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# Phosphatidylethanolamine Distribution and Fluidity in Outer and Inner Membranes of the Gram-Negative Bacterium *Erwinia carotovora*

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1. The distribution of phosphatidylethanolamine, the major lipid of *Erwinia carotovora*, was investigated in intact bacteria, spheroplasts and outer- and inner-membrane preparations, with the amino-group reagent 2,4,6-trinitrobenzenesulphonic acid. Only 4% was found on the external surface of the outer membrane with 30% on the internal surface, whereas the inner membrane had 27 and 38% on its external and internal surfaces respectively. Some comparative studies were made with three other bacteria. 2. The fluidity of the membranes of *E. carotovora* was studied by using the fluorescent probe 1,6-diphenylhexa-1,3,5-triene. Results were consistent with the hydrocarbon region of the outer membrane bilayer being less fluid than that of the inner one. 3. On the basis of these and other results a model for the outer- and inner-membrane structures of *E. carotovora* is proposed.

Some information on the arrangement of lipids in the membranes of bacteria is now available. Thus studies with membranes of the Gram-positive bacteria *Micrococcus lysodeikticus* (Barsukov *et al.*, 1976), *Bacillus subtilis* (Bishop *et al.*, 1977) and *Bacillus megaterium* (Rothman & Kennedy, 1977) have indicated that phospholipids are asymmetrically distributed. However, no similar studies have been reported for Gram-negative bacteria, which have a more complex system of inner and outer membranes (Costerton *et al.*, 1974; Salton & Owen, 1976; Braun, 1978). We have therefore investigated the distribution of phosphatidylethanolamine by using 2,4,6-trinitrobenzenesulphonic acid labelling, and fluidity by using a fluorescent probe, in outer- and inner-membrane preparations from the Gram-negative bacterium *Erwinia carotovora*. This bacterium is a plant pathogen (Starr & Chatterjee, 1972) and an opportunistic human pathogen (von Graevenitz, 1977) containing about 95% of its total phospholipid as phosphatidylethanolamine (Shukla *et al.*, 1978; Shukla & Turner, 1979). Preliminary results with other Gram-negative bacteria are also discussed.

## Materials and Methods

### *Materials*

*Escherichia coli* (N.C.I.B. 8114) and *Flavobacterium rhenanum* (N.C.I.B. 9157) were obtained as freeze-dried cultures from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K., and are referred to by the N.C.I.B. catalogue numbers. *Er. carotovora* (N.C.P.P.B. 1280) was obtained from the National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, Herts., U.K., and is referred to by its N.C.P.P.B. catalogue number. *Mycobacterium smegmatis* (N.C.T.C. 7017), which was obtained from the National Collection of Type Culture, Central Public Health Laboratory, Colindale Avenue, London NW9, U.K., is similarly referred to by the N.C.T.C. catalogue number. The fluorescent probe 1,6-diphenylhexa-1,3,5-triene was supplied by Ralph N. Emmanuel, Wembley, Middx., U.K. All other chemicals were purchased either from Sigma Chemicals (Kingston upon Thames, Surrey, U.K.) or from BDH Chemicals (Poole, Dorset, U.K.) and were of analytical-reagent grade.

### *Methods*

*Growth of micro-organisms.* Liquid cultures (1 litre) were grown in 2 litre conical flasks. The standard medium contained 3.0g of glycerol, 7.0g of $K_2HPO_4$, 3.0g of $KH_2PO_4$, 1.0g of $Na_2SO_4$,$7H_2O$ and 0.1g of $MgSO_4$,$7H_2O$, made up to 1 litre with glass-distilled water. Ethanolamine was used as a nitrogen source in the medium at a concentration of 100mg of nitrogen/litre. For *Esch. coli* the medium was supplemented with 40$\mu$g of vitamin $B_{12}$/litre. The medium was adjusted to pH 7.0 by HCl or 20% (w/v) NaOH as appropriate. In the case of *Myc. smegmatis* the bacteria were grown on the standard

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medium (200ml) supplemented with 2% (w/v) Bacto-agar, in Roux bottles at 37°C, as surface cultures. All media were sterilized by autoclaving.

*Preparation of spheroplasts, outer and inner membranes.* Spheroplasts from *Esch. coli* and *F. rhenanum* were prepared by a lysozyme/EDTA treatment similar to that described for *Er. carotovora* (Shukla & Turner, 1975), except that the concentrations of lysozyme used for *Esch. coli* and *F. rhenanum* were 20 $\mu$g/ml and 180 $\mu$g/ml respectively. Outer and inner membranes from *Er. carotovora* spheroplasts were isolated by sonic disruption and density-gradient centrifugation as described by Shukla & Turner (1975).

*Labelling of phosphatidylethanolamine with trinitrobenzenesulphonic acid.* The procedure for labelling with 2,4,6-trinitrobenzenesulphonic acid was essentially that of Roseman *et al.* (1975).

*Labelling of external phosphatidylethanolamine.* To 0.6ml of sample suspension in 0.05M-NaCl (containing 200–300nmol of reactive phosphatidylethanolamine), 0.2ml of 0.8M-$NaHCO_3$ (pH8.5) and 20$\mu$l of 1.5% (w/v) trinitrobenzenesulphonic acid in water were added. With spheroplast suspensions, a concentration of 0.75M-sucrose was maintained to avoid lysis. The reaction was allowed to proceed at 30°C for 30min in the dark, then terminated by the addition of 1.2ml of 1.06% (v/v) Triton X-100 in 1.5M-HCl. Appropriate blanks were run in parallel to test the possibility of trinitrobenzenesulphonic acid reaction continuing after the termination of the reaction: no labelling was observed. Reaction mixtures containing intact cells and spheroplasts were centrifuged at 5000*g* for 20min and the pellets washed twice with water (0.75M-sucrose for spheroplasts). Lipid was extracted from these pellets immediately. In the case of outer and inner membranes, the lipids were extracted directly from the reaction mixtures. The extracted lipid was then dried under $N_2$ and dispersed in a solution containing 0.6ml of 0.05M-NaCl, 0.2ml of 0.8M-$NaHCO_3$ (pH8.5) and 1.22ml of 1.06% (v/v) Triton X-100 in 1.5M-HCl. The absorbance of this suspension was recorded at 410nm.

*Labelling of total phosphatidylethanolamine.* In this case, the total lipid was first extracted from 0.5ml of the sample suspension and dried under $N_2$. Approx. 200–300nmol of phosphatidylethanolamine was taken for labelling. To this lipid, 0.6ml of 1.6% (v/v) Triton X-100 in 0.8M-$NaHCO_3$ (pH8.5), 0.6ml of 0.05M-NaCl and 20$\mu$l of 1.5% (w/v) trinitrobenzenesulphonic acid in water were added and the mixture was incubated for 30min at 30°C. The reaction was terminated by the addition of 0.8ml of 0.4% (v/v) Triton X-100 in 1.5M-HCl and the absorbance at 410nm was recorded. Under these conditions, an absorbance of 1.0 corresponded to 0.368$\mu$mol of phosphatidylethanolamine.

Analysis of trinitrobenzenesulphonic acid-labelled lipids on silica gel G (0.5mM) in a solvent system of chloroform/methanol/1M-$NH_4OH$ (80:36:5, by vol.) showed that phosphatidylethanolamine was the only lipid that reacted with trinitrobenzenesulphonic acid. It was also established that trinitrophenylated phosphatidylethanolamine was not hydrolysed by purified phospholipases C (*Bacillus cereus* and *Clostridium perfringens*).

*Measurement of fluidity.* Outer- and inner-membrane preparations from *Er. carotovora* were separately suspended in 10–15ml of water and sonicated at 0–4°C to decrease turbidity. Diphenylhexatriene (0.33ml of a solution containing 2.5mg/20ml of ethanol, stored at 4°C in the dark) was mixed with 1ml of membrane suspension and kept for 10min at 30°C. After this time the polarization of the fluorescence emitted at 430nm was recorded at 20–21°C with a Perkin–Elmer fluorescence spectrophotometer model MPF-2A (Hitachi, Tokyo, Japan). The excitation wavelength for 1,6-diphenylhexa-1,3,5-triene was 380nm. No correction for light scattering was necessary. The magnitude of the recorder-pen deflection for the vertically and horizontally polarized emission beams was determined by setting the excitation and emission beam polarizers successively at 0° and 90°. The degree of fluorescence polarization (*P*) was calculated from the equation:

$$P\ (\text{corrected}) = \frac{V_v - L_v \dfrac{V_h}{L_h}}{V_v + L_v \dfrac{V_h}{L_h}}$$

where *V* and *L* are the vertical and horizontal components respectively. The subscripts v and h indicate the direction of polarization, vertical and horizontal, of the light incident on the sample.

*Analytical methods.* Lipids were extracted by the method of Folch *et al.* (1957) and their lipid phosphorus was assayed by the procedure of Fiske & Subbarow (1925). Protein was estimated by the method of Lowry *et al.* (1951).

## Results

### *Trinitrobenzenesulphonic acid labelling of intact cells and spheroplasts*

Intact bacteria of four different genera were labelled with trinitrobenzenesulphonic acid. The results are shown in Table 1. It was apparent that in *Er. carotovora*, *Esch. coli*, *Flavobacterium rhenanum* and *Myc. smegmatis* only 3–9% of the total phosphatidylethanolamine was labelled, the remaining 91–97% of phosphatidylethanolamine being inaccessible to trinitrobenzenesulphonic acid. When

trinitrobenzenesulphonic acid-labelled lipids from the above bacteria were analysed by t.l.c., phosphatidylethanolamine was found to be the only phospholipid labelled.

When spheroplasts of *Er. carotovora*, *Esch. coli* and *F. rhenanum* were exposed to trinitrobenzenesulphonic acid, there was a slight increase in the labelling of phosphatidylethanolamine compared with intact bacteria (Table 2). However, most of the phosphatidylethanolamine (80–87%) still remained unlabelled, i.e. was inaccessible to trinitrobenzenesulphonic acid.

*Trinitrobenzenesulphonic acid labelling of outer- and inner-membrane vesicles of Er. carotovora*

Outer and inner membranes of *Er. carotovora*, isolated from their spheroplasts (Shukla & Turner, 1975), were labelled with trinitrobenzenesulphonic acid and the results are shown in Table 3. Of the total phosphatidylethanolamine of the outer-membrane vesicles, about 13% was accessible to trinitrobenzenesulphonic acid and 87% remained unlabelled. However, in the inner-membrane vesicles, 41% of the total phosphatidylethanolamine was labelled with trinitrobenzenesulphonic acid and the remaining 59% was not accessible to the reagent. Of the total membrane phosphatidylethanolamine in *Er. carotovora*, 35 and 65% were present in the outer and inner membranes respectively. Thus the amounts of phosphatidylethanolamine apparently present on the external and internal surfaces of outer- and inner-membrane vesicles could be estimated and are shown in Table 4.

*Fluidity in membranes of Er. carotovora*

Fluorescent probes have been widely used in

Table 1. *Labelling of intact cells of different bacteria with trinitrobenzenesulphonic acid*

The bacteria were grown and labelled with trinitrobenzenesulphonic acid as described in the Materials and Methods section. Lipids were extracted from trinitrobenzenesulphonic acid-treated cells and the amount of labelled phosphatidylethanolamine (PE) was determined from the absorbance at 410nm (see the Materials and Methods section). Values are means (± S.D.) from two experiments, where each determination was done in duplicate. Variation between duplicates was ±3%. Numbers in parentheses are for trinitrobenzenesulphonic acid-reactive phosphatidylethanolamine as percentages of the total phosphatidylethanolamine.

| Bacterium | Total PE (nmol/ml of suspension) | Trinitrobenzenesulphonic acid-reactive PE (nmol/ml of suspension) |
|---|---|---|
| *Er. carotovora* | 4158 ± 18 | 123 ± 3 (3) |
| *Esch. coli* | 3537 ± 29 | 164 ± 2 (5) |
| *F. rhenanum* | 2790 ± 33 | 255 ± 5 (9) |
| *Myc. smegmatis* | 238 ± 2 | 9 ± 1 (4) |

Table 2. *Labelling of spheroplasts of different bacteria with trinitrobenzenesulphonic acid*

The procedures for the preparation of spheroplasts from bacteria and their treatment with trinitrobenzenesulphonic acid were as described in the Materials and Methods section. Values are means (± S.D.) from two experiments, where each determination was done in duplicate. Variation between duplicates was ±3%. Results in parentheses are percentages of the total phosphatidylethanolamine (PE) present.

| Bacterial source of spheroplasts | Total PE (nmol/ml of suspension) | Trinitrobenzenesulphonic acid-reactive PE (nmol/ml of suspension) |
|---|---|---|
| *Er. carotovora* | 2597 ± 3 | 436 ± 35 (16) |
| *Esch. coli* | 2194 ± 24 | 446 ± 4 (20) |
| *F. rhenanum* | 2524 ± 64 | 323 ± 7 (13) |

Table 3. *Labelling of outer- and inner-membrane vesicles of Er. carotovora with trinitrobenzenesulphonic acid*

Outer- and inner-membrane vesicles were treated with trinitrobenzenesulphonic acid, their lipid extracted and the amount of labelled phosphatidylethanolamine (PE) was determined (see the Materials and Methods section). Data from two experiments (means ± S.D.) are shown, where each determination was done in duplicate. Variation between duplicates was ±3%. Results in parentheses are percentages of the total phosphatidylethanolamine present.

| Membrane fraction | Total PE (nmol/ml of suspension) | Trinitrobenzenesulphonic acid-reactive PE (nmol/ml of suspension) |
|---|---|---|
| Outer membrane | 2756 ± 34 | 366 ± 4 (13) |
| Inner membrane | 3303 ± 14 | 1338 ± 2 (41) |

Table 4. *Estimated distribution of phosphatidylethanolamine (PE) in the membranes of Er. carotovora*
The content of PE was estimated by labelling with trinitrobenzenesulphonic acid (see Table 3). The distribution of PE on different surfaces of outer and inner membrane was calculated from the proportion of PE accessible to trinitrobenzenesulphonic acid in the outer- and inner-membrane vesicles as described in the text. The values are represented as percentages of the total PE in the membranes.

| | Total PE (%) | Estimated distribution of PE (%) | |
|---|---|---|---|
| | | External surface | Internal surface |
| Outer membrane | 35 | 4.6 | 30.3 |
| Inner membrane | 65 | 26.4 | 38.5 |

Table 5. *Fluidity in outer and inner membranes of Er. carotovora*
The membranes were prepared from spheroplasts as described by Shukla & Turner (1975). The outer and inner membranes were treated with the fluorescent probe diphenylhexatriene and the degree of fluorescence polarization (*P*) was calculated as described in the Materials and Methods section. Results for two separate experiments are presented.

| | Degree of fluorescence polarization | |
|---|---|---|
| | Outer membrane | Inner membrane |
| Expt. I | 0.24 | 0.19 |
| Expt. II | 0.26 | 0.23 |

measurements of membrane fluidity (Radda & Vanderkooi, 1972). In the present study diphenylhexatriene was incorporated into the outer- and inner-membrane vesicles of *Er. carotovora* and the degree of polarization of the fluorescence emission (*P*) was calculated. The results are shown in Table 5. It was clear that the fluorescent probe gave a lower fluorescence-emission value for the inner-membrane vesicles than for the outer-membrane vesicles (Table 5).

## Discussion

Treatment of *Er. carotovora* and three other bacteria with the amino-group reagent trinitrobenzenesulphonic acid resulted in little labelling of phosphatidylethanolamine. This confirmed that the reagent does not penetrate intact bacteria as observed previously for other species (Litman, 1973; Carraway, 1975; Marinetti & Love, 1976). It also showed that there was little phosphatidylethanolamine present on the external surface of the bacteria. The small amount of phosphatidylethanolamine (3–9%) that was labelled by trinitrobenzenesulphonic acid might be due to some degree of cell lysis. It may be concluded that almost all the phosphatidylethanolamine found after cell disruption is located in the internal surfaces of the intact bacteria. It is relevant to recall that treatment of *Salmonella typhimurium* with phospholipase C showed that very little of the phospholipids was accessible to attack by this enzyme at the external surface (Kamio & Nikaido, 1976), an observation in keeping with our labelling experiments with Gram-negative bacteria.

Labelling of spheroplasts of *Er. carotovora*, *Esch. coli* and *F. rhenanum* with trinitrobenzenesulphonic acid indicated that they were only slightly permeable. Here again a major proportion of phosphatidylethanolamine was inaccessible to the reagent, giving support to the conclusion, from intact cell labelling, that this phospholipid is predominantly present on membrane surfaces other than the external surface of the cell.

An interesting pattern of distribution of phosphatidylethanolamine emerged from the labelling of outer- and inner-membrane vesicles of *Er. carotovora* (see Table 4). Of the total phosphatidylethanolamine of the membranes (outer + inner), a negligible amount (4%) was present on the outer surface of the outer membrane, whereas about 30% was on the internal surface. This, together with the results from intact cell labelling, leads to the conclusion that the outer membrane has phosphatidylethanolamine present only in its inner half (inner leaflet), confirming previous suggestions (see Salton & Owen, 1976; Braun, 1978). In the case of the inner membrane 27% of the phosphatidylethanolamine is present on its external surface and 38% on the internal surface. This suggests that in the inner membrane, phosphatidylethanolamine is in a bilayer.

It is likely that the acyl chains of phosphatidylethanolamine molecules present in the inner half of the outer membrane interact with the acyl chains of externally oriented lipopolysaccharides. This is supported by the observation that the numbers of fatty acids present in the phospholipids of the outer membrane are equal, within the limits of detection, to those provided by the lipid A portion of the lipopolysaccharides (Braun, 1978).

Fluorescent hydrocarbon probes have been used in various membrane studies (Rudy & Gitler, 1972; Radda & Vanderkooi, 1972; Shinitzky & Barenholz, 1974). Their incorporation into outer and inner

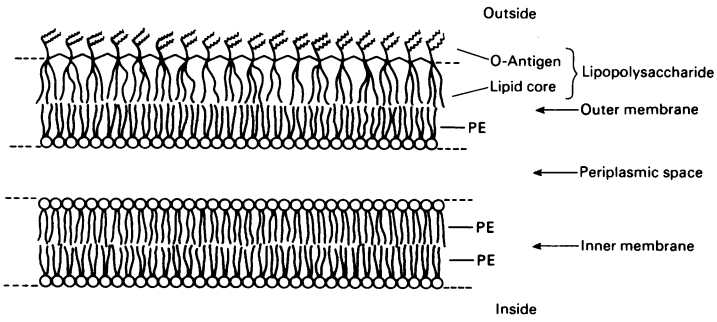


Fig. 1. *Possible arrangement of phosphatidylethanolamine in membranes of Er. carotovora*
Abbreviation: PE, phosphatidylethanolamine.

membranes of *Er. carotovora* indicates the presence of hydrophobic environments in both membranes. Although the results obtained with such probes depend on several factors (Hare & Lussan, 1977), the results are consistent with the hydrocarbon region of the bilayer of the outer membrane being less fluid than that of the inner one. That this is the case is further suggested by the findings (*a*) that as in other Gram-negative bacteria (Osborn *et al.*, 1972; Hasin *et al.*, 1975) the protein/lipid ratio of the outer membrane is higher (Shukla *et al.*, 1978) and (*b*) that its content of short-chain and unsaturated fatty acids is lower (S. D. Shukla, unpublished work) than that of the inner membrane. The relatively greater rigidity of the outer membrane could arise from the presence of lipid A (containing saturated acyl chains; Rottem, 1978) and probably also from immobilization of lipids around proteins (Hubbell & McConnell, 1971).

From the results, a model for the molecular distribution of phosphatidylethanolamine in the outer and inner membranes of *Er. carotovora* can be constructed (Fig. 1). Since intact cells and spheroplasts of different bacteria showed a pattern of trinitrobenzenesulphonic acid labelling similar to that of *Er. carotovora*, it is possible that their membranes may have similar distributions of phosphatidylethanolamine.

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