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Outer Membrane of *Salmonella typhimurium:* Accessibility of Phospholipid Head Groups to Phospholipase C and Cyanogen Bromide Activated Dextran in the External Medium†

Yoshiyuki Kamio and Hiroshi Nikaido*

ABSTRACT: Whole cells of *Salmonella typhimurium* were treated with *Bacillus cereus* phospholipase C or with CNBr-activated dextran. If phosphatidylethanolamine head groups are exposed and accessible on the outer surface of the outer membrane of these cells, it was expected that these groups would be hydrolyzed by the former agent, and become covalently coupled to the latter agent. With strains producing lipopolysaccharides of S or Rc type, results did not indicate the presence of any accessible head groups on the outer surface. In contrast, with strains that produce outer membranes containing less complete lipopolysaccharides (Rd or Re type) and reduced amounts of proteins, both methods clearly showed the presence of exposed phosphatidylethanolamine head groups. These data can be most easily explained by assuming that the outer membrane of S and Rc strains either contains all phospholipid molecules in its inner leaflet or has proteins that completely cover up the head groups at its outer surface. In either model, the reduction in the amount of outer membrane proteins in Rd or Re mutants would produce membranes with exposed phospholipid head groups. CNBr-activated dextran can be easily prepared, and reacts with high efficiency under near-physiological conditions. Its additional advantage as a nonpenetrating membrane-labeling reagent is that we can be quite confident on its impermeability because of its size, in contrast with most other reagents whose presumed impermeability is dependent only on the presence of charged groups.

The cytoplasm of gram-negative bacteria is enclosed by two membranes (Glauert and Thornley, 1969). The inner membrane corresponds to the cytoplasmic membrane, and the outer membrane, together with the underlying peptidoglycan layer, comprises the cell wall. The outer membrane is similar to many other biological membranes in that it contains phospholipids and proteins and that it gives a typical "unit membrane" profile in thin sections. The outer membrane also contains a third component, lipopolysaccharide (LPS).[1] LPS was shown to form mixed bilayers with phospholipids (Rothfield and Horne, 1967), and these results led to the hypothesis that the outer membrane is basically a mixed LPS–phospholipid bilayer with intercalated protein molecules (Schnaitman, 1971; Nikaido, 1973; Costerton et al., 1974).

Other results suggest, however, that the outer membrane is very different from phospholipid bilayer-type membranes. Specifically, both phospholipid bilayer model membranes (Mueller and Rudin, 1969; Bangham, 1972) and plasma membranes (Collander and Bärlund, 1933) allow rapid passive diffusion of hydrophobic substances, but the outer membranes of wild type *Salmonella* or *Escherichia coli* cells apparently act as penetration barriers against hydrophobic compounds (reviewed in Nikaido, 1973; see also Nikaido, 1976). Furthermore, this barrier property is influenced by the nature of LPS produced: outer membranes of "deep rough" mutants

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[1] Abbreviations used are: LPS, lipopolysaccharide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_i, inorganic phosphate.

TABLE I: Bacterial Strains Used.

Strain	Genotype	LPS Type Produced	References
LT2	*S. typhimurium* wild type	S	
HN202	LT2 *galE503*	Rc	Strain Ml of Fukasawa and Nikaido (1961)
TA2167	LT2 *galE506 hisC3076*	Rc	Ames et al. (1974)
SL1004	LT2 *rfaG571 rfb-430 metA22 trpB2 flaA66 str*	Rd_1	Wilkinson and Stocker (1968)
SL1181	LT2 *rfaF511 metA22 trpB2 flaA66 str*	Rd_2	Wilkinson et al. (1972)
HN504	LT2 *rfa-4311 galE506 hisC3076*	Rd_2	This paper
HN505	LT2 *rfa-4312 galE506 hisC3076*	Rd_2	This paper
TA2168	LT2 *rfa-1009 galE506 hisC3076*	Re	Ames et al. (1974)
HN501	LT2 *rfa-4308 galE506 hisC3076*	Re	This paper
HN502	LT2 *rfa-4309 galE506 hisC3076*	Re	Smit et al. (1975)
HN503	LT2 *rfa-4310 galE506 hisC3076*	Re	This paper
TA2169	LT2 *omp-1010 galE506 hisC3076*	Rc(Omp$^-$)	Ames et al. (1974)
TA2171	LT2 *omp-1011 galE506 hisC3076*	Rc(Omp$^-$)	Ames et al. (1974)

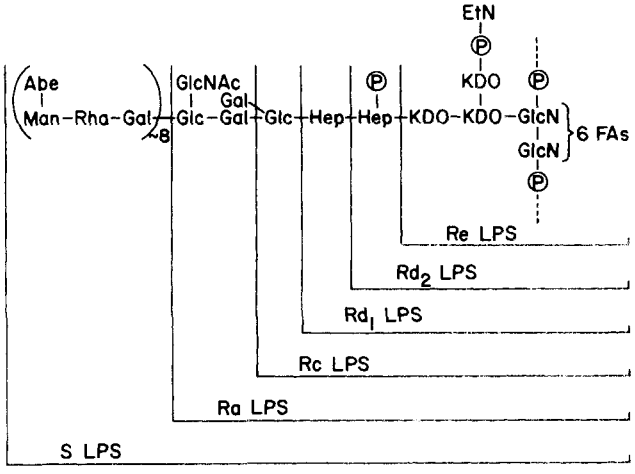

FIGURE 1: Structure of LPS produced by the *S. typhimurium* strains. The scheme shows a "monomer" unit; a molecule of LPS is thought to contain, on an average, three such units linked through pyrophosphate linkages (dotted line). S, Ra, Rc, etc. refers to the structural type of LPS produced. Other abbreviations: Abe, abequose; Rha, L-rhamnose; Hep, L-*glycero*-D-*mannno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic acid; EtN, ethanolamine; FA, fatty acid.

with very incomplete LPS (Rd or Re type, see Figure 1) are apparently much more permeable to hydrophobic antibiotics and dyes (Schlecht and Schmidt, 1969; Roantree et al., 1969; Gustafsson et al., 1973) and thus behave more like phospholipid-bilayer-type membranes. This difference was difficult to explain on the basis of the thickness of hydrophilic saccharide layer on cell surface, as S or wild type strains with LPS containing 40 or more monosaccharide units/chain and Rc mutants with LPS containing only 6 sugar residues/chain showed similar barrier properties against hydrophobic molecules, but Rd or Re mutants with 3-5 sugar residues/chain of LPS suddenly produced very permeable membranes. More recently, however, a drastic reduction in outer membrane protein has been discovered in Rd and Re, but not in Rc mutants (Ames et al., 1974); thus, the hydrophobic permeability of the outer membrane seems to be directly correlated with the protein content of the membrane.

These observations led us to the hypothesis that the outer membrane of S and Rc strains constitutes a hydrophobic penetration barrier either because its phospholipid bilayer regions are completely covered up by other membrane components, presumably proteins, or because it does not contain a phospholipid bilayer structure at all (Smit et al., 1975). In Rd and Re mutants, the amount of proteins is reduced, and there is a compensatory increase in the phospholipids in the outer membrane (Smit et al., 1975). These changes would either expose preexisting phospholipid bilayer regions or produce new phospholipid bilayer regions, with the resultant increase in hydrophobic permeability.

This work tests this hypothesis by looking for the presence of phospholipid head groups exposed on the surface of intact bacterial cells, i.e., on the outer surface of the outer membrane. Such head groups were indeed found on the surface of Rd_1, Rd_2, and Re mutants, but not on Rc mutants or the wild type (S) strain.

Materials and Methods

Bacterial Strains. Salmonella typhimurium LT2 and its derivatives were used. The properties of these strains are shown in Table I, and the structure of LPS produced by these strains in Figure 1. HN501 through 505 are spontaneous "deep rough" mutants isolated from TA2167 by selection with phage C21 (Ames et al., 1974). TA2169 and TA2167 are crystal violet sensitive, C21-resistant mutants that produce outer membranes with reduced protein content, yet with normal amounts of apparently normal Rc-type LPS (Ames et al., 1974); we propose to call them *omp* (for outer membrane protein) mutants.

Other Materials. Phospholipase C from *Bacillus cereus* was purchased from Calbiochem (lot 300485) and was partially purified by gel filtration through a 1.25 × 63 cm column of Sephadex G-75 in 50 mM Tris-HCl buffer (pH 7.6)–5 mM $CaCl_2$–50% glycerol (Zwaal et al., 1971). The specific activity of the purified enzyme was 250 μmol of P_i liberated mg^{-1} min^{-1} at 37 °C, using phosphatidylethanolamine from *S. typhimurium* as the substrate. The enzyme also hydrolyzed phosphatidylglycerol rapidly. In contrast to the crude enzyme, the purified enzyme never caused lysis of deep rough mutants when added to suspensions of whole cells.

Phospholipase C from *Clostridium perfringens* was obtained from Sigma (lot 32C-160). Alkaline phosphatase from *E. coli* (specific activity 12 μmol min^{-1} mg^{-1} of protein) was also a Sigma product.

CNBr-activated dextran was prepared as follows (Kågedal and Åkerström, 1971). Dextran T-10 (9740 daltons) from Pharmacia (0.2 g) was dissolved in 10 ml of water, and to this, 10 ml of aqueous solution of CNBr (11 mg/ml) was added under stirring, in two portions at an interval of 1 min. The mixture was stirred vigorously in the hood, and the pH was maintained at 10.7 by the addition of 4 N and then 1 N NaOH.

After 30 min, the solution was adjusted to pH 8.5 with 1 N HCl and was used immediately for coupling experiments.

^{14}C phospholipids for activated dextran coupling experiments were prepared from LT2 cells grown in L broth (Bertani, 1951) containing [G-^{14}C]glucose, by extraction with chloroform–methanol (Folch et al., 1957). The organic phase was washed twice with the "theoretical upper phase" of Folch et al. (1957), and was stored at −70 °C. ^{3}H phospholipids were prepared similarly from cells grown in the presence of [2-^{3}H]glycerol (see below).

$H_3{}^{32}PO_4$, [2-^{3}H]glycerol, and [1-^{14}C]oleic acid were purchased from New England Nuclear Corp., and D-[G-^{14}C]-glucose from Schwarz/Mann.

Cultivation of Bacteria. Cells were grown in L broth (Bertani, 1951) (glucose omitted) at 37 °C with aeration by shaking, and were always harvested during the mid-exponential phase of growth. ^{32}P-Labeled cells were prepared by diluting 0.1 ml of an overnight culture into 5 ml of fresh broth, and adding 0.5 mCi of carrier-free [^{32}P]phosphoric acid after 30 min. The cells were harvested when the density reached 0.2 mg (dry weight)/ml and washed twice with ice-cold 10 mM Hepes buffer, pH 7.4. Some batches of cells were specifically labeled in glycerophospholipids as follows. A portion (0.5 ml) of an overnight culture was diluted into 5 ml of prewarmed fresh broth, and 200–300 μCi of [2-^{3}H]glycerol (30–45 nmol) was added after 30 min. After 2 h of growth, the cells were centrifuged at room temperature and washed twice with fresh L broth. The cells were then resuspended in 10 ml of prewarmed L broth containing 0.05% glycerol, and the suspension was shaken for 1 h at 37 °C. Finally, the cells were harvested by centrifugation, and were washed twice with ice-cold 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 8.5) before use.

Isolation of Outer and Inner (Cytoplasmic) Membranes. This was carried out essentially as described by Koplow and Goldfine (1974), except that the cells were centrifuged rapidly (5 min), washed rapidly with ice-cold 10 mM Hepes buffer, pH 7.4, and disrupted at 0 °C in a French pressure cell in this buffer. Thus, the material was never exposed to Tris buffer or EDTA during the preparation. The degree of contamination of the outer membrane by the inner membrane was less than 5%, and that of the inner membrane by the outer membrane was 10–30% (Smit et al., 1975).

Identification of Hydrophilic Degradation Products after Phospholipase C Digestion. Cells were grown overnight in a synthetic medium "63" (Cohen and Rickenberg, 1956) containing 50 μg/ml of L-histidine and 0.1% glucose. One milliliter of this culture was diluted into 20 ml of the fresh medium, which had the identical composition except that it contained 2 μCi/ml of [G-^{14}C]glucose. The cells were harvested when the density of the culture reached 0.25 mg dry weight/ml, and were washed three times with cold 10 mM Hepes buffer, pH 7.4. Cells (1.5 mg dry weight) were treated with 100 μg of *B. cereus* phospholipase C in 0.2 ml of 10 mM Hepes buffer, pH 7.4, for 15 min at 37 °C. Portions (0.15 ml) of the incubation mixtures were applied to paper strips, and were separated by descending paper chromatography with 1-butanol–acetic acid–water (62:15:25, v/v) as solvent. After 48 h at room temperature, the paper strips were dried, and the portions corresponding to glycerol phosphate (and ethanolamine phosphate, see Results) were cut out and the labeled substances were eluted with water. The eluates (1 ml) were made 10 mM in Tris-HCl buffer, pH 8.5, and were treated with 120 μg of *E. coli* alkaline phosphatase at 37 °C for 1 h. These preparations were again separated by paper chromatography (15 h) in the solvent mentioned above. Paper strips were then cut into 2.5-cm-wide segments and the radioactivity was determined by liquid scintillation counting.

As controls, phospholipids were extracted from the same amounts (1.5 mg) of cells with chloroform–methanol (2:1) (Folch et al., 1957), dried under a stream of N_2, and resuspended in 0.2 ml of 10 mM Hepes buffer, pH 7.4. This suspension was treated with phospholipase C, and the hydrophilic reaction products were separated exactly as described above.

Coupling of Phosphatidylethanolamine with CNBr-Activated Dextran. Labeled cells (1–2 mg dry weight), membrane vesicles (containing 1 mg of protein), or phospholipids (0.2–0.5 mg) were suspended in 1 ml of 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 8.5. To this, 5 ml of CNBr-activated dextran, prepared as described above, was added with stirring. If we assume that all of CNBr added had reacted with dextran, the number of imido ester groups present was at least a thousandfold excess over the total number of phosphatidylethanolamine molecules in the reaction mixture. The reaction mixture was kept at 20 °C, and the reaction was stopped usually after 1 h by the addition of 0.1 volume of 1 M ethanolamine (pH 7.4). Samples were often frozen at this stage and kept at −70 °C until analysis.

Unreacted phospholipids were separated from the covalently linked dextran–phosphatidylethanolamine complex as follows. In method A, the samples were applied to paper strips, which were irrigated with 1-butanol–pyridine–water (6:4:3, v/v) as solvent. The complex remained at the origin, whereas free phospholipids migrated close to the solvent front. In method B, we first solubilized membrane components by adding sodium dodecyl sulfate to the reaction mixture to a final concentration of 2% and heating the mixture at 100 °C for 2 min. The solution was then applied to a 1.27 × 53 cm column of Bio-Gel P-100 (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.), and the column was eluted with 2% sodium dodecyl sulfate. Fractions (1 ml) were collected and assayed for radioactivity.

Other Methods. Radioactivity was determined with an Amersham-Searle Isocap-300 liquid scintillation spectrometer, by using a scintillator fluid described by Bray (1960).

Results

Treatment of Intact Cells with Phospholipase C. Outer membrane constitutes the outermost layer in the cell envelope of *Salmonella,* which usually does not produce capsular material. The outer membrane contains phospholipids (mainly phosphatidylethanolamine and phosphatidylglycerol) (Osborn et al., 1972). If many of the phospholipid head groups are exposed on the outer surface of the membrane, it may be possible to degrade these groups by treating intact cells with a phospholipase.

Among phospholipases, phospholipase C is preferable to phospholipase A because of the following reasons. (a) Phospholipase A produces lysophosphatides which act as detergents and disaggregate the membrane structure, whereas phospholipase C produces hydrophilic phosphorylated compounds that diffuse out into the medium and diglycerides that stay in the membrane but have no detergent activity (Glaser et al., 1970). (b) In the phospholipid molecules in the membrane, the hydrophilic head group which is the substrate for phospholipase C would be more exposed to the outside aqueous medium than the fatty acid ester linkages that are hydrolyzed by phospholipase A. Consequently, phospholipids in membranes appear to be more vulnerable to the attack by phospholipase C (Woodward and Zwaal, 1972).

When phospholipase C from *Clostridium perfringens* was

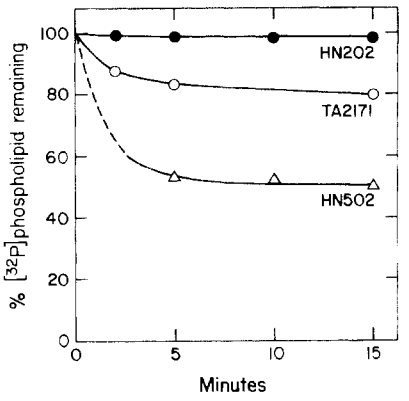

FIGURE 2: Digestion of phospholipids by treatment of intact cells with phospholipase C. ^{32}P-labeled cells (about 2 mg dry weight), prepared as described in Methods, were treated with 12 μg of the partially purified phospholipase C (Methods) in 1.0 ml of 10 mM Hepes buffer, pH 7.4, at 28 °C. At times indicated, 0.20 ml of the reaction mixture was added to 0.2 ml of cold 10% trichloroacetic acid. After centrifugation, the pellet was extracted twice with 1.5 ml of ethanol–ether (3:1), and the radioactivity in the extract, corresponding to the amount of [^{32}P]phospholipids remaining at each time, was determined.

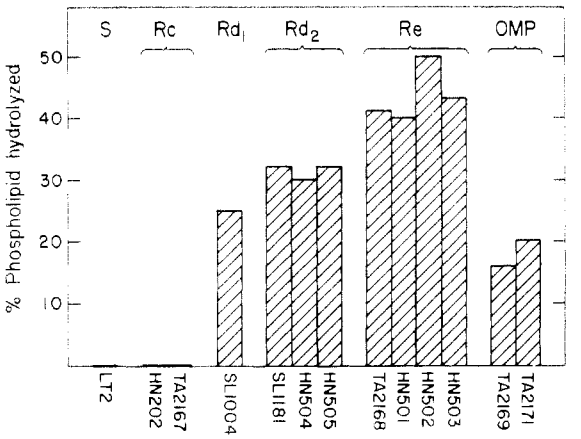

FIGURE 3: Phospholipase C digestion of phospholipids in various strains of *S. typhimurium*. The bars indicate the extents of digestion of [^{32}P]-phospholipids when intact cells of various strains were treated with phospholipase C for 15 min under the conditions described in the legend to Figure 2. TA2169 and TA2171 are *omp* mutants that incorporate less than normal amounts of proteins into the outer membrane; their LPS is of Rc type (see Methods).

incubated in the presence of 2 mM $CaCl_2$ with the crude phospholipids of *Salmonella*, which mainly contained phosphatidylethanolamine and phosphatidylglycerol, we found that its activity toward these lipids was less than 0.1% of that measured by the use of phosphatidylcholine. In contrast, phospholipase C from *Bacillus cereus* rapidly hydrolyzed both phosphatidylethanolamine and phosphatidylglycerol, and we used a partially purified preparation of this enzyme (see Methods) for digestion of phospholipids on the surface of intact cells.

The results of such an experiment are shown in Figure 2. It is seen that phospholipids in an Rc mutant are not degraded. Since the "polysaccharide" side chain in Rc LPS contains only six monosaccharide residues (Figure 1), it is difficult to ascribe this resistance to the steric hindrance by the saccharide chains of LPS. It should also be noted that the activity of phospholipase C in the incubation mixture was in great excess, at least one hundred times more than necessary for the complete digestion of all phospholipids present during the incubation period.

It is known that phospholipids of intact erythrocytes are not digested by phospholipases unless the membrane is stretched by suspending the cells in slightly hypotonic media (Woodward and Zwaal, 1972). The outer membrane of our cells, however, must be in a significantly stretched state, as our cells were grown in L broth (osmolarity higher than 0.1 M) and were resuspended in 0.01 M Hepes buffer; we have seen that the cell volume significantly increases under these conditions (G. Decad and H. Nikaido, unpublished observation).

In contrast to the results with the Rc strain, phospholipids in some other mutants were rapidly digested by phospholipase C (Figure 2). Since the digestion appeared to reach a plateau by 15 min, the extent of digestion after 15 min treatment was measured with various strains (Figure 3). Clearly, S and Rc strains were completely resistant to phospholipase C, whereas significant hydrolysis was observed in all "deep rough" (Rd and Re) mutants as well as in *omp* mutants. These results suggest that phospholipid molecules are present in the outer half of the outer membrane in "deep rough" and *omp* mutants, but are either absent in the outer half or present but completely hidden underneath the proteins in S and Rc strains. An alternative interpretation is that the difference is due to different degrees of steric hindrance by the saccharide chains of LPS, but this is unlikely in view of the sudden change in phospholipase sensitivity accompanying the loss of a single monosaccharide residue, from the complete resistance of Rc strains to the marked sensitivity of Rd_1 strain (Figure 3). Furthermore, the sensitivity of *omp* mutants in spite of the presence of the normal amounts of Rc-type LPS is in direct contradiction to the steric hindrance hypothesis.

We then examined the nature of phospholipids that are exposed on the surface of Re cells. This was done by treating ^{14}C-labeled cells with phospholipase C and separating the hydrophilic degradation products by paper chromatography. Difficulties were encountered, however, because large amounts of labeled, low-molecular-weight compounds were excreted by the cells during incubation, and these compounds produced a broad, background smear on paper chromatography. We therefore adopted the following two-step procedure. First we chromatographed the supernatant from incubation mixture in 1-butanol–acetic acid–water (62:15:25, v/v), in which ethanolamine phosphate (the degradation product from phosphatidylethanolamine) and glycerol phosphate (the degradation product from phosphatidylglycerol) have almost identical R_f values. The band containing these phosphorylated compounds, as well as other ^{14}C-labeled excreted compounds, was eluted, and the eluate was chromatographed in the same solvent after treatment with alkaline phosphatase. Most of the contaminating compounds apparently were not phosphorylated and migrated with the same rate, but glycerol and ethanolamine now moved much faster and were separated from contaminants. As shown in Figure 4, when ^{14}C-labeled phospholipid liposomes were digested, both [^{14}C]ethanolamine and [^{14}C]glycerol were produced after digestion with phospholipase C and phosphatase. In contrast, digestion of intact cells of an Re mutant produced only [^{14}C]ethanolamine, a result suggesting the presence of phosphatidylethanolamine, but not phosphatidylglycerol, molecules on the outer surface of the outer membrane of this strain. This result is also consistent with the finding of Osborn et al. (1972) that the outer membrane contains less phosphatidylglycerol than the inner, cytoplasmic membrane.

Coupling of CNBr-Activated Dextran with Phosphatidylethanolamine. The outer membrane of *S. typhimurium*

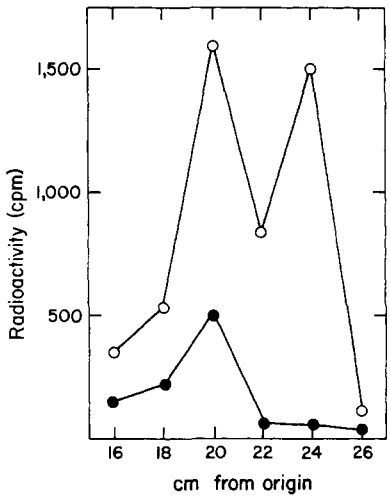

FIGURE 4: Paper chromatographic separation of hydrophilic degradation products after phospholipase C digestion. ^{14}C-Labeled cells of TA2168, an Re mutant, and phospholipids extracted from these cells were treated with *B. cereus* phospholipase C, and the hydrophilic degradation products were separated by paper chromatography. The band containing both ethanolamine phosphate and glycerophosphate was eluted, and the eluate was rechromatographed in the same solvent after treatment with alkaline phosphatase. Ethanolamine and glycerol standards are located at 20 and 24 cm from origin, respectively. O—O, Products from phospholipid liposomes; ●—●, products from intact cells. For details see text.

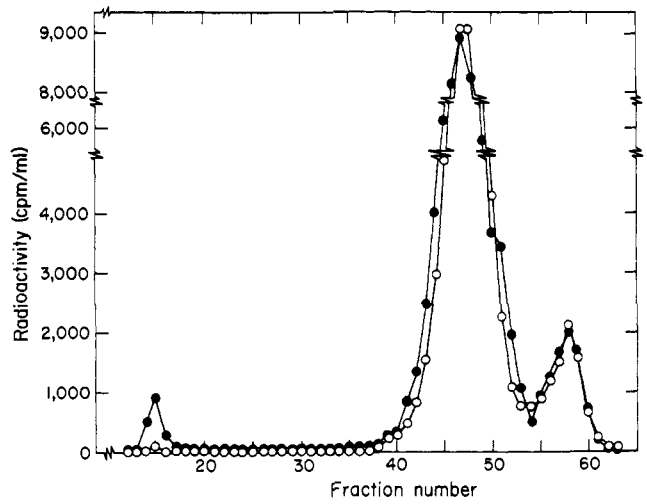

FIGURE 5: Coupling of CNBr-activated dextran to [^3H]phospholipids in intact cells of SL1004. Phospholipids were specifically labeled by growing cells in the presence of [2-^3H]glycerol (Methods). The cells were treated with activated dextran for 1 h, and the remaining activated dextran was inactivated by adding excess ethanolamine. The cells were then solubilized in 2% sodium dodecyl sulfate, and ^3H phospholipids (fractions 39 through 60) were separated from ^3H-phospholipid–dextran complex (eluted before fraction 35, mostly in fractions 13 through 17) by gel filtration in 2% sodium dodecyl sulfate (see Methods). ●—●, Material from cells treated with activated dextran; O—O, control in which the dextran was inactivated by 0.1 M ethanolamine (pH 8.5) prior to the addition of ^3H-labeled cells.

does not allow the penetration of dextrans (Nakae and Nikaido, 1975). Thus, the CNBr-activated dextran can be used as the nonpenetrating coupling reagent for phosphatidylethanolamine head groups (Axén et al., 1967).

In a preliminary experiment, liposomes made from ^{14}C phospholipids of *S. typhimurium* were incubated with the activated dextran in 0.1 M $NaHCO_3$–Na_2CO_3 buffer at pH 8.5, and portions of the reaction mixture were fractionated by paper chromatography (Methods). When the reaction was performed at 0 °C there was a slow and steady increase in the amount of ^{14}C-labeled substance at the origin, and after 7 h 1.7% of the total [^{14}C]phospholipids was coupled to the activated dextran. At 20 °C, the coupling reaction proceeded more rapidly, and 5.2% of the total ^{14}C phospholipids was converted to the dextran complex already after 1 h, with a much slower increase afterwards, approximately at the rate of 1% of the remaining phospholipids becoming coupled with each additional hour of incubation.

Since the paper chromatography method gave too high blank values with intact cells, we tested another method for the detection of the coupling product. The reaction mixture was solubilized in 2% sodium dodecyl sulfate, and the solution was fractionated by gel filtration in 2% sodium dodecyl sulfate (see Methods). When ^3H phospholipid liposomes, treated with ethanolamine-inactivated CNBr-dextran, were fractionated on this column, all radioactivity was eluted just before the complete inclusion volume. In contrast, with liposomes treated with activated dextran, 3–10% of the radioactivity was eluted at the exclusion and partial inclusion volumes. These results establish clearly that a covalently linked dextran–phospholipid complex was produced with the use of activated dextran. However, they were somewhat puzzling on first sight, because the dextran–phospholipid complex containing 10 000-dalton dextran was expected to be eluted rather close to the inclusion volume, from the known molecular sieving properties of Bio-Gel P-100. Apparently this unexpectedly large size of the complex is mainly due to the fact that dextran molecules become polymerized during "activation", as already noted by Kågedal and Åkerström (1971); thus, with one batch of our activated dextran, about 40% of the material was eluted with the K_{av} values of 0–0.5 on the same Bio-Gel P-100 column. If each sodium dodecyl sulfate micelle contains more than one molecule of the phospholipid coupled to such a polymerized dextran, most of the micelles will be eluted before the elution of the micelles containing free phospholipids.

Reaction of Phospholipids Exposed on the Cell Surface. A Rd_1 mutant, SL1004, was specifically labeled in glycerophospholipids by growing it in the presence of [2-^3H]glycerol (Methods). These cells were reacted with CNBr-activated dextran at 20 °C under standard conditions (Methods). After 1 h, the reaction was stopped by adding 0.1 ml of M ethanolamine. The samples were then dissociated with sodium dodecyl sulfate and were fractionated by gel filtration (Methods) (Figure 5). In the control tube the cells were incubated with the activated dextran which was pretreated with 0.1 M ethanolamine; in this case most of the radioactivity was eluted just before the inclusion volume, i.e., in fractions 40 through 52 (Figure 5). However, 0.9% of the total radioactivity was found in fractions 13 through 35. We do not know the identity of labeled compounds eluted in these fractions.

In cells that had been reacted with activated dextran, a larger fraction of the total radioactivity was eluted at exclusion volume and at partially excluded volumes (fractions 13 through 35). The fraction of radioactivity appearing in these excluded fractions, when corrected for the fraction of radioactivity found in the control, corresponds to the fraction of ^3H in the total cellular phospholipids that was covalently coupled to the activated dextran.

That the ^3H-labeled compound in this fraction corresponds to phosphatidylethanolamine covalently linked to dextran was suggested by the following observations. (a) Since the cells were grown in the presence of [2-^3H]glycerol, the only cellular component radioactively labeled to a significant extent was glycerophospholipids, which contained, under our conditions of experiment, at least 98% of the radioactivity incorporated.

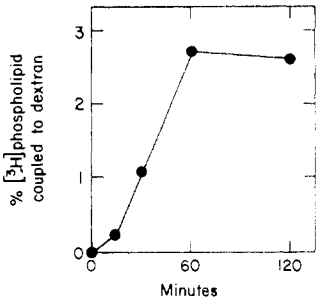

FIGURE 6: Time course of coupling with CNBr-activated dextran. TA2168 cells (about 2 mg dry weight) specifically labeled in glycerophospholipids with ^3H (see Methods) were treated with CNBr-activated dextran at 20 °C under standard conditions (Methods). At times indicated, portions of the reaction mixture were taken out, and the extent of phospholipid-dextran coupling was determined by gel filtration in sodium dodecyl sulfate as in Figure 5.

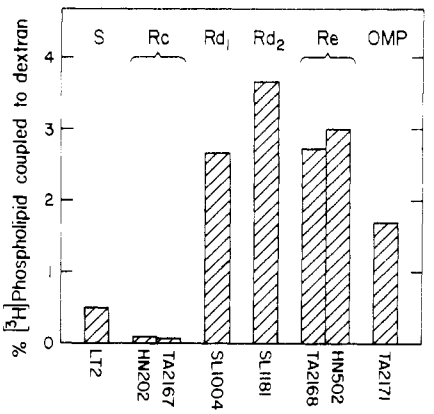

FIGURE 7: Coupling of activated dextran to ^3H phospholipids on the surface of cells of various strains. Experiments were performed as described in the legend to Figure 5 (also see Methods), and the fraction of radioactivity eluted before fraction 35 was calculated after correction for the radioactive material eluted in this region in the control.

(b) When this excluded fraction was dialyzed against water for 4 days, and then was hydrolyzed in 4 N HCl for 4 h at 100 °C, the only radioactive compound recovered had the same paper chromatographic mobility as glycerol in three solvent systems. (c) When the dialyzed preparation of this fraction was shaken at 37 °C for 10 h with an equal volume of diethyl ether in the presence of 180 μg of partially purified phospholipase C from *Bacillus cereus* (Methods), 91% of the radioactivity became partitioned into the ether phase. This material had identical mobility with diglyceride on silica gel thin-layer chromatography in three solvent systems. (d) When the isolated outer membrane was incubated with the activated dextran, only phosphatidylethanolamine, but not phosphatidylglycerol or cardiolipin, formed a complex with the dextran (see below).

The time course of coupling (Figure 6) indicates that the reaction is complete within 1 h; in all other experiments we therefore reacted cells and membranes with activated dextran for 1 h at 20 °C.

When various strains were examined by treating intact cells with CNBr-activated dextran, the results shown in Figure 7 were obtained. Clearly, very little, if any, phospholipids react with the nonpenetrable reagent in strains producing S and Rc type LPS, but a significant fraction gets coupled in strains producing Rd_1, Rd_2, and Re LPS or in *omp* strains.

Significant amounts of ^3H-labeled material were eluted, in the experiment of Figure 5, at the complete inclusion volume

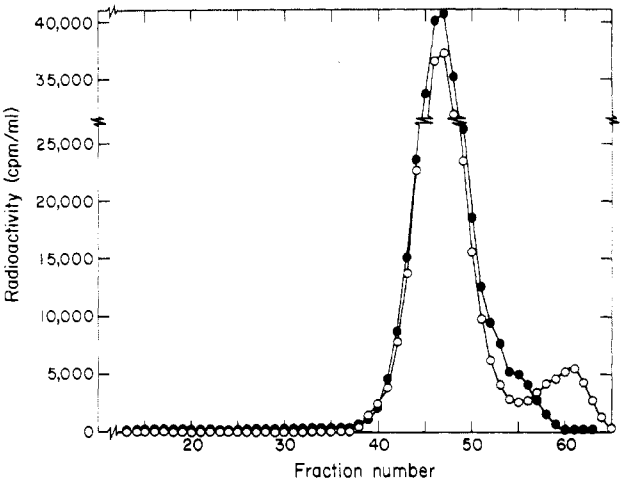

FIGURE 8: Effect of incubation at pH 8.5 on ^3H phospholipids in *S. typhimurium* cells. HN202 cells were grown in the presence of [2-^3H]-glycerol as described in Methods. One portion of the cell suspension was incubated at 20 °C for 1 h in 0.1 M Tris-HCl buffer, pH 7.0; the other in 0.1 M Tris-HCl buffer, pH 8.5. The pH of both samples was then adjusted to 7.0, the cells were solubilized in 2% sodium dodecyl sulfate, and ^3H-labeled compounds were separated by gel filtration on a column of Bio-Gel P-100, as in Figure 5. ●—●, cells incubated at pH 7.0; ○—○ cells incubated at pH 8.5. Cells before incubation give a pattern similar to cells incubated at pH 7.0 (not shown).

(fractions 54–63). This material presumably corresponds to low-molecular-weight, hydrophilic compounds that do not form mixed micelles with sodium dodecyl sulfate, as control experiments showed that all ^3H phospholipids were eluted in fractions 40 through 52. Since the final stage of cell culture was done in the presence of unlabeled glycerol (see Methods), specifically to chase radioactivity in the glycerol phosphate pool into phospholipids, the existence of this labeled material at the inclusion peak was rather puzzling. The experiment shown in Figure 8 shows that the "chase" was indeed successful and the cells initially contained all their ^3H in phospholipids, but incubation of the cells at pH 8.5 produced, from some of the ^3H phospholipids, low-molecular-weight, hydrophilic compounds labeled with ^3H. This observation makes one suspect that the CNBr-activated dextran might be reacting with such low-molecular weight compounds rather than with phosphatidylethanolamine. However, an Rc mutant that produces large amounts of the low-molecular-weight compounds at pH 8.5 (Figure 6) did not produce significant amounts of ^3H-labeled, exclusion volume material upon incubation with CNBr-dextran at pH 8.5 (Figure 7). Thus the low-molecular-weight products apparently cannot react with extracellular, activated dextran because of either their location or their chemical structure.

Activated Dextran Reacts only with Outer Membrane Components. The CNBr-activated dextran was assumed to be impermeable through the outer membrane. However, we could not rigorously rule out the possibility that the coupling of dextran to outer membrane components would destabilize the membrane structure so as to allow, eventually, the penetration of the reagent. Furthermore, since the outer membrane of Re mutants is known to allow, at least occasionally, the penetration of the proteins such as lysozyme (Tamaki and Matsuhashi, 1973) and periplasmic proteins (Lindsay et al., 1973), the possibility remained that the phospholipid coupling seen in deep rough strains was a result of penetration of the dextran reagent through the outer membrane, followed by its reaction with phospholipids in the cytoplasmic membrane.

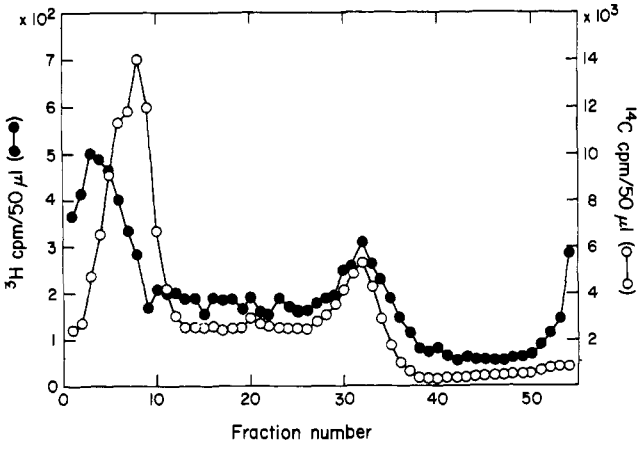

FIGURE 9: Sucrose density gradient centrifugation of membranes from cells treated with activated dextran. Strain TA2168 was grown in the presence of [2-^{3}H]glycerol as described in Methods. Another batch of the cells was grown by diluting 0.1 ml of an overnight culture into 5 ml of L broth containing 20 μCi of [1-^{14}C]oleate (372 nmol), followed by shaking for 4 h at 37 °C. The ^{3}H-labeled cells were treated with CNBr-activated dextran (see Methods) for 1 h at 20 °C, and then the dextran was inactivated by the addition of 0.1 M (final concentration) ethanolamine. The cells were then washed twice with 0.1 M ethanolamine–0.01 M Hepes, pH 7.4, and then were mixed with the ^{14}C-labeled cells. The cells were than disrupted and membranes were fractionated as described in Methods.

In order to rule out these uncertainties, we prepared membranes from activated-dextran-treated Re cells and fractionated the membranes on a sucrose-density gradient. As seen in Figure 9, the density of the outer membranes has become much higher as a result of the attachment of dextran, but the density of the inner, cytoplasmic, membrane fraction was unaltered. The results show that the reagent attached itself only to the components of the outer membrane under our conditions of experiment.

Although arguments can still be made that the results of Figure 9 may be obtained even if the activated dextran reacted mainly with inner membrane phospholipids, we do not believe that such interpretations are correct. (a) One can argue that the bacterial population was heterogeneous, and massive penetration of activated dextran through outer membrane occurred only in a small fraction of cells. In these cells, then, the cytoplasmic membrane lipids were labeled heavily with dextran, and the labeled membrane became so dense that it sedimented with the labeled outer membrane. This hypothesis, however, can be excluded because we know that the isolated cytoplasmic membrane, when coupled maximally with dextran, still has a density low enough so that it never enters the 2.02 M sucrose layer (not shown). (b) One can also argue that only small numbers of activated dextran penetrate through the outer membrane of each cell and become coupled to the cytoplasmic membrane phospholipids. The labeled cytoplasmic membrane would still have a practically unaltered buoyant density, because the amount of dextran coupled was so small. We can, however, exclude this hypothesis, because when "the cytoplasmic membrane band" and "outer membrane band" from the experiment of Figure 9 were dissociated by sodium dodecyl sulfate and fractionated by gel filtration, the dextran–phospholipid complex was found only in the material from the "outer membrane band" (not shown).

Coupling with Isolated Outer and Inner Membranes. In order to make sure that the activated dextran can become coupled to phosphatidylethanolamine molecules in the outer membrane if their head groups are made accessible to the reagent, we isolated outer membrane from [2-^{3}H]glycerol-labeled cells of HN202 as described in Methods. The preparation containing about 1 mg of protein was suspended in 3 ml of 5 mM Tris-HCl buffer, pH 8.0, and was treated with egg white lysozyme (150 μg) for 4 h at 37 °C. In the course of incubation, the preparation was sonicated once for 90 s with a Biosonik IV sonic oscillator. The suspension was then centrifuged at 100 000g for 30 min, and the pellet washed twice and finally resuspended in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 8.5. The suspension was divided in two equal parts, which were treated with activated dextran, and with activated dextran pretreated with 0.1 M ethanolamine, respectively, for 1 h at 20 °C. The reaction was stopped by adding one-tenth volume of 1 M ethanolamine, then each reaction mixture was divided again into two equal parts, which were used for the quantitation of dextran–phospholipid complex (by gel filtration on Bio-Gel P-100, see Methods), and for the extraction and quantitation of unreacted phospholipids (by thin-layer chromatography), respectively. Table II shows that as much as 26% of total phospholipids became coupled to dextran, thus proving that phospholipids in the outer membrane, if exposed, can react with the activated dextran. Possibly many of the outer membrane vesicles were leaky (Nakae and Nikaido, 1975) or inverted,[2] or extensive scrambling of the membrane components took place during the membrane preparation. Table II furthermore indicates that only phosphatidylethanolamine decreased significantly (by 36%) after the treatment with activated dextran, and that the amount lost from this fraction (11 300 − 7200 = 4080 cpm) was entirely recovered as the excluded volume fraction from the Bio-Gel P-100 gel filtration (4350 cpm). This result establishes that the ^{3}H-labeled substance in the latter fraction corresponds to phosphatidylethanolamine coupled to dextran.

TABLE II: Fractionation of [^{3}H]Phospholipids from Outer Membrane Vesicles That Had Been Treated with Activated Dextran.[a]

Fraction	Radioactivity Recovered in Each Fraction (cpm)	
	Control	Membrane Treated with Activated Dextran
Cardiolipin	587	514
Phosphatidylglycerol	2 400	2 140
Phosphatidylethanolamine (A)	11 300	7 220
Phospholipid–dextran complex (B)	130	4 350
A + B	11 430	11 570

[a] Suspensions of HN202 outer membrane, specifically labeled in phospholipids by growth in the presence of [2-^{3}H]glycerol, were incubated with CNBr-activated dextran and with the activated dextran pretreated with 0.1 M ethanolamine ("control"), respectively. Each suspension was then divided into two equal parts, each containing 16 400 cpm. Phospholipids were extracted from one part by the method of Bligh and Dyer (1959), and were separated by chromatography on Merck silica gel H plates, with chloroform–methanol–acetic acid (65:25:10) as the solvent. The other part was solubilized with sodium dodecyl sulfate, and the "phospholipid–dextran complex" was isolated by gel filtration as shown in Figure 5.

[2] French pressure cell treatment, which was used for the isolation of outer membrane, is known to produce inside-out vesicles of the inner membrane (Altendorf and Staehelin, 1974).

Isolated cytoplasmic (inner) membranes from LT2, similarly labeled in phospholipids by growth in the presence of [2-^{3}H]glycerol, were also treated with activated dextran under the standard conditions. The excess activated dextran was inactivated with ethanolamine, and membrane components were separated by gel filtration in sodium dodecyl sulfate. In a typical experiment, the dextran–phosphatidylethanolamine complex contained 3956 cpm out of the total of 23 185 cpm applied to the column. Osborn and co-workers (1972) showed that 60% of the [2-^{3}H]glycerol label incorporated into the cytoplasmic membrane is located in phosphatidylethanolamine, and we have also obtained a very similar figure (unpublished). If we assume that most of the cytoplasmic membrane fragments exist as closed vesicles (see Osborn et al., 1972) and that phosphatidylethanolamine molecules are distributed more or less symmetrically, then 23 185 cpm × (60/100) × (½) = 6956 cpm of [^{3}H]phosphatidylethanolamine should be available for coupling with the activated dextran. The amount of the dextran–[^{3}H]phosphatidylethanolamine complex actually obtained (3956 cpm) therefore corresponds to 57% of the theoretical value. Of course, the efficiency could be as low as 24% if the distribution of phosphatidylethanolamine is strongly asymmetrical or most of the vesicles are open. In any case, there is no doubt that the efficiency of coupling is extremely high.

Discussion

The outer membrane of *Salmonella typhimurium* contains three components, i.e. LPS, phospholipids, and proteins. Since series of mutants producing defective LPS are available (for review, see Nikaido, 1973), one can examine the effect of alteration of LPS structure on the supramolecular organization and functions of the outer membrane. Available evidence suggests that no radical changes occur in the organization of membrane when the length of the polysaccharide chain of LPS becomes reduced from more than 40 monosaccharide units (in S-form or wild type) down to 6 monosaccharide residues (in Rc mutants). However, further reduction in the length of "polysaccharide" chain produces "deep rough" mutants (Rd or Re mutants) that are very different from S or Rc strains. The outer membrane of deep rough mutants, in comparison with that of S or Rc strain, (a) contains reduced amounts of proteins (Ames et al., 1974; Koplow and Goldfine, 1974; Smit et al., 1975) and (b) allows a much more rapid diffusion of hydrophobic compounds (Roantree et al., 1969; Schlecht and Schmidt, 1969; Nikaido, 1976). The organization of the outer membrane of deep rough mutants thus seems to be very different from that of S or Rc strains.

We have shown in this paper, by treating intact cells of various strains with phospholipase C, that phospholipid head groups are accessible for enzymatic attack on the outer surface of the outer membrane in Rd_1, Rd_2, and Re, but not in Rc or S strain (Figure 3). These experiments, however, suffered from two drawbacks. First, we cannot completely exclude the possibility that steric hindrance by the "polysaccharide" portion of LPS is responsible for the resistance of phospholipids of Rc and S strains to digestion, although this seems unlikely in view of the arguments presented in the Results. The second, more serious objection is related to the observation that the outer membrane of "deep rough" mutants allows some penetration of enzymes, both the inward penetration by lysozyme added to the medium (Tamaki and Matsuhashi, 1973) and the outward leakage of periplasmic enzymes (Lindsay et al., 1973). Even a small number of molecules of phospholipase C, if they get into the periplasmic space of Rd or Re cells, should be able to produce extensive degradation of phospholipids by the catalytic nature of their action. Thus, the sensitivity of Rd and Re mutants to phospholipase C digestion might reflect the permeability of outer membrane, rather than the location or accessibility of phospholipid head groups.

The covalent labeling experiments with CNBr-activated dextran were designed to circumvent these difficulties. First, the dextran with flexible polysaccharide chains would be much less susceptible to steric hindrance than enzyme proteins with their rigid secondary and tertiary structure. Second, it would be very difficult for such a large, extremely hydrophilic molecule to diffuse through the outer membrane. Even if a few molecules did, they would not affect the results significantly because their action is stoichiometric rather than catalytic. Furthermore, we have demonstrated that CNBr-activated dextran did not become attached to the inner membrane of Re mutants, if the coupling is done with intact cells (Figure 9).

The treatment of intact cells with CNBr-activated dextran thus showed conclusively that very few, if any, phosphatidylethanolamine head groups are exposed on the outer surface of the outer membrane in S or Rc strains. The coupling technique cannot detect the phosphatidylglycerol head groups; however, it is very unlikely that such groups were present, since they should have been digested with phospholipase C in the experiment of Figure 4. In contrast, significant amounts of phosphatidylethanolamine in the intact Re cells reacted with the nonpenetrating coupling reagent, as predicted by our hypothesis. From the data presented by Osborn et al. (1972), we can assume that about 40% of the total cellular phospholipids are located in the outer membrane. Our quantitative assay showed that the amounts of phospholipid in the outer membrane of deep rough mutants are enough to cover the entire inner surface and about 33% of the outer surface of the outer membrane (Smit *et al.*, 1975). From these results one can expect roughly (0.33/1.33) × 40 = 10% of the total cellular phospholipids to be exposed on the cell surface or the outer surface of the outer membrane. The coupling experiment with inner surface and about 33% of the outer surface of the outer membrane (Smit et al., 1975). From these results one can expect roughly (0.33/1.33) × 40 = 10% of the total cellular can therefore expect 2.4–5.7% of the total cellular phospholipids getting coupled to the dextran reagent in deep rough mutants. The actual values obtained (2.7–3.7%) were very close to the expected value.

In deep rough mutants, phospholipase C hydrolyzed much larger fractions (20–50%) of cellular phospholipids (Figure 3) than those that became coupled to dextran. It seems likely that, once the exposed phospholipids are hydrolyzed, the architecture of the membrane becomes so destabilized that eventually all of the outer membrane phospholipids get degraded by the enzyme.

Phospholipase C and activated dextran thus react with a substantial fraction of phospholipid head groups in the outer membrane of deep rough mutants, but *not* of S or Rc strains. The presence of reactivity obviously suggests the exposed location of the head groups, but its absence is more difficult to interpret. For example, the lack of reactivity to activated dextran might be a result of a specific, local interference by neighboring groups, e.g., amino groups of proteins. Although this possibility cannot be excluded completely, we believe it is rather unlikely in view of the high reactivity of phosphatidylethanolamine head groups in the isolated outer and inner membranes. Thus, the difference in the reactivity of head groups can be explained most simply, we believe, by either of the following models. In the first model, the outer membranes

of all strains contain phospholipid bilayer regions. However, in S or Rc strains, the head groups of phospholipids are covered by proteins at the outside surface of the membrane. The reduction in protein content, seen in deep rough and *omp* mutants, simply exposes the previously hidden head groups. This model can explain the low permeability of S or Rc outer membrane toward hydrophobic molecules, which may encounter difficulty in diffusing through the protein layer. In the second model, the outer membrane normally (i.e., in S and Rc strains) has an extremely asymmetric structure, in which practically all phospholipid molecules are located in the inner half of the membrane, and its outer half is mainly composed of proteins and LPS. The absence of phospholipid bilayer regions in this membrane would be consistent with the lack of permeability toward hydrophobic substances. In the deep rough and *omp* mutants, the outer membrane contains less proteins and more phospholipids (Smit et al., 1975). This alteration in composition produces outer membrane with phospholipid bilayer regions, which would allow a rapid penetration of hydrophobic molecules.

Although asymmetric distribution of membrane components has been described (Bretscher, 1972; Marchesi et al., 1972), the asymmetry postulated in our second model is extreme, and it is not known whether such an arrangement of component molecules produces a stable membrane structure. Nevertheless, we tend to favor the second model slightly, because it is consistent with some other observations of ours (Smit et al., 1975). First, the phospholipid content of S or Rc outer membrane is quite low, barely enough to cover only one side of the membrane, but in deep rough mutants it increases to a level which can cover one side completely and 25–50% of the other side too. Secondly, freeze-fracturing cleaves easily the outer membrane of deep rough mutants, but not of S or Rc strains, suggesting that the former, but not the latter, has the characteristics of a phospholipid-bilayer-type membrane. Thirdly, the outer half of the "deep rough" outer membrane, when observed by freeze-fracture electron microscopy, contained flat, particle-free areas which are usually assumed to correspond to halves of phospholipid bilayers, whereas such areas were not found in the outer halves of Rc outer membrane, which were filled by a dense packing of particles.

Although several "nonpenetrating" reagents have been described for the labeling of exposed membrane components (Berg, 1969; Bretscher, 1972; Rifkin et al., 1972), in most cases the impermeant nature of the reagents is dependent on the presence of charged groups, and these low-molecular-weight reagents are expected to penetrate readily the membranes containing large hydrophilic pores. Indeed, some of these reagents were shown to penetrate the plasma membrane of some animal cells (see Wallach and Winzler, 1974). In contrast the nonpenetrability of the activated dextran is based on its very large molecular size and its very hydrophilic nature. Thus, for the labeling of the surface of specialized membranes such as the outer membrane, one has to use a high-molecular-weight reagent such as activated dextran; even for the more usual kind of membranes, activated dextran may be preferable because one can be more confident about its nonpenetrability. Furthermore, the CNBr-activated dextran is easy to prepare, reacts very efficiently at low temperatures (20 °C or even lower) and at a close-to-neutral pH, and does not alter the charges of the head groups after conjugation. Its major drawback as the phospholipid-labeling reagent is the fact that it reacts only with primary amino groups. The reagent, however, also reacts with amino groups of exposed membrane proteins, and thus is very useful in determining whether any membrane protein is exposed on the outside surface of the membrane. When used for this purpose, the reagent in fact should give a much less equivocal result than many other methods, for example, lactoperoxidase-catalyzed iodination procedure that suffers from uncertainties on the permeability of H_2O_2, I^-, and I_3^-. We are currently applying this method to determine the localization of outer membrane proteins.

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Effect of Triton X-100 on the Hydrolysis of Sphingomyelin by Sphingomyelinase of Rat Brain

Saul Yedgar and Shimon Gatt*

ABSTRACT: Mixed dispersions of the nonionic detergent Triton X-100 and sphingomyelin were used as substrate for sphingomyelinase of rat brain. The dependence of the rate of hydrolysis on the concentration of sphingomyelin was measured in two ways: at a fixed concentration of Triton X-100 or at varying concentrations of this detergent, while maintaining a fixed molar ratio of Triton X-100 to sphingomyelin. In either case, the v vs. S curves deviated from the hyperbolic shape predicted by the Michaelis–Menten kinetic theory. These deviations are discussed and interpreted on the basis of the physicochemical properties of the mixed dispersions of detergent and lipid studied in previous papers.

In previous papers (Cooper et al., 1974; Yedgar et al., 1974, 1975), the physico-chemical properties of aqueous dispersions of sphingomyelin (SM)[1] and the nonionic detergent Triton X-100 (TR) were investigated. Below about 0.2 mM Triton, sphingomyelin was dispersed as bilayered liposomes. At greater concentrations of this detergent, the lipid was solubilized and mixed micelles of Triton X-100 and sphingomyelin were formed. Once formed, the nature of these mixed micelles depended on the molar ratio of detergent to lipid. When the ratio of TR to SM was between 0.5 and 4.0, the system was homogeneous and monodispersed. In this region the aggregation number of sphingomyelin, i.e., the number of SM molecules in the mixed micelle, varied from about 50 to 440, as the TR to SM ratio varied from about 4 to 0.5, respectively.

At the same time the aggregation number of TR remained practically constant, about 200, irrespective of the TR to SM ratio. This suggests that the concentration of the mixed micelles is a linear function of the detergent concentration only. The micellar concentration can therefore be obtained by dividing the molar concentration of TR by 200, its aggregation number.

In this paper the effect of Triton X-100 on the enzymatic hydrolysis of sphingomyelin by sphingomyelinase of rat brain was investigated. Deviations from the hyperbolic shapes of v vs. S curves are interpreted on the basis of the physicochemical properties of the mixed dispersions of the detergent and the lipid elaborated in the above publications.

Materials and Methods

Sphingomyelin was prepared from bovine brain by extraction with mixtures of chloroform and methanol, followed by chromatography on alumina and silicic acid. The purity of the preparation was tested on thin-layer plates of silica gel. Tritium-labeled sphingomyelin of spinal cord was prepared by catalytic hydrogenation with tritium gas in the presence of palladium on charcoal (Gatt et al., 1973) and was diluted with nonradioactive SM[1] of bovine brain. Triton X-100 was purchased from BDH and one lot (No. 30532) was used throughout. Tritium-labeled Triton X-100 was a generous gift of Rohm and Haas; it was further purified and the purity of the two preparations was checked chromatographically in two solvent systems as described (Yedgar et al., 1975).

Sphingomyelinase of rat brain was an extract of lysosomes of brains of 14 day old rats (Gatt and Gottesdiner, 1976). Its specific activity was about 1000 nmol of sphingomyelin hydrolyzed by 1 mg in 1 h.

The rate of enzymatic hydrolysis of sphingomyelin was followed by the method of Barenholz et al. (1966) as modified for assaying the hydrolysis of lecithin (Gatt, 1968). The assay mixtures were prepared by method B of Yedgar et al. (1975). Incubation time was 1 h at 37 °C. The results reported here are the average of two or more determinations.

Results

Detergents have been used extensively to facilitate enzymatic utilization of lipid substrates (reviewed by Gatt, 1973). Two approaches have been used to study the effect of a detergent on v vs. S curves. In the first, the concentration of the substrate was varied, but that of the detergent was maintained at a fixed concentration. In the second, the concentrations of both substrate and detergent were varied simultaneously while maintaining a fixed ratio between these two components.

The experiment of Figure 1 shows v vs. S curves using the first approach. The concentration of sphingomyelin was varied and that of Triton X-100 was constant; this was repeated at several fixed concentrations of the detergent. All curves were biphasic, exhibiting a hyperbolic region at low substrate concentrations, a maximal value ("inversion point") followed by

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[1] Abbreviations used are: SM, sphingomyelin; TR, Triton X-100.