifugation (10,000 x g for 20 minutes) and washed twice in 0.15 M saline (pH 7.6). Washed cells were then suspended in 5 ml of 0.5 M perchloric acid and left for 30 minutes in an ice bath.
Insoluble materials were collected by centrifugation and suspended in 5 ml of 0.5 M perchloric acid at 80°C for about 30 minutes. The hot perchloric acid insoluble material was collected by centrifugation. DNA and RNA in the acid soluble fraction were estimated colorimetrically with diphenylamine (Burton, 1956) and orcinol (Aswell, 1957) respectively using DNA (from calf thymus gland, B.D.H., England) and RNA (from Yeast, B.D.H., England) as standards. The hot perchloric acid insoluble material was digested overnight in 3 ml of 1M NaOH at 37°C. Protein was estimated from it by the method of Lowry et al (1951) using crystalline 'Bovine serum albumin' as standard.

2.13 Characterization of protein component of V. parahaemolyticus

Protein composition of V. parahaemolyticus was determined by Sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis following, in general, the method of Weber and Osborn (1969).

(a) Preparation of protein solutions

For determination of molecular weight of the protein contents of V. parahaemolyticus, some marker proteins were used. The marker proteins included transferrin, Bovine serum albumin, Ovalbumin, Pepsin, B-lactoglobulin, RNAse and Cytochrome C. About 0.5 mg amounts of each of these marker proteins were incubated at 37°C for 2 hours in sodium phosphate buffer containing 7.8 gm of NaH₂PO₄, H₂O (B.D.H.) and 38.6 gm of Na₂HPO₄, 7H₂O (S. Merck) in 1000 ml of redistilled water (pH 8.0). The buffer also contained 0.1% (W/V) sodium dodecyl sulfate (SDS) (BDH) and 0.1% (V/V) β-mercaptoethanol (S. Merck).

For preparation of protein samples from V. parahaemolyticus, vibrios were harvested from logarithmic growth phase in PW medium by centrifugation and washed twice in 0.15 M saline (pH 8.0). The vibrios were then incubated at 37°C for 2 hours in sodium phosphate buffer (pH 8.0) containing 1% (W/V) SDS and
1% (V/V) β-mercaptoethanol. After incubation, the insoluble materials were separated by centrifugation. The clear supernatant was dialysed for 48 hours at room temperature against sodium phosphate buffer containing 0.1% (W/V) SDS and 0.1% (V/V) β-mercaptoethanol.

(b) SDS - polyacrylamide gel electrophoresis of proteins

The following solutions were prepared:

(i) Acrylamide solution - 3.56 gm of acrylamide (B.D.H.) and 96 mg of \( \text{N}_2 \text{H}_2 \) - methylenebisacrylamide (B.D.H.) were dissolved in 20 ml of redistilled water. Insoluble materials were removed by filtration through Whatman No. 1 filter paper and stored at 4°C in a dark bottle.

(ii) Ammonium persulfate solution - This solution was prepared on the day of use. 75 mg of ammonium persulfate (A.R., B.D.H.) was dissolved in 5 ml of redistilled water.

(iii) Gel buffer - This buffer contained 7.8 gm of \( \text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} \); 38.6 gm of \( \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \) and 2 gm of SDS in 1000 ml of redistilled water.

For preparation of gels, 15 ml of gel buffer was first de aerated and mixed with 13.5 ml of acrylamide solution. The mixture was again de aerated; 1.5 ml of fresh solution of ammonium persulfate and 0.045 ml of TEMED (\( N_2N\text{H}_2N\text{H}_2 \) - Tetramethylethylenediamine, Fluka) were added and thoroughly mixed. Gel tubes were made of glass of dimensions 12 cm length and 0.6 cm internal diameter. Lower end of the tubes were sealed watertight with parafilm "M" (Marathon, a division of American Can Company, Wisconsin, U.S.A.). Each tube was filled with 2 ml of the mixture and redistilled water was carefully layered over the mixture. The tubes were allowed to stand 30 minutes at room temperature for polymerization after which water was drawn off and samples applied over the polymerized gels in the tubes.
Before application, sucrose (A.R., B.D.H.) (8% W/V) and suitable amount of tracking dye (Bromophenol blue, E. Merck, 0.05% W/V) were mixed with each sample. After mixing, samples were applied on the gels and gel buffer, diluted 1:1 with redistilled water, was carefully layered on top of each sample to fill the tubes. The parafilm sealing was taken off from each tube and the tubes were fitted in the holes of the upper tank of electrophoresis apparatus in a water tight manner with the help of rubber caps. The two tanks (upper and lower) of the electrophoresis apparatus were filled with gel buffer, diluted 1:1 with redistilled water. The upper tank was connected with the negative electrode while the positive electrode was connected to the lower tank. Electrophoresis was carried out at a constant current of 8 mA/tube for 4 hours after a brief prerun at a current of 2 mA/tube for 30 minutes.

After electrophoresis, length of each gel and distance moved by the tracker dye in it were measured.

Staining and destaining of the gels were performed following the method of Martonosi and Halpin (1971). The gels were taken out of glass tubes and stained for 18 hours at room temperature with 0.1% (W/V) amido black (E. Merck) dissolved in a mixture of methanol (A.R., B.D.H.) : water : Acetic acid (A.R., B.D.H.) (50 : 50 : 10; V/V). Destaining was performed with methanol : water : acetic acid (50 : 50 : 10; V/V) followed by 10% (V/V) acetic acid.

Final length of the gels after destaining and distance moved by different protein bands were measured. Mobility of each protein band was calculated following Weber and Osborn (1969) as follows:

\[ \text{Mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}} \times \frac{\text{Length before staining}}{\text{Length after destaining}} \]

Mobilities of marker protein samples, obtained in this study, were plotted against their known molecular weights on a semi-log scale. Unknown molecular
weights of protein bands obtained from the *V. parahaemolyticus* sample were determined from the standard graph with the help of their mobility values.

2.14 **Characterization of lipopolysaccharide of *V. parahaemolyticus***

(a) **Preparation of acetone dried vibrios**

*V. parahaemolyticus*, 28E/68 was grown at 37°C for 18 hours on PA medium. The vibrios were collected from the agar surface in 0.15 M saline (pH 8.0), centrifuged, and finally obtained as a very thick suspension in saline. This suspension was added dropwise with constant stirring to a large volume of acetone which had been cooled for 1 hour at 0°C. Stirring was stopped after addition of the entire suspension and the whole was allowed to settle for 15 minutes. Acetone was decanted and the bacterial mass was spread on a watch glass and dried to a constant weight.

(b) **Extraction of crude lipopolysaccharide (C-LPS) from the acetone dried vibrios**

Lipopolysaccharide (LPS) was extracted from the acetone dried vibrios (Mergenhagen et al., 1961; Nelson and Roantree, 1967; Raff and Wheat, 1968; Penson and Gray, 1969; Quirk et al., 1976) using, in general, the hot phenol-water method of Westphal et al. (1952). To one gram of acetone dried vibrios in redistilled water (30 ml) was added 30 ml of phenol-water (9:1). The mixture was heated at 70°C with constant stirring for 10 minutes in a water bath. After cooling, it was centrifuged at 1500 x g for 60 minutes. The aqueous phase at the top was removed with a capillary pipette. The remaining interphase and nonsolubly cell residue were subjected to phenol extraction twice in succession. The aqueous phases collected from the three preparations were pooled and subjected to dialysis against distilled water at 4°C for 72 hours. The material obtained was termed as crude LPS-preparation (C-LPS).

This method is presented schematically in Fig. 2.2.
Acetone dried cells
Phenol, 45% (W/V) in distilled water,
10 mins at 70°C, cooled, centrifuged

Aqueous layer
(Top)

Interphase
(Middle)

Cell residue
(Bottom)

Pooled, treated with
Phenol 45% (W/V) in dist. water, 70°C, 10 mins,
Cooled, centrifuged.

Aqueous layer
(Top)

Interphase
(Middle)

Cell residue
(Bottom)

Pooled and dialysed at 4°C
for 72 hrs.

Discarded

Dialysand
(Crude lipopolysaccharide)

Dialysate
discarded

(C-LPS)

Fig. 2.2 Isolation of lipopolysaccharide of V. parahaemolyticus cells
(c) **Preparation of nucleic acid free lipopolysaccharide**

The crude LPS preparation obtained was freed of nucleic acids following, in general, the method of Mergenhagen et al. (1961). The pH of the crude preparation was adjusted to 7.7 with 0.5 N NaOH. RNase (from Bovine pancreas, 4x times crystallized salt free, protease free, B.D.H., England) was added to the material (0.5 mg/ml) and incubated for 24 hours at 60°C. It was then dialysed against distilled water at 4°C for 72 hours. Phenol (A.R., B.D.H.) was added to this dialysed solution to a final concentration of 0.55 g/ml and incubated at 70°C for 10 minutes. The mixture was then centrifuged and the top aqueous phase was removed with the help of a capillary pipette. Pure lipopolysaccharide was precipitated from this aqueous phase by addition of two volumes of cold acetone and a small amount of sodium chloride (A.R., B.D.H.). The precipitate was dried over calcium chloride in vacuum. The final material obtained was termed pure lipopolysaccharide (P-LPS). The method is shown schematically in Fig. 2.3.

(d) **Chemical characterization of P-LPS**

(i) **Test for the presence of nucleic acid in C-LPS and P-LPS**

Appropriate amounts of C-LPS and P-LPS were dissolved separately in redistilled water. Optical density of the solutions were measured in the wavelength range 220-300 nm by a Beckman DU spectrophotometer against redistilled water as blank.

(ii) **Estimation of carbohydrate material in P-LPS**

Carbohydrate material in the purified lipopolysaccharide preparation (P-LPS) was estimated by the method of Dubois et al. (1956) using glucose (A.R., B.D.H.) as standard.

The following reagents were used:

Concentrated H$_2$SO$_4$ - reagent grade, specific gravity 1.84 (A.R., B.D.H.)
**Crude LPS preparation**

pH adjusted to 7.7; treated with HMA-se (0.5 mg/ml) for 24 hrs at 60°C

Dialysed at 4°C for 72 hrs

&

Dialysand

Dialysate

Dialysate

Dialysate

Discarded

Discarded

Discarded

Discarded

Treated with phenol (0.55 gm/ml) for 10 mins at 70°C; cooled, centrifuged

Aqueous phase (Top)

Interphase

Bottom phase

Discarded

Treated with acetone (2 Vols) and trace of NaCl at 0°C; kept at 4°C overnight, centrifuged

Precipitate

Supernatant

Supernatant

Discarded

Discarded

Dried in vacuum

(Yellowish white amorphous powder)

(Pure lipopolysaccharide)

(P -LPS)

Fig. 2.3  **Purification of crude lipopolysaccharide preparation**
Phenol 80% (W/W) – solution in water prepared by adding 80 gms of phenol (A.R., B.D.H.) to 20 gms of redistilled water.

Phenol solution (0.05 ml) was added to 2 ml of P-LPS or standard solution (of known concentration) in redistilled water followed by rapid addition of conc. H₂SO₄ (5 ml). The mixture was allowed to stand at room temperature for 30 minutes. Optical density was then measured against a blank (similarly treated redistilled water) at 480 - 490 nm in a photoelectric colorimeter.

The calibration of the colorimeter scale with known amounts of glucose is presented in Fig. 2.4 (Table 2.1).

(iii) Estimation of total hexose in P-LPS

The amount of total hexose in P-LPS was estimated by the anthrone method using glucose as standard (Boa, 1955).

The anthrone reagent was prepared in the following way: concentrated H₂SO₄ (720 ml) was added to redistilled water (280 ml). While the mixture was still warm, anthrone (B.D.H.) (500 mg) and thiourea (B.D.H.) (10 gm) was added and mixed until dissolved. This mixture was cooled, stored in a refrigerator and used after at least 14 hours.

To one ml of P-LPS or standard solution (of known concentration) in redistilled water was added 5 ml of cold anthrone reagent, shaken vigorously and heated in a boiling water-bath for 15 minutes. The mixture was cooled and absorbancy measured at 620 nm after 20 minutes.

The calibration of the colorimeter scale with different amounts of the standard (glucose) is shown in Fig. 2.5 (Table 2.2).

(iv) Estimation of hexosamine in P-LPS

Hexosamine in the P-LPS was estimated by the method of Gatt and
Table 2a

Calibration of the colorimetre scale. Results were obtained as average of two or more independent experiments.

<table>
<thead>
<tr>
<th>Observed O. D.</th>
<th>O. D. after dilution</th>
<th>Dilution factor</th>
<th>Corrected O. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.27</td>
<td>2</td>
<td>0.54</td>
</tr>
<tr>
<td>0.75</td>
<td>0.40</td>
<td>2</td>
<td>0.80</td>
</tr>
<tr>
<td>1.00</td>
<td>0.35</td>
<td>4</td>
<td>1.40</td>
</tr>
<tr>
<td>1.40</td>
<td>0.30</td>
<td>5</td>
<td>1.50</td>
</tr>
<tr>
<td>1.60</td>
<td>0.30</td>
<td>7</td>
<td>2.10</td>
</tr>
<tr>
<td>1.90</td>
<td>0.30</td>
<td>8</td>
<td>2.40</td>
</tr>
<tr>
<td>3.10</td>
<td>0.40</td>
<td>10</td>
<td>4.00</td>
</tr>
<tr>
<td>4.00</td>
<td>0.40</td>
<td>12</td>
<td>4.80</td>
</tr>
<tr>
<td>5.00</td>
<td>0.40</td>
<td>16</td>
<td>6.40</td>
</tr>
</tbody>
</table>

Table 2.1

Calibration data for the estimation of Carbohydrate.

<table>
<thead>
<tr>
<th>Glucose (/ug/ml)</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1.2</td>
</tr>
<tr>
<td>25</td>
<td>1.9</td>
</tr>
<tr>
<td>50</td>
<td>3.4</td>
</tr>
<tr>
<td>80</td>
<td>4.7</td>
</tr>
<tr>
<td>100</td>
<td>5.4</td>
</tr>
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</table>
Fig. 2.4. Calibration of the colorimeter scale with known amounts of glucose (A.R., B.D.H.) for the estimation of carbohydrate by the method of Dubois et al. (1956).

Suitable amount of P-LPS was hydrolyzed by 2N HCl at 100°C for 10 hours and then neutralized with 2 M Na₂CO₃. 2% solution of acetylacetone in 1.5 Na₂CO₃ was prepared. 0.5 ml of this solution was added to 1 ml of the neutralize P-LPS, sample or standard solution, final pH being adjusted between 9.6 and 9.8. Mixture was then taken in glass vials, sealed and heated in boiling water bath for 20 minutes. It was then cooled and 1.0 ml of ethanol was added followed by 0.5 ml of Ehrlich's reagent (1 gm of p-dimethyl amino benzaldehyde in a mixture of 15 ml of concentrated HCl and 15 ml of ethanol). CO₂ was expelled from the mixture by vigorous shaking. Optical density was measured at 530 nm after about 5 minutes.

The calibration of the colorimeter scale with different amounts of standard (glucosamine) is shown in Fig. 2.6 (Table 2.3).

(v) Estimation of fatty acid in P-LPS

Fatty acid in the P-LPS preparation was estimated by the method of Haskins (1961) using palmitic acid (K. Light & Co., England) as standard.

The following reagents were used:

Stock ferric perchlorate - Ferric perchlorate (5 gm) was dissolved in 10 ml of 70% HClO₄ and 10 ml water, diluted to 100 ml with cold absolute ethanol finally stored in refrigerator. Ferric perchlorate reagent was prepared by mixing stock ferric perchlorate (4 ml) with 70% HClO₄ (3 ml) and then diluting this mixture to 100 ml with cold absolute ethanol.

Alkaline hydroxylamine - 4% ethanolic hydroxylamine solution (prepared by dissolving 2.0 gm hydroxylamine in 2.5 ml of redistilled water, diluted to 50 ml with absolute ethanol) was mixed with an equal volume of 8% ethanolic NaOH (prepared by dissolving 4 gm of NaOH in 2.5 ml of redistilled water, diluted to 50 ml with absolute ethanol) in a stoppered cylinder. The NaCl formed was separated by cent
Fig. 2.5, Calibration of the colorimeter scale with known amounts of glucose (A, B, C, D, E) for the estimation of hemase by the method of Roe (1955).
Table 2.2
Calibration data for the estimation of hexose

<table>
<thead>
<tr>
<th>Glucose (ug/ml)</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.35</td>
</tr>
<tr>
<td>40</td>
<td>0.71</td>
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<tr>
<td>80</td>
<td>1.2</td>
</tr>
<tr>
<td>100</td>
<td>1.54</td>
</tr>
<tr>
<td>150</td>
<td>2.15</td>
</tr>
<tr>
<td>200</td>
<td>2.75</td>
</tr>
</tbody>
</table>

Table 2.3
Calibration data for the estimation of hexosamine

<table>
<thead>
<tr>
<th>Glucosamine (ug/ml)</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1.3</td>
</tr>
<tr>
<td>25</td>
<td>2.24</td>
</tr>
<tr>
<td>50</td>
<td>3.7</td>
</tr>
<tr>
<td>80</td>
<td>4.9</td>
</tr>
<tr>
<td>100</td>
<td>5.41</td>
</tr>
</tbody>
</table>
Fig. 2.6. Calibration of the colorimeter scale with known amounts of glucosamine (Note: 1 mg/ml) for the estimation of hexosamine by the method of Gott and Berman (1966).
gation and the supernatant taken for use.

Solution of P-LPS or standard in ethanol (1 ml) was mixed with 2 ml of freshly prepared alkaline hydroxylamine reagent and the mixture was kept in a tightly sealed leakproof test tube. The tube was heated at 67°C for 5 hours in a water bath. It was then cooled to room temperature and ferric perchlorate reagent (5 ml) added. The purple colour formed was read at 530 nm against a blank (similarly treated ethanol) after 30 minutes.

The calibration of the colorimeter scale with different amounts of the standard (palmitic acid) is shown in Fig. 2.7 (Table 2.4).

(vi) Estimation of uronic acid in P-LPS

The uronic acid content of P-LPS was estimated following, in general, the modified carbazole method of Bitter and Wyns (1961) using glucuronic acid (L. Light & Co., England) as standard.

To 1 ml of P-LPS or standard solution in redistilled water was added 6 ml of sulphuric acid reagent (195 ml of concentrated H₂SO₄ + 5 ml of M Na₂B₄O₇, 10 H₂O) at 0°C. The mixture was gently shaken for a few minutes and then vigorously. The mixture was then heated in a sealed tube for 15 to 20 minutes in a boiling water bath, cooled and 0.2 ml of carbazole reagent (0.1% carbazole in ethanol) was added. This mixture was then shaken, heated for another 10 minutes at 100°C and finally kept in dark for 3 hours. The optical density was measured after that period at 530 nm.

The calibration of the colorimeter scale with different amounts of the standard (glucuronic acid) is shown in Fig. 2.8 (Table 2.5).

(vii) Estimation of protein in P-LPS

The amount of protein in P-LPS was estimated by the method of Lowry et al. (1951) using crystalline 'Bovine serum albumin (Fraction V, L. Light & Co.,
Fig. 2.7. Calibration of the colorimeter scale with known amounts of palmitic acid (K. Light & Co., England) for the estimation of fatty acid by the method of Haskins (1961).
### Table 2.4

Calibration data for the estimation of fatty acid

<table>
<thead>
<tr>
<th>Palmitic acid (/μg/ml)</th>
<th>Optical density (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td>100</td>
<td>0.12</td>
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<tr>
<td>200</td>
<td>0.26</td>
</tr>
<tr>
<td>300</td>
<td>0.40</td>
</tr>
<tr>
<td>400</td>
<td>0.50</td>
</tr>
<tr>
<td>500</td>
<td>0.61</td>
</tr>
</tbody>
</table>

### Table 2.5

Calibration data for the estimation of uronic acid

<table>
<thead>
<tr>
<th>Glucuronic acid (/μg/ml)</th>
<th>Optical density (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.25</td>
</tr>
<tr>
<td>20</td>
<td>0.55</td>
</tr>
<tr>
<td>40</td>
<td>1.10</td>
</tr>
<tr>
<td>60</td>
<td>1.65</td>
</tr>
<tr>
<td>80</td>
<td>2.05</td>
</tr>
<tr>
<td>100</td>
<td>2.70</td>
</tr>
</tbody>
</table>
Fig. 2.8. Calibration of the colorimeter scale with known amounts of glucuronide acid (L. Light & Co., England) for the estimation of uronic acid by the method of Bitter and Ewins (1961).
The following reagents were used:


Solution B - 0.5% CuSO$_4$, 5H$_2$O (G.R., E. Merck) in 1% sodium potassium tartrate (G.R., E. Merck).

Solution C - Alkaline copper solution: 50 ml of solution A + 1 ml of solution B.

Folin Ciocalteu phenol reagent (E. Merck) - diluted to make it 1 N in acid.

Solution C (5 ml) was added to 1 ml of P-LPS or standard solution (of known amount) in redistilled water and allowed to stand for at least 10 minutes at room temperature. Folin ciocalteu reagent (0.5 ml) was then rapidly added and immediately mixed. The mixture was then allowed to stand at room temperature for at least 30 minutes. Optical density of the mixture was finally measured against a blank at 680 nm with the help of a photoelectric colorimeter.

The calibration of the colorimeter scale with known amounts of the standard (Bovine serum albumin) has been presented in Fig. 2.9 (Table 2.6).

(viii) Estimation of phosphorus in P-LPS

Phosphorus in the P-LPS preparation was estimated quantitatively by the method of Fiske and Subbarow (1925) using K$_2$HPO$_4$ (Graded reagent, E. Merck) as standard. The following reagents (analytical grade) were used:

(i) sulphuric acid, 5N, (ii) ammonium molybdate, 2.5% (W/V) in H$_2$O and (iii) reducing reagent prepared by thoroughly mixing 0.2 gm of 1-amino-2-naphthol-4-sulphonic acid with 1.2 gm of sodium bisulfite and 1.2 gm of sodium sulfite in powdered form and then dissolving 0.25 gm of this mixture in 10 ml of redistilled water.

To 1 ml of solution of deproteinised P-LPS or standard in redistilled water, 1 ml of sulphuric acid was added followed by 1 ml of molybdate. After mixing,
### Table 2.6
Calibration data for the estimation of protein

<table>
<thead>
<tr>
<th>Bovine serum albumin (μg/ml)</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.18</td>
</tr>
<tr>
<td>25</td>
<td>0.68</td>
</tr>
<tr>
<td>50</td>
<td>1.22</td>
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<tr>
<td>100</td>
<td>2.31</td>
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<tr>
<td>150</td>
<td>3.33</td>
</tr>
<tr>
<td>200</td>
<td>4.61</td>
</tr>
</tbody>
</table>

### Table 2.7
Calibration data for the estimation of phosphorus

<table>
<thead>
<tr>
<th>Phosphorus (μg/ml)</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.4</td>
</tr>
<tr>
<td>20</td>
<td>1.12</td>
</tr>
<tr>
<td>30</td>
<td>1.41</td>
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<tr>
<td>40</td>
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<td>50</td>
<td>2.29</td>
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<tr>
<td>80</td>
<td>3.95</td>
</tr>
<tr>
<td>100</td>
<td>4.65</td>
</tr>
</tbody>
</table>
Fig. 2.9. Calibration of the colorimeter scale with known amounts of bovine serum albumin (Fraction V, L. Light & Co., England) for the estimation of protein by the method of Lowry et al. (1951).
Reducing solution (0.1 ml) was added and the volume was made up to 10 ml. After proper mixing, the absorbancy was measured at 660 nm after 10 minutes.

The calibration of the colorimeter scale with known amounts of the standard ($K_2HPO_4$) is shown in Fig. 2.10 (Table 2.7).

(ix) Detection of heptose in P-LPS

Presence of heptose in the P-LPS preparation was detected by the cysteine-sulfuric acid method of Dische (1955).

P-LPS solution (1 ml) in redistilled water was placed in ice water. 4.5 ml of a mixture of 6 parts of concentrated sulfuric acid and 1 part of water was added to the P-LPS solution. After one minute, the reaction mixture was shaken in ice water and then transferred to tap water. It was kept under tap water for 2 minutes and finally in a boiling waterbath for 3 minutes. It was then cooled under tap water and 0.1 ml of 3% solution of cysteine hydrochloride ($B_dH_4$) was added. The mixture was shaken for a brief period and then allowed to stand at room temperature. After a few hours, the colour of the mixture was noted and its optical density at different wavelengths in the range 450-610 nm was measured in a photoelectric colorimeter. A similarly treated redistilled water (used as control) was used as blank.

(x) Detection of 2-keto-3-deoxy-octonic acid (KDO) in P-LPS

The detection of KDO in the P-LPS preparation was carried out following in general the thiobarbituric acid test described by Weissbach and Hurwitz (1959).

P-LPS solution in redistilled water (0.2 ml) was added to 0.25 ml of 0.025 N HIO$_4$ in 0.125 N $H_2SO_4$ and incubated at room temperature for 20 minutes. Then 0.5 ml of 2% sodium arsenite solution in 0.5 N HCl was added with shaking and the final mixture allowed to stand for two minutes. 2 ml of 0.3% thiobarbituric acid (pH 2.0) was then added to it and after stirring, the mixture was heated for 10 minutes at $10^\circ C$. It was cooled and the optical density measured at different wavelengths.
Fig. 2.9. Calibration of the colorimeter scale with known amounts of $\text{KH}_2\text{PO}_4$ (G.R., E. Merck) for the estimation of phosphorus by the method of Fiske and Subbarow (1925).
between 450 and 610 nm against a similarly treated redistilled water (used as control) in a photoelectric colorimeter.

(xi) **Paper chromatography of P-LPS**

For paper chromatography, P-LPS samples were hydrolysed in sealed tubes at 100°C for 10 hours in 2 N HCl. Hydrolysates were neutralized by repeated evaporation to dryness in vacuo over KOH pellets (Humphrey and Vincent, 1969). Solutions of the residue in 10% (V/V) isopropanol (A.R., B.D.H.) were used for ascending paper chromatography on Whatman no. 1 paper. The solvent systems used were as follows:

(i) isopropanol - water (4:1, by volume),

(ii) n-butanol - ethanol - water (5:1:4, by volume, upper layer).

(A.R., B.D.H.)

Paper chromatograms were developed by the method of Smith (1960) using the silver nitrate reagent. The reagents used were as follows:

(a) AgNO₃ saturated solution in water - 0.1 vol.
Acetone - 20 vol.

(b) NaOH - 0.5% (W/V) in ethanol.

The paper was first dipped through the silver nitrate reagent and the acetone was blown off. After drying, the paper was dipped through the alkali and again the solvent (ethanol) was blown off. All spots appeared within 10 minutes at room temperature, while the background changed to light brown.

For identification of sugars, solution of glucose (A.R., B.D.H.) in 10% (V/V) isopropanol was applied for parallel run with the LPS samples. After development of the paper chromatograms, distances moved by glucose and sugars in the P-LPS sample were measured. Rg values of sugars were then determined as follows:

\[
Rg = \frac{\text{distance moved by sugar from origin}}{\text{distance moved by glucose from origin}} \times 100
\]
Individual sugars in P-LPS sample were identified by comparing their Rg values with known Rg values of standard sugars in corresponding solvent systems. The standard values used were verified in the laboratory by similar chromatographic method.

(xii) Infrared spectrum of P-LPS

Infrared spectrum of P-LPS preparation was taken in the 'nujol' phase with the help of a Perkin Elmer infrared spectrophotometer provided with an automatic recorder.