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Transmembrane Permeability Channels across the Outer Membrane of *Haemophilus influenzae* Type b

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Outer membranes of *Haemophilus influenzae* type b were fractionated to yield Triton X-100-insoluble material and lipopolysaccharide and phospholipids. Liposomes reconstituted from lipopolysaccharide and phospholipids were impermeable to sucrose (M_r, 342) and to a high-molecular-weight dextran (average M_r, 6,600). When the Triton X-100-insoluble material was introduced into the reconstituted liposomes, the vesicles became permeable to sucrose, raffinose (M_r, 504), and stachyose (M_r, 666) and fully retained dextrans of M_r greater than 1,500. Inulin (average M_r, 1,400) was tested for its efflux from the reconstituted outer membrane vesicles; 62% of the added inulin was trapped. The molecular weight exclusion limit for the outer membrane of *H. influenzae* type b was therefore estimated at approximately 1,400. A protein responsible for the transmembrane diffusion of solutes was purified from *H. influenzae* type b by extraction of whole cells with cetyl trimethyl ammonium bromide. When this extract was passed over DEAE-Sepharose, three protein-containing peaks (I, II, and III) were eluted. Peaks I and II contained mixtures of proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; when tested for their pore-forming properties, these proteins were unable to render liposomes of lipopolysaccharide and phospholipid permeable to sucrose. Peak III contained only one molecular species of protein of molecular weight 40,000; this protein acted as a porin in reconstituted vesicles. The molecular weight exclusion limit for 40,000-molecular-weight protein matched the estimate of approximately 1,400 which was determined for outer membranes. A series of homologous saccharides of increasing degree of polymerization was prepared from agarose by hydrolysis with β-agarase and fractionation on gel filtration chromatography. These oligosaccharides of M_r 936, 1,242, 1,548, and 1,854 were assayed for retention by the complete vesicles containing 40-kilodalton protein and lipopolysaccharide and phospholipids. All of these oligosaccharides were lost by efflux through the porin. Since the molecular conformation of the largest oligosaccharide is an elongated semirigid helix, it is suggested that the pore formed by the 40-kilodalton protein does not act as a barrier to the diffusion of this compound.

The outer membrane of *Haemophilus influenzae* type b shares a number of characteristics that are common to gram-negative bacteria. One such characteristic is the presence of a small number of proteins that are found in apparently high numbers in the membrane and are termed "major" outer membrane proteins. In *Escherichia coli*, a major protein of molecular weight 36,000 was termed "matrix protein" because of its ability to self-assemble into a regular paracrystalline array (25). This protein is also known as "porin" since it forms water-filled channels that allow the permeation of low-molecular-weight solutes (23). The functional properties of porin proteins have been investigated for only a few bacterial species. In *E. coli* K-12 (22) and in *Salmonella typhimurium* (21), porins were reconstituted into liposomes consisting of lipopolysaccharide (LPS) and phospholipids. When such reconstituted vesicles were tested for their ability to retain solutes of different sizes, the pores showed a molecular weight exclusion limit of about 600. By comparison, when porin protein F from *Pseudomonas aeruginosa* was reconstituted into liposomes, the molecular weight exclusion limit was found over a range of 3,000 to 9,000 (13, 14, 28). This difference in molecular weight exclusion limits is thought to reflect some fundamental aspect of the structure and composition of the porins.

In addition to the in vitro studies of pore-forming properties of the outer membrane, several reports have focused upon the permeability of the outer membrane in whole cells. A simple model for estimating the ease of penetration of a β-lactam antibiotic across the outer membrane was developed by Zimmermann and Rosselet (29). Comparison of the rate of hydrolysis of the drug by intact cells which harbor a β-lactamase versus the rate of hydrolysis of the drug by sonic extracts of the organism permits an estimate of the permeability coefficient, C, for many β-lactams used against gram-negative bacteria. For *E. coli*, the values of C were on the order of 10^{-4}, whereas for *P. aeruginosa*, a permeability coefficient at least 10-fold smaller than that for *E. coli* was determined (2). Although the outer membrane of *E. coli* acts as a permeability barrier to the diffusion of β-lactam antibiotics, the outer membrane of *Pseudomonas* is even less permeable.

We recently evaluated the role of the outer membrane of *H. influenzae* type b as a permeability barrier against three members of the penicillin family and seven cephalosporins (7). Our data generated values of C between 3.7×10^{-3} and 5.0×10^{-2} for the 10 β-lactams and led to the conclusion that the outer membrane of this bacterium provides very little barrier against the free diffusion of these compounds.

To characterize further the apparently high permeability by low-molecular-weight solutes and to identify a pore-forming protein in *H. influenzae* type b, we describe a method for the isolation of a major protein of molecular weight 40,000 (40K) from the outer membrane of this organism. The 40K protein could be reconstituted into liposomes of LPS and phospholipid to form sucrose-permeable vesicles. The molecular weight exclusion limit was determined for both outer membranes and for 40K protein.

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MATERIALS AND METHODS

Bacterial strain and culture conditions. *H. influenzae* type b ATCC 9795 was obtained from the American Type Culture Collection, Rockville, Md. Bacteria were grown at 37°C with vigorous agitation in CY^+ medium (8) supplemented with 0.10 M sodium phosphate (pH 7.8). Cells were harvested at late exponential phase and stored at −70°C before use.

Preparation of outer membranes, lipopolysaccharide, and phospholipids. Outer membranes were prepared as follows. Bacteria were passed three times through a French pressure cell to obtain a cell lysate, and this material was centrifuged to pellet the cell envelopes. Cytoplasmic membrane proteins were solubilized from envelopes with 2% Triton X-100 to yield Triton X-100-insoluble (TI) material containing outer membrane proteins (8). The detergent concentration was reduced by batchwise dialysis against Bio-Beads SM-2 (Bio-Rad Laboratories, Mississauga, Ontario, Canada) (16). Protein content was estimated by the dye-binding assay (6) with the reagent from Bio-Rad; bovine serum albumin was the standard protein. Lipopolysaccharide (LPS) was collected from *H. influenzae* type b by the technique of Johnson and Perry (18). Confirmation of the purity of LPS was obtained by the carbocyanine dye assay (17) such that a single symmetrical peak was found at 460 nm when the sample was scanned from 400 to 700 nm. Total lipids were extracted from *H. influenzae* type b by the method of Kates (19). Because neutral lipids interfered with formation of liposomes, they were removed by binding to a silicic acid column and by elution with chloroform. Phospholipids were then displaced from the column with methanol and were quantitated by measuring phosphate content with the ashing technique of Ames and Dubin (1).

Radioactive compounds. Methoxy-[^{3}H]dextrans (0.175 mCi/mg), [^{3}H]inulins (0.139 mCi/mg), and [^{14}C]sucrose (0.10 mCi/mg) were purchased from New England Nuclear (Lachine, Quebec, Canada). Because of the heterogeneity of the molecular weights of the radiolabeled polymers, it was necessary to fractionate the material to obtain defined size classes. A Bio-Gel P-10 column (200-400 mesh; 1.2 by 48 cm) in 0.5 M NH_4HCO_3 was calibrated with sucrose (M_r, 342), raffinose (M_r, 504), stachyose (M_r, 666), a dextran fraction with average M_r 1,300 (supplied by B. E. Holbein of this department), and Dextran T-10 (M_r, 10,000; Pharmacia Fine Chemicals, Dorval, Quebec, Canada). Sugars were assayed by the phenol-sulfuric acid method (10), and their K_{av} values were calculated according to their elution position from the column. With this calibrated column, [^{3}H]dextran fractions with estimated average M_r values of 1,500, 2,500, 3,800, 5,200, 6,600, and 9,400 were obtained. The same column was used to prepare [^{3}H]inulin with average M_r 1,400. Fractions of radiolabeled dextran of a specific size class or of radiolabeled inulin were pooled and concentrated to 1.0 ml by rotary evaporation. Each pooled fraction was rechromatographed on the same column. Peaks from this second gel filtration step were collected, rotary evaporated, suspended in 100 mM NaCl–1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2), and used in the reconstitution of outer membranes.

Preparation of radiolabeled oligosaccharides. Raffinose (50 mg; Sigma Chemical Co., St. Louis, Mo.) and stachyose (50 mg; Sigma) were labeled with tritiated borohydride by a method similar to that described by Decad and Nikaido (9). A reaction mixture containing oligosaccharide, galactose oxidase (225 U; P-L Biochemicals, Inc., Dorval, Quebec, Canada), and 0.07 ml of toluene was incubated overnight at 37°C. After adjustment of the pH of the sample to 10.0, potassium boro[^{3}H]hydride (2.25 mg) was added; incubation was for 6 h at ambient temperature. Unlabeled potassium borohydride (20 mg) was then added, followed by a further incubation for 3 h. The reactions were stopped by addition of acetic acid, and the sample was applied to a Bio-Gel P-2 column (1.6 by 85 cm) run at 65°C and eluted with distilled water. Fractions containing the labeled [^{3}H]raffinose or [^{3}H]stachyose, well separated from the other reaction products, were collected and concentrated by rotary evaporation. The labeled oligosaccharide eluted from the gel filtration column at a K_{av} identical to that of the unlabeled sugar as assayed by the phenol-sulfuric acid method.

For generation of a series of homologous saccharides of increasing molecular weights, the following strategy was adopted. Agarose is composed of a repeating disaccharide containing alternating residues of (1 → 4)-linked 3,6 anhydro-α-L-galactose and (1 → 3)-linked β-D-galactose (10). When this polymer was digested with an excess of β-agarase that was purified from the marine bacterium *Pseudomonas atlanticum*, fractions were obtained by gel filtration chromatography that were identified as a series of polymerized saccharides of increasing molecular weight. The disaccharide DP1 (DP = degree of polymerization of the disaccharide) (M_r, 324) could be separated from the tetrasaccharide DP2 (M_r, 630), the hexasaccharide DP3 (M_r, 936), the octasaccharide DP4 (M_r, 1,242), the decasaccharide DP5 (M_r, 1,548), and the dodecasaccharide DP6 (M_r, 1,854). The identity of each was confirmed by proton nuclear magnetic resonance and by ^{13}C-nuclear magnetic resonance (W. Yaphe, personal communication). Preparation of the radiolabeled derivatives of each member of the DP*n*-series of saccharides was the above-described method, using tritiated borohydride. The reaction products were separated on a calibrated Bio-Gel P-2 column by elution with distilled water. The [^{3}H-labeled]DP*n* fractions gave K_{av} values that were the same as those for unlabeled DP*n*.

Fractionation of outer membrane proteins of *H. influenzae* type b. Whole cells were washed with 0.10 M phosphate buffer (pH 7.4) containing 10 μM phenylmethylsulfonyl fluoride and centrifuged at 8,000 × *g* for 15 min. The cell pellet was suspended in 9 volumes of water containing 2% (wt/vol) cetyl trimethyl ammonium bromide (CTB; Sigma) and stirred for 1 h at ambient temperature. The extract was centrifuged at 17,000 × *g* for 15 min. The supernatant was discarded, and 9 volumes of water was added to the pellet. The ionic strength of the mixture was raised by adding 4 M $CaCl_2$ to a final concentration of 1.0 M. After stirring for 60 min, the suspension was made to 20% (vol/vol) ethanol; the precipitated nucleic acids were removed by centrifugation at 17,000 × *g* for 15 min. The ethanol concentration of the supernatant was then raised to 80% (vol/vol), and the resulting precipitate was recovered by centrifugation. The pellet was washed twice with 100 volumes of ethanol and once with acetone and was either lyophilized or used immediately. This material was designated the CTB extract.

CTB extract was suspended at a concentration of 2% (wt/vol) in 50 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA–0.5 M NaCl–5% (wt/vol) *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonia-1-propanesulfonate (Zwittergent Z-3,14; Calbiochem-Behring Corp., La Jolla, Calif.). The mixture was stirred for 60 min and centrifuged at 17,000 × *g* for 15 min. The pellet was reextracted with the same detergent, and the supernatants were pooled. The Zwittergent-solubilized mixture was dialyzed exhaustively against buffer containing 50 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA–0.05%

TABLE 1. Requirements for reconstitution of outer membrane vesicles of *H. influenzae* type b

Composition of vesicles	% Radiolabeled saccharide retained[a]							
	With Mg^{2+} (10 mM)				Without Mg^{2+}			
	[^{3}H] dextran[b]	[^{14}C] sucrose	[^{3}H] raffinose	[^{14}C] sucrose	[^{3}H] dextran[b]	[^{14}C] sucrose	[^{3}H] raffinose	[^{14}C] sucrose
TI material or LPS or phospholipid	0.06	0.05			0.05	0.02		
LPS + phospholipid	0.15	0.04	0.06	0.05	1.56	0.65	0.51	0.60
Complete (TI material + LPS + phospholipid)	ND[c]	ND	ND	ND	1.30	0.16	0.11	0.16

[a] Values were determined by comparing the amount of radiolabel that was retained by the vesicles after gel filtration with the amount of radiolabel added to the reconstitution mixture.
[b] Average M_r 6,600.
[c] ND, Not determined.

(wt/vol) Z-3,14 and applied to a DEAE-Sepharose CL-6B (Pharmacia) column (2.6 by 22 cm) equilibrated with the same buffer. The column was washed with 3 bed volumes of buffer to elute some proteins. All fractions eluted from the ion-exchange matrix were monitored for absorbance at 280 nm and for the identity of protein bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20).

Reconstitution of outer membrane vesicles. Reconstitution experiments were adapted from the protocols described by Nakae (21, 22). Phospholipids (0.5 μmol) in chloroform were dried in borosilicate tubes to a thin film under a stream of nitrogen and allowed to stand in an evacuated desiccator for 30 min. LPS (0.7 mg), suspended by heating at 60°C for 30 min in water, was added to the phospholipids. As a source of outer membrane proteins, 10 μg of protein from TI material was added. Components were sonicated for 60 s in a Disontegrater water bath sonicator (Ultrasonic Industries Inc., Hicksville, N.Y.). After the materials were dried under nitrogen at 45°C, the tubes were placed again in an evacuated desiccator for 30 min. To this sample was added 200 μl of 100 mM NaCl–1 mM HEPES (pH 7.2) containing 2.0×10^5 cpm of [^{14}C]sucrose and 2.0×10^5 cpm of a given ^{3}H-saccharide. Sonication for 60 s disrupted the sample and trapped the radiolabels inside the resealed vesicles. The tubes were equilibrated for 30 min in a 45°C water bath and allowed to cool to room temperature over 2 h. The reconstitution mixture was chromatographed on a Sepharose 4-B column (1 by 28 cm), using 100 mM NaCl–1 mM HEPES (pH 7.2). The column was monitored for (i) absorbance at 280 nm for the presence of intact vesicles and (ii) radioactivity of [^{14}C]sucrose and ^{3}H-saccharide; samples were counted in a Beckman LS 8000 liquid scintillation counter.

Amino acid analysis. Samples of 40K protein were hydrolyzed in vacuo at 100°C for 18 h in 6 N HCl. Amino acid analysis was performed with a Beckman 6000 series high-performance analyzer.

RESULTS

Reconstitution of vesicles with outer membrane proteins of *H. influenzae* type b. The requirements for formation of intact vesicles from membrane components of *H. influenzae* type b were established by comparing the retention of a high-molecular-weight radiolabeled dextran (average M_r, 6,600) versus radiolabeled raffinose (M_r, 504) by different combinations of TI material and LPS and phospholipid. The reconstitution systems were tested in the presence and the absence of 10 mM $MgCl_2$ (Table 1). None of the components alone was able to retain high amounts of dextran or sucrose. Liposomes formed by LPS plus phospholipid produced leaky or unstable vesicles in the presence of magnesium. In the absence of magnesium, the liposomes were intact and sealed, and a higher retention of [^{3}H]dextran was observed as compared with either [^{14}C]sucrose or [^{3}H]raffinose. When TI material was incorporated into the liposomes, the high-molecular-weight dextran was retained but sucrose and raffinose were lost by efflux from the vesicles. Because liposomes and complete vesicles were best reconstituted in the absence of magnesium, this cation was not added to the buffers in subsequent reconstitution experiments.

The phenomenon whereby magnesium destabilizes the membrane vesicles has been noted by others using different bacterial outer membranes (24). We previously reported (7) that magnesium was needed to prevent the leakage of β-lactamase from the periplasm of whole cells. It may be that the concentrations of ions are important. The presence of excess magnesium may destabilize the membrane by forming single associations with negatively charged groups on the LPS. Lower concentrations of the ion might cross-link the LPS, thereby stabilizing the outer membrane or the liposomes.

The amount of ^{3}H-saccharide that was retained by the complete vesicles varied according to the molecular weight of the saccharide. The maximum retention of [^{3}H]dextran (average M_r, 6,600) was 1.56% of the amount added to the reconstituted vesicles containing outer membrane fragments as TI material (Table 1). One [^{3}H]inulin fraction of average M_r 1,400 and five [^{3}H]dextran fractions of average M_r 1,500, 2,500, 3,800, 6,600, and 9,400 were tested for their retention by reconstituted vesicles. Dextrans which had an average M_r greater than 1,500 showed more than 80% retention by vesicles reconstituted with TI material and LPS and phospholipids (Fig. 1A). When [^{3}H]raffinose and [^{3}H]stachyose were tested in the same manner, they were lost from the reconstituted mixture; the amount remaining associated with the vesicles was similar to the amount of sucrose (20 to 30%). The amount of [^{3}H]inulin (average M_r, 1,400) that was retained by the complete vesicles containing TI material was intermediate to that shown by stachyose and dextran (average M_r, 1,500). These data suggested that (i) liposomes of LPS and phospholipids could be made permeable to sucrose, raffinose, and stachyose by the presence of TI material from *H. influenzae* type b; and (ii) the molecular weight exclusion limit of these reconstituted vesicles was between M_r 666 and 1,500.

Purification of a porin. The pore-forming activity of the TI material described above was identified as follows. Membrane proteins were extracted from whole cells with cetyl trimethyl ammonium bromide and suspended in buffer containing 50 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA, 0.5 M NaCl, and 5% (wt/vol) Zwittergent Z-3,14. This

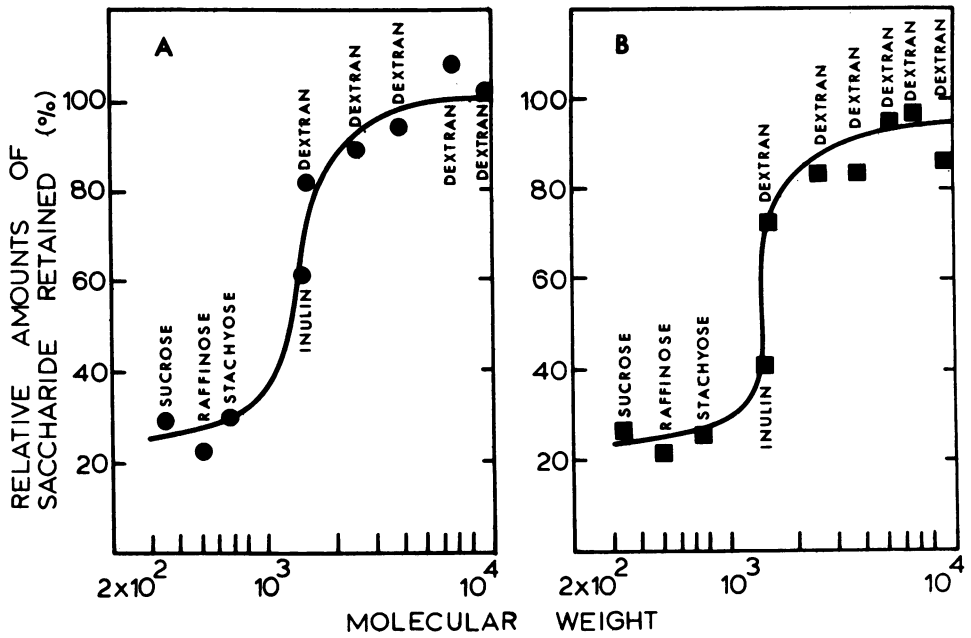

FIG. 1. Molecular weight exclusion limit for saccharides in reconstituted outer membrane vesicles of *H. influenzae* type b. Control vesicles formed by sonication of LPS and phospholipid were able to trap the labeled saccharide; this value was taken as 100% retention. (A) Reconstitution of TI material into liposomes of LPS and phospholipid. The amount of retention of each saccharide is expressed relative to the value for control vesicles. Each point on the graph represents between 3 and 10 complete vesicle experiments and the same number of control experiments. Mean values are shown. (B) Reconstitution of 40K protein from the outer membrane of *H. influenzae* type b into liposomes. The saccharides were tested for their retention by reformed vesicles; data are expressed as percent retention relative to controls to which no pore-forming protein was added. The saccharides were sucrose, raffinose, stachyose, inulin (average M_r, 1,400), and dextran fractions (average M_r, 1,500, 2,500, 3,800, 5,200, 6,600, and 9,400).

material was passed over DEAE-Sepharose CL-6B. Proteins were retarded by the gel, and some could be eluted without an increase in the ionic strength of the buffer. Three peaks (I, II, and III) of protein-containing material were found (Fig. 2). The peak containing the most protein eluted between fractions 150 and 162. When peak III was applied to a gel filtration column of Sephacryl S-200, a single symmetrical peak was observed (data not shown).

A comparison was made of the proteins in the TI material, CTB extract, and peaks I, II, and III from the DEAE column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed the protein pattern of the TI material shown previously (8; Fig. 3, lane A) and indicated that one of

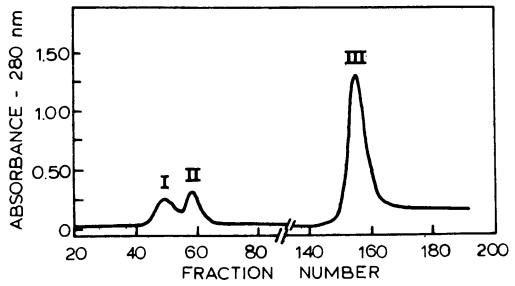

FIG. 2. Ion-exchange chromatography of the CTB extract of *H. influenzae* type b. CTB extract (65 mg of protein) was fractionated at room temperature on a DEAE-Sepharose CL-6B column as described in the text. The eluting buffer contained 50 mM Tris-hydrochloride (pH 8.0), 0.5 M NaCl, and 0.05% (wt/vol) Zwittergent Z-3,14. Fractions of 1 ml were collected. No salt gradient was necessary to elute peaks I, II, and III. Samples were pooled, concentrated, dialyzed, and analyzed for protein.

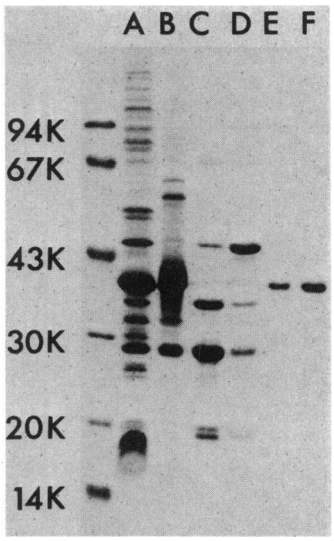

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins from *H. influenzae* type b. Proteins used as molecular weight standards: phosphorylase b (94K), bovine serum albumin (68K), ovalbumin (43K), carbonic anhydrase (30K), and soybean trypsin inhibitor (21K), all from Pharmacia. Lane A, TI material (10 μg) shows the major outer membrane proteins of molecular weights 46,000, 40,000, 36,000, 27,000, and 16,000. Lane B, CTB extract from whole cells of *H. influenzae* (10 μg). Lane C, peak I from the fractionation of CTB extract on the DEAE-Sepharose column (10 μg). Lane D, peak II from DEAE-Sepharose (10 μg). Lanes E and F, peak III from DEAE-Sepharose (0.5 and 1.0 μg, respectively). The samples were boiled for 5 min before being loaded onto the gel. After electrophoresis, the gel was stained with Coomassie blue.

the proteins of apparent molecular weight 40,000 was considerably enriched after CTB extraction (Fig. 3, lane B). Further purification of the 40K protein was successful when DEAE-Sepharose was used. Peaks I and II (Fig. 3, lanes C and D, respectively) showed complex mixtures of proteins; all of the major proteins of the TI material except 40K protein were found in these peaks. Peak III (Fig. 3, lanes E and F) appeared to contain only 40K protein. This sample (yield, 8.5 mg from 17 g [wet weight] of cells) was the purest preparation obtained in this study and was used for all subsequent experiments.

Each of the protein-containing samples from peaks I, II, and III was tested for pore-forming activity in the reconstitution system. When 10 μg of protein from peaks I and II was combined with LPS and phospholipid, sucrose-permeable vesicles could not be formed. The same amount of protein from peak III rendered the LPS-phospholipid liposomes permeable to sucrose, raffinose, and stachyose (Fig. 1B). Because 41% of the [^{3}H]inulin (average M_r, 1,400) and 73% of the [^{3}H]dextran (average M_r, 1,500) were retained by the complete vesicles containing 40K protein, it appeared that the exclusion limit fell close to 1,400 daltons.

Properties of the porin of *H. influenzae* type b. In an attempt to provide a complementary assessment of the value for the molecular weight exclusion limit that the 40K protein from *H. influenzae* conferred to the reconstituted liposomes, we prepared a number of oligosaccharides from the natural polymer, agarose. This material is composed of a repeating disaccharide of M_r 324. By enzymatic hydrolysis of agarose with β-agarase (11), a family of saccharides was prepared which were of increasing molecular weights: 324, 630, 936, 1,242, 1,548, and 1,854. These values spanned the range of molecular weights for the exclusion limit that was derived for the TI material and for 40K protein. The elution of each of the radiolabeled fractions from a calibrated gel filtration column is shown in Fig. 4 and is compared with the elution positions for unlabeled glucose, sucrose, raffinose, and stachyose. The curve for the elution of each member of the DP*n* series displayed remarkable linearity when K_{av} values were plotted against their molecular weights. Some deviation from this linear relationship was found for glucose, sucrose, raffinose, and stachyose.

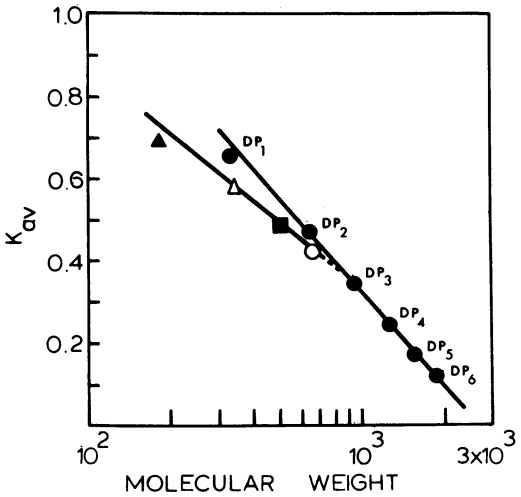

FIG. 4. Fractionation of radiolabeled oligosaccharides obtained by enzymatic hydrolysis of agarose and reaction with tritiated borohydride. A gel filtration column of Bio-Gel P-2 was calibrated with unlabeled glucose, sucrose, raffinose, stachyose, and two dextran fractions of average M_r, 1,200 and 10,000. The tritiated saccharides of the DP*n* series were loaded separately onto the column and eluted with water. The elution position of DP*n* was determined by counting the fractions for radioactivity and is expressed as K_{av}. Each DP*n* was collected, concentrated, and used in reconstitution experiments. The elution positions of unlabeled glucose (▲), sucrose (△), raffinose (■), and stachyose (○) are also indicated.

TABLE 2. Assays of the efflux of radiolabeled oligosaccharides from reconstituted vesicles containing 40K protein from *H. influenzae* type b

Oligosaccharide	M_r	% Retention[a]	No. of expts
[^{3}H]DP_3	936	28 ± 13	6
[^{3}H]DP_4	1,242	42 ± 12	3
[^{3}H]DP_5	1,548	30 ± 9	5
[^{3}H]DP_6	1,854	24 ± 20	6
[^{3}H]dextran	1,500	73 ± 22	10
[^{3}H]dextran	2,500	84 ± 24	8

[a] Values are means ± standard deviation; retention is relative to the amount of oligosaccharide trapped by liposomes of LPS and phospholipid.

The 40K protein from *H. influenzae*, incorporated into reconstituted vesicles, was tested for the retention or loss of the tritiated oligosaccharides DP3, DP4, DP5, and DP6 and compared with the percent retention of [^{14}C]sucrose. When each member of the DP*n* series was tested for its retention by complete vesicles reconstituted with 40K protein, low retention of all compounds was observed (Table 2).

Amino acid analysis of 40K protein from *H. influenzae* type b was performed in triplicate (Table 3). When compared with porins from other bacterial species, the composition of porin from *H. influenzae* showed (i) a markedly elevated content of basic amino acids; (ii) a lower level of neutral residues, especially glycine and alanine; and (iii) decreased amounts of methionine. The average hydrophobicity of the protein was estimated to be 960 cal/mol (4,019.5 J/mol) according to the calculation of Bigelow and Channon (4), suggesting that 40K protein is only moderately hydrophobic.

DISCUSSION

The outer membrane of gram-negative bacteria is a highly specialized structure which acts as a permeability barrier to the free diffusion of compounds across the cell envelope. This membrane must also be selectively permeable to permit the uptake of nutrients into the cell. There are now ample suggestions in the literature that the molecular sieving properties of the cell wall of *H. influenzae* type b are markedly different from those of the cell walls of other gram-negative bacteria. *H. influenzae* which does not harbor a β-lactamase is exquisitely sensitive to β-lactam antibiotics and requires MICs of ampicillin and penicillin G as low as 0.02 and 0.06 μg/ml, respectively. This contrasts with the MIC values of ampicillin against *E. coli*, about 1.0 μg/ml, and against *P. aeruginosa*, >500 μg/ml. Our evaluation of the role of the outer membrane of *H. influenzae* as a barrier against 10 β-lactam antibiotics was another indication of a very permeable cell wall (7). The concentration of one of these antibiotics in the periplasm was estimated at 77 μM when the external concentration in the surrounding medium was fixed at 100 μM. Calculation of the permeability coefficient *C* for β-lactams led to our proposal that the pores of *H. influenzae* are part of an "open" wall structure compared with the cell walls of other gram-negative bacteria examined to date.

This paper extends the above observations and further characterizes the outer membrane permeability of *H. influenzae*. An estimate of the molecular weight exclusion limit for native outer membranes was made by measuring the

TABLE 3. Amino acid composition of porin proteins from bacterial species

Amino acid	Amino acid content (mol of residue per mol of protein)[a]			
	H. influenzae 40K protein[b]	*E. coli* matrix protein[c]	*S. typhimurium* 36K porin[d]	*Neisseria gonorrhoeae* 34K protein I[e]
Basic	(0.18)	(0.09)	(0.09)	(0.10)
Lysine	34	18	17	18
Histidine	10	2	1	6
Arginine	17	12	13	6
Dicarboxylic	(0.24)	(0.24)	(0.26)	(0.23)
Aspartic, asparagine	33	54	64	33
Glutamic, glutamine	49	27	29	36
Neutral	(0.58)	(0.66)	(0.66)	(0.67)
Theronine	19	21	27	15
Serine	13	17	22	27
Proline	7	5	4	12
Glycine	27	45	49	42
Alanine	24	30	26	27
Half cystine		1		1
Valine	29	22	18	27
Methionine	2	4	4	4
Isoleucine	15	11	10	6
Leucine	31	21	22	18
Tyrosine	23	25	31	15
Phenylalanine	15	18	20	12
Tryptophan		3	4	

[a] Values in parentheses indicate the fraction of the total number of residues of each class.
[b] This study.
[c] Reference 25.
[d] Reference 27.
[e] Reference 5.

retention or loss of radiolabeled sugars and oligosaccharides. We initially fractionated a heterodisperse preparation of [^{3}H]dextran of average M_r 5,000 into subclasses of dextrans, each with a narrowly defined molecular weight range. In 22 experiments, control liposomes containing LPS and phospholipids trapped an average of 1.16% of the added tritium-labeled saccharide (about 225,000 cpm) and 0.66% of the added [^{14}C]sucrose (also 225,000 cpm). Vesicles reconstituted with outer membranes prepared as TI material retained relatively high amounts of all the dextrans prepared. The smallest [^{3}H]dextran tested, average M_r 1,500, showed 84% retention by the complete reconstituted vesicles with TI material by comparison with control liposomes. Radiolabeled derivatives of sucrose, raffinose, and stachyose diffused out of the reconstituted vesicles; 62% of the added [^{3}H]inulin was trapped by the vesicles containing TI material. From these data, we conclude that the molecular weight exclusion limit of the outer membrane of *H. influenzae* type b is approximately 1,400.

This estimate of molecular weight exclusion limit was supported by (i) the isolation of a pore-forming protein from the outer membrane of *H. influenzae*, and (ii) the reconstitution of this porin into liposomes of LPS and phospholipid. The radiolabeled dextrans were efficiently trapped by the reconstituted vesicles, inulin showed only 41% retention, and the remaining three low-molecular-weight saccharides were lost by efflux (Fig. 1B). The molecular weight exclusion limit for the isolated, reconstituted 40K protein corresponded to the value of approximately 1,400 determined for outer membranes.

An unexpected observation was made when a series of saccharides of increasing degree of polymerization (DP*n*) was tested for retention by reconstituted vesicles containing the 40K protein. The DP*n* series of homologous oligosaccharides used in the reconstitution system ranged in M_r from 936 to 1,854. Each of DP3, DP4, DP5, and DP6 was efficiently trapped within liposomes of LPS and phospholipid but was lost from the complete vesicles to which 40K protein was added. Information concerning the structure of the repeating saccharides suggests that these derivatives from the agarose family differ in conformation from members of the dextran family. DP3 makes one turn of a helix and has an axial periodicity of 0.95 nm; DP6 makes two turns of a helix and two chains of DP6 are predicted to be arranged as a double helix 1.90 nm in length (3). The width of an agarose molecule has been estimated between 1.0 and 1.6 nm (15). These data characterize DP6 as a distinctly linear polymer. Thermodynamic considerations of bending such an elongated structure would prevent DP6 from assuming a random coil. To account for the efflux of DP6 from the reconstituted vesicles, one might imagine a channel of sufficient diameter to accommodate the shell of hydration of this oligosaccharide and of sufficient length to span the membrane. The pathway through the channel is not likely S-shaped because a linear, semirigid helix could not readily traverse an aqueous pore which formed a barrier to free diffusion of DP6. By comparison with the DP*n*-series, dextrans from Leuconostoc are a family of branched glucans containing the α-1,6 linkage. The extensive branching of these molecules contributes to their more spherical conformation in solution. Such a globular structure for dextran (M_r, 1,500) would have a greater hydrodynamic diameter than the diameter of the pore, thereby preventing its diffusion. This fundamental difference in structure of the uncharged permanent molecules is consistent with the idea of porin as a hollow cylinder which spans the outer membrane of *H. influenzae* type b.

We were also interested in determining whether only one species of protein from the outer membrane of *H. influenzae* showed porin activity or whether there might be more than one molecular species of pore-forming protein in the membrane. Fractionation of the CTB extract on DEAE-Sepharose yielded three distinctly separated peaks, all of which could be recovered without increasing the salt concentration

of the eluting buffer. When each of the three protein preparations was tested for pore-forming activity, only peak III containing the 40K protein was found to render liposomes of LPS and phospholipids permeable to [^{14}C]sucrose. This absence of channel-forming activity by proteins from peaks I or II in reconstituted vesicles suggests that none of the other major proteins that we have detected in the outer membrane of *H. influenzae* serves as a channel for the diffusion of low-molecular-weight solutes. The possibility cannot be excluded that some pore-forming protein may be induced under growth conditions different from those that we used; such a mechanism would be analogous to the induction of the LamB pore of *E. coli* for maltose and maltodextrins (26) or the induction of protein D1 in the outer membrane of *P. aeruginosa* by glucose (12).

In summary, the 40K porin protein from *H. influenzae* type b is characterized by three features which distinguish it from other bacterial porins studied to date. First, the molecular weight exclusion limit of approximately 1,400 lies between the value for the *Enterobacteriaceae* and the pseudomonads. By this criterion, *H. influenzae* has intermediate sized pores compared with these bacterial species. This parameter may therefore account for the ready penetration by β-lactam antibiotics and low-molecular-weight solutes. Second, the porin of *H. influenzae* has a markedly higher content of basic amino acids than do other porins. If these positively charged residues were to form part of the channel and were directed towards the lumen of the pore, it would contribute to greater hydrophilicity of the porin. Third, the 40K protein is not a peptidoglycan-associated protein (8); this is similar to porin proteins F (13) and D1 (12) of *P. aeruginosa*, neither of which is operationally defined as peptidoglycan-associated. These latter two properties are probably responsible for the ease of extraction by CTB and the ease of elution from an anion-exchange column. Our current efforts are directed towards understanding other properties of the porin of *H. influenzae* type b, including its size and behavior in black lipid membranes.

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