THE EXCLUSION LIMIT OF THE OUTER MEMBRANE OF *Haemophilus influenzae* TYPE b

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ABSTRACT

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 and to high molecular weight dextrans. When the Triton X-100-insoluble material was introduced into the reconstituted liposomes, the vesicles became permeable to sucrose, raffinose, and stachyose and fully retained dextrans of molecular weight greater than 1,500. Inulin (molecular weight 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 1,400 daltons.

RESUME

La fraction insoluble dans le Triton X-100 ainsi que le lipopolysaccharide et les phospholipides ont été purifieé à partir de la membrane externe de Haemophilus influenzae du type b. Des liposomes reconstitués avec le lipopolysaccharide et les phospholipides étaient imperméable au sucrose et aux dextrans de poids moléculaires élevés. Lorsque le materiél insoluble dans le Triton X-100 fut introduit dans les liposomes reconstitués, ces derniers devinrent perméables au sucrose, au raffinose et au stachyose, mais retinrent complètement les dextrans de poids moléculaires supérieurs à 1,500. La rétention de l'inuline (poid moléculaire 1,400) par des vésicules reconstituées de la membrane externe a également été testée; 62% de l'inuline ajoutée aux vésicules a été retenue par celles-ci. La limite d'exclusion de la membrane externe de H. influenzae du type b est donc estimée à 1,400 daltons.

TABLE OF CONTENTS

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I. INTRODUCTION

1.1 The Outer Membrane

Gram-negative bacteria possess a multilayered cell envelope which is composed of three structures: a cytoplasmic membrane, a thin peptidoglycan layer, and an external lipid bilayer called the outer membrane. A zone called the periplasm lies between the outer and the cytoplasmic membrane. In the periplasm is found the peptidoglycan, a network of sugar-peptides that gives strength and shape to the cell wall (Braun and Hantke, 1981; Benz, 1985).

Gram-positive bacteria do not possess an outer membrane and were found to be more sensitive than gram-negative bacteria to a wide range of compounds such as bile salts, detergents, dyes and large antibiotics (Nikaido and Nakae, 1979). The disruption of the outer membrane of gram-negative bacteria such as Escherichia coli and Klebsiella aerogenes with ethylenediamine tetraacetate (EDTA) resulted in an increased sensitivity to actinomycin D and benzylpenicillin (Hamilton-Miller, 1965; Lieve, 1965; Lieve, 1968). The primary function of the outer membrane is thus to act as a barrier to the passage of molecules into the cell (Lieve, 1974; Osborn, Rick, Lehmann, Rupprecht, and Singh, 1974).

The outer membrane cannot, however, be an absolute barrier since it must also allow the passage of nutrients

into the cell and the loss of metabolic wastes from the cell. The sensitivity of gram-negative bacteria to antibiotics also depends on the ability of molecules to penetrate the outer membrane. Unlike the cytoplasmic membrane, the outer membrane does not possess any known active transport mechanisms. Water-soluble molecules are able to cross the outer membrane without active transport mechanisms because the outer membrane is a permeable barrier.

The permeability of the outer membrane has been attributed to the presence of water-filled channels or pores which are formed by outer membrane proteins called "porins" (Nakae, 1976a). Since the movement of molecules through the pores is due to passive diffusion, no energy has to be provided by the cell. The channels can only accommodate molecules smaller than a certain size. Larger molecules are excluded from entering the cell through the pores. The pore has an exclusion limit which is defined in terms of the molecular weight of the largest molecule that can diffuse through it. The outer membrane is thus a molecular sieve.

Large molecules that cannot permeate through the pores may be able to cross the membrane via specialized facilitated transport mechanisms. One such example is the transport of vitamin B-12 across the outer membrane of E. coli by the outer membrane protein BtuB (White, DiGirolamo, Fu, Preston, and Bradbeer, 1973).

1.2 Identification of Porins

Porin proteins in bacteria have been identified by a variety of methods, all of which demonstrated pore-forming ability. The following sections describe techniques which have been used to identify these proteins and to characterize some of their properties.

1.2.1 The Efflux of Radiolabels from Vesicles

The first technique used to identify porins was the radiolabel efflux method described by Nakae (1975). Phospholipid and lipopolysaccharide (LPS) purified from a gram-negative bacterium were mixed by mild sonication in the presence of radiolabelled sugars to form liposomes. Liposomes are membrane-enclosed structures that are impermeable to both low and high molecular weight saccharides. Any saccharides enclosed in the liposomes were trapped inside as indicated by high retention of radiolabels by the liposomes. Vesicles were formed by the mild sonication of a mixture of phospholipid, lipopolysaccharide and porin proteins in the presence of a radiolabelled sugar. Thus, vesicles contain protein reconstituted into LPS and phospholipids, whereas liposomes contain only LPS and phospholipids. The vesicles were permeable to water-soluble molecules because of the channels formed in the membrane by the porins. The reconstitution mixture was chromatographed on a gel-filtration column to allow both for the separation of vesicles from untrapped labels and for any efflux of

radiolabels from the vesicles through the pores. The retention of radiolabelled sugars was demonstrated by (i) measuring the amount of radioactivity associated with the vesicles that eluted from the column, and (ii) comparing this value with the retention of the same radiolabel by the control liposomes. Low retention of low molecular weight saccharides by vesicles in comparison with liposomes was an indication of the presence of porins.

The exclusion limit of the pores was measured by utilizing radiolabelled saccharides of increasing molecular weights (Nakae, 1976). When the size of the saccharide exceeded the exclusion limit of the pore, it was retained by the vesicles to the same degree as control liposomes. The experimental system was particularly useful because it permitted the measurement of the exclusion limit of the pores without actually having to isolate the porin. This was based on the assumption that the general permeability of the outer membrane was dependent on the pores formed by its major porins. The system was not, however, designed to give information on the kinetics of permeation through the pores. Diffusion occurred too rapidly for the time course of the efflux of radiolabels from the vesicles to be followed even when the temperature was lowered to 0°C (Nakae, 1976).

1.2.2 Porin-deficient Mutants

The second method that helped to identify the porins of gram-negative bacteria was the isolation of mutant strains

that could no longer produce a major porin. The comparison of the outer membrane protein compositions of the mutant with the wild type by SDS-polyacrylamide electrophoresis aided in the identification of possible porin proteins.

Porin-deficient mutants were identified by the loss of permeability to a wide variety of substrates such as glucose and copper ions (Bavoil, Nikaido, and von Meyenberg, 1977; Lutkenhaus, 1977). When a minimal medium was used, porin-deficient mutants of E. coli B were slow growers by comparison with the wild type. Solutes were, therefore, still capable of crossing the membrane to supply nutrients to the cell; the outer membrane of the mutant remained permeable. The permeability of the outer membrane of the mutant can be due to several factors. Some porin-deficient mutants were capable of producing residual amounts of porin. The residual number of porins was, however, insufficient to accommodate the required influx of nutrients to maintain the normal growth rate. Another explanation is that solutes are able to penetrate through pores formed by alternative porins. Spontaneous revertants of porin-deficient mutants of E. coli were observed and were attributed to a new porin, NmpC (Hindahl, Crockford, and Hancock, 1984; Pugsley and Schnaitman, 1978). The bacterium had an alternative pore which was used to ensure the permeability of the outer membrane. Permeation of solutes through these pores was, however, too slow to allow for a normal growth rate.

When a rich medium was used, the growth rate of the porin-deficient mutant became similar to that of the wild type. The rich medium provided a high external

concentration of solutes that resulted in a steep concentration gradient across the outer membrane. This increased the rate of permeation of solutes through the residual and alternative pores, causing enough quantities of solutes to cross the membrane and to sustain a normal growth rate in the mutant. The overall permeability of the outer membrane was, therefore, dependent not only on the presence of pores but also on the number of pores and on the concentration gradient across the membrane.

The decreased permeability of the outer membrane of these mutants also resulted in a loss of sensitivity to certain antibiotics. Many porin-deficient mutants were selected by their acquired resistance to various antibiotics. These antibiotics may still be able to gain access to the periplasm through the residual or alternative pores but did so at too slow a rate for the antibiotic to be effective. For example, the rates of diffusion of β-lactams across the outer membrane of a porin-deficient mutants of Salmonella typhimurium was reduced to less than 10% of that of the wild type (Nikaido, Ah Song, Shaltiel, and Nurminen, 1977). An exception to this is the permeation of aminoglycosides. The decreased number of pores in porin-deficient mutants does not limit the rate with which the aminoglycosides appear in the periplasm (Nakae and Nakae, 1982). Aminoglycosides must penetrate through the pores very efficiently.

1.2.3 Conductivity Experiments

The third method used to identify porins relied upon conductivity experiments. A black-lipid membrane was formed over an aperture which separated two solution-filled chambers (Benz, Ishii, and Nakae, 1980). Porin molecules deposited near the membrane inserted spontaneously into the bilayer to form pores. The formation of pores in the membrane allowed the flow of ions from one chamber to another; pore formation was measured as an abrupt increase in conductivity. When two pores inserted into the membrane at the same time, an increment in conductivity was observed that was twice the size of that for one pore. If the protein used in these experiments was not able to form pores, no increase in conductivity was observed. Using this experimental system, porins can be readily identified.

The diameter of pore can be calculated from the conductivity since the amount of conductivity is dependent on the area of the pore. The calculation of the diameter is dependent on the measurement of single events: the insertion of one pore. The increments in conductivity can be measured in nSiemens. The use of outer-membrane extracts was not feasible since this would result in the insertion of large membrane fragments including a large number of porin proteins at one time. The protein to be tested must, therefore, be isolated before it can be used.

1.2.4 Liposome Swelling Assays

The fourth technique used to identify a porin molecule is the liposome swelling assay (Luckey and Nikaido, 1980). Liposomes made of phospholipids and protein were formed in the presence of large dextrans and then diluted in an isotonic solution of a low molecular weight sugar. If the protein tested formed pores, the vesicles swelled because water and solute entered the vesicles. The swelling of the vesicles was observed with a spectrophotometer as a decrease in the optical density. In the absence of pores, no swelling was seen. The liposome swelling assay is a relatively rapid technique to test for pore-forming ability of an isolated protein.

The technique was also useful for measuring the relative rates of diffusion of various solutes through the pores since the rate of diffusion is inversely proportional to the initial rate of swelling. The rate of diffusion calculated was not an absolute rate since no proportionality constant was used to describe the relationship between the two rates. The relative rates of diffusion of a variety of small sugars through the pores can be used to calculate the pore size (Nikaido and Rosenberg, 1983).

1.3 Porins

The principal porins of several species of bacteria have been identified using a combination of the four

techniques described in the previous section. This section discusses properties of porins. The porins of E. coli, S. typhimurium, and Pseudomonas aeruginosa have been studied extensively. The porins of the two enteric bacteria (E. coli and S. typhimurium) have many similarities and all three bacteria share several common characteristics.

The principal porins of all three bacteria are "major" outer membrane proteins, signifying that a large number of copies of these proteins is present in the outer membrane. In E. coli B, there is an estimated 100,000 to 500,000 copies of porin molecules per cell (Rosenbusch, 1974); in P. aeruginosa there are about 200,000 copies per cell (Angus, Carey, Caron, Kropinski, and Hancock, 1982). All porins identified thus far have a molecular weight between 35 to 44 Kdaltons. Porins are non-covalently associated with the underlying peptidoglycan layer (Lugtenberg, Bronstein, van Selm, and Peters, 1977; Hancock, Irvin, Costerton, and Carey, 1981).

Porin proteins of the enteric bacteria are refractory to solubilization by sodium dodecylsulphate (SDS) at low temperatures and in buffer of low ionic strength. To solubilize the porins of the enteric bacteria in SDS, one must either heat the preparation above 60°C or increase the ionic strength of the solution by the addition of 0.1 M NaCl. Such SDS treatment of the porins of E. coli and S. typhimurium does not denature the protein and the protein remains functional as a pore.

The porin of the pseudomonad is more readily solubilized by SDS than the porins of the enteric bacteria.

Treatment with SDS in low ionic conditions denatured the porin of P. aeruginosa and rendered it non-functional. However, the porin is highly stable when treated with SDS in high ionic conditions (Yoshimura, Zalman, and Nikaido, 1983).

The porin monomers of E. coli and S. typhimurium aggregate into oligomers to form functional pores. Frequently, trimers and hexamers of porins can be identified in SDS-polyacrylamide gels of outer membrane protein extracts from E. coli (Rosenbusch, 1974). The smallest functional oligomer in the enteric bacteria appears to be the trimer since only the reconstitution of a trimer of porins into vesicles resulted in the formation of pores (Tokunaga, Tokunaga, Okajima, and Nakae, 1979). The use of monomers and dimers did not result in the formation of pores in the vesicles but this may be due to the process by which the monomer and dimer were purified. If trimers are the smallest functional oligomer, then in E. coli B where there were about 100,000 to 500,000 copies of porins per cell, there must be about 33,000 to 160,000 transmembrane channels per cell.

The porins of P. aeruginosa do not have to aggregate into oligomers to be functional (Yoshimura et al., 1983). No multimers were observed in SDS-polyacrylamide gels of outer membrane extracts of P. aeruginosa but this may be due to the easy solubilization of the porin of this bacteria by SDS.

Porins are also receptors for colicins and bacteriophages (Braun and Hantke, 1981). Some porins were

first identified as phage receptors before their pore-forming activity was discovered. Such properties are useful in the selection of porin-deficient mutants.

1.3.1 Escherichia coli

E. coli B has only one constitutive major porin, the 36.5 Kdalton protein. The 36.5 K protein was also termed the "matrix" protein because it formed a highly organized network or lattice which was observable by electron microscopy (Rosenbusch, 1974). The formation of this matrix was attributed to the association of the porin with the underlying peptidoglycan layer and neighbouring outer membrane components. In vitro reconstitution of this lattice was dependent on the presence of peptidoglycan, lipoprotein and lipopolysaccharide. The absence of any one of the components resulted either in an absence of the lattice or in the formation of a lattice with a periodicity smaller than the 7.5 nm normally observed (Rosenbusch, 1974). The interaction of the porin with the peptidoglycan is responsible for (1) maintaining the attachment of the outer membrane to the cell; (2) preventing random diffusion of the porin within the lipid bilayer; and (3) maintaining the organization and integrity of the outer membrane. Porin-deficient mutants treated with 150 mM Tris displayed structural changes in their outer membrane and loss of viability. The same structural changes were not seen in

wild type cells that underwent the same treatment (Nogami and Mizushima, 1983).

E. coli K-12 differs from E. coli B in that it possesses two major constitutive porins: OmpF and OmpC. Both these proteins form a matrix in the outer membrane of this bacterium. A lattice with a periodicity of 7 nm was observed by electron microscopy; this lattice was smaller than that formed by the 36.5 K protein of E. coli B (Yamada and Mizushima, 1978). OmpF and OmpC proteins probably have a structural role similar to that of the 36.5 K protein.

A comparison of the amino acid sequences of OmpF and OmpC proteins demonstrated a high degree of homology (Nikaido and Wu, 1984). Furthermore, there is considerable sequence homology between the OmpF and the 36.5 K protein of E. coli B. The high degree of homology amongst these three porins strongly suggest that all three are derived from the same ancestral protein.

The exclusion limits of the pores formed by these three porins lie between 600 and 700 daltons (Nakae, 1976). The diameters of the pores of all three lie between 1.3 and 1.4 nm (Benz and Hancock, 1981).

1.3.2 Salmonella typhimurium

The three major constitutive species of porins of S. typhimurium (OmpD, OmpF and OmpC or 34K, 35K and 36K respectively) have been characterized chemically (Tokunaga,

Tokunaga, and Nakae, 1979). The amino acid compositions and trypsin digest maps showed that the three proteins share a significant degree of homology.

A comparative study of the peptide maps of the porins of S. typhimurium and E. coli K-12 indicated some limited homology between the porins of the two species (Lee and Schnaitman, 1980). The data suggested that the porins of both bacteria arose from one original porin protein. A common ancestral porin may explain why the exclusion limits and diameters of both species are similar.

E. coli K-12 and S. typhimurium have multiple species of porin molecules. The monomer composition of the trimers was shown to be homogenous by the following procedure (Ishii and Nakae, 1980): The different populations of porin trimers were separated by electrofocusing. The isolated trimers were then dissociated into monomers and they were electrophoresed on a SDS-polyacrylamide gel. For each trimer only one protein band was observed indicating that the composition of trimers was homogenous.

1.3.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa has only one major constitutive porin, protein F, which forms a large pore in the outer membrane. The molecular weight exclusion limit of the pores formed by protein F was estimated to be between 3,000 to 9,000 daltons. A diameter of 2.2 nm was measured for the

pore (Benz and Hancock, 1981; Hancock, Decad, and Nikaido, 1979).

Protein F was shown to form 100 to 300 times fewer pores per unit area than the porins of E. coli. Consequently there may be only about 300 functional pores in the outer membrane of P. aeruginosa. This estimate is considerably lower than the calculation of 30,000 functional pores for E. coli (Angus et al., 1982). Conductivity experiments also showed less pore-forming activity per unit weight of porin for P. aeruginosa compared to either E. coli or S. typhimurium (Benz and Hancock, 1981). An important consequence of this property is a lower degree of outer membrane permeability for P. aeruginosa by comparison with the enteric bacteria.

1.4 Specialized Pores

The porins discussed thus far formed pores through which a wide variety of water-soluble molecules can cross the outer membrane. The porins discussed in this section form pores that participate in the uptake of specific molecules. The selectivity may be due to (1) the chemical nature of the channel wall; (2) the configuration of the channel wall; and (3) the association with the porin of a protein that has a high affinity for specific molecules.

1.4.1 Pores Selective for Charged Molecules

The PhoE (OmpE or e) protein of *E. coli* and the P protein of *P. aeruginosa* were induced when the bacteria underwent phosphate starvation. The PhoE protein formed a pore with a diameter (1.2 nm) which is similar in size to the diameter of OmpC pores (1.3 nm) (Benz, Darveau, and Hancock, 1984). Water-soluble molecules possessing one or two negative charges diffused rapidly through the PhoE pore, whereas, such molecules diffused through OmpF and OmpC pores only at very slow rates (Korteland, Tommassen, and Lugtenberg, 1982). The PhoE protein thus has a role in the uptake of negatively charged solutes. Though the production of PhoE is derepressed by phosphate starvation, the pore has no preference for molecules that have a phosphate moiety over molecules possessing other anionic groups.

The P protein of *P. aeruginosa* formed a pore with a diameter of 0.7 nm (Hancock, Poole, and Benz, 1982). The P channel cannot accommodate saccharides because of its small size. Like PhoE, the P channel showed a preference for anions. The selectivity of the two pores is thought to be due to a net positive surface charge in the channel. The selectivity of P pores for anions was greater than that of the PhoE pore and this was thought to be due to the smaller size of the P pore which allowed for greater interaction between the solute and the channel (Benz *et al.*, 1984).

1.4.2 Pores Selective for Saccharides

The LamB protein of E. coli is produced when maltose is present and glucose is absent from the environment. The protein was first identified as the receptor for the lambda phage but its role for the cell is the specific uptake of maltose and maltodextrins (Szmelcman and Hofnung, 1975).

The LamB protein was shown to be a porin by radiolabel efflux and conductivity experiments (Boehler-Kohler, Boos, Dieterle, and Benz, 1979; Luckey and Nikaido, 1980; Nakae and Ishii, 1980). The diameter of the LamB pore was calculated to be 1.6 nm by conductivity experiments and is larger than that of the OmpF pore (1.4 nm) (Boehler-Kohler et al., 1979).

The selectivity of the pore was illustrated by the following observations. Maltose, a disaccharide, permeated through LamB pores 40 times faster than another disaccharide, sucrose (Luckey and Nikaido, 1980). The LamB pore permitted the rapid passage of maltodextrins up to six residues in length, yet stachyose, a tetrasaccharide diffused through only at a lower rate. Thus LamB formed a pore that is large enough for the passage of different sugars but is highly discriminating for maltose and maltodextrins.

Isolated LamB protein demonstrated selectivity for maltose and maltodextrins when reconstituted into vesicles (Bavoil and Nikaido, 1981). The selectivity of the pore is, therefore, attributed to the configuration of the channel rather than to the periplasmic maltose-binding protein with

which the LamB protein may be associated.

The 44K protein of S. typhimurium was also induced by the presence of maltose. The protein formed a pore which was involved in the specific uptake of maltose (Palva, 1978; Tokunaga, Tokunaga, and Nakae, 1979).

Protein D1 of P. aeruginosa was induced specifically by glucose. The protein formed a pore which had a role in the high affinity uptake of glucose. The D1 protein is associated with a periplasmic glucose-binding protein (Hancock and Carey, 1980) and may account for the specificity of the pore.

Protein K was found exclusively in pathogenic strains of E. coli K-12 (Paakkenen, Gotshlich, and Makela, 1979). Protein K was present in addition to the two major porins, OmpF and OmpC. The protein shared a high degree of amino acid homology with OmpF and like OmpF it formed pores that allowed for the rapid permeation of nutrients (Sutcliffe, Blumenthal, Walter, and Foulds, 1983; Whitfield, Hancock, and Costerton, 1983). The specific function of the pores formed by protein K is unknown. It may have a role in the transport of components of the capsule to the cell surface since the protein is found only in encapsulated strains.

1.4.3 Pores Selective for Amino Acids and Nucleosides

Mutants of E. coli K-12 that lacked both the OmpF and OmpC porins still allowed certain amino acids (serine, glycine and phenylalanine) to penetrate the outer membrane.

The ability of these amino acids to cross the outer membrane was thought to be due to the Tsx protein since mutants that lacked the OmpF, OmpC and Tsx proteins were no longer permeable to these amino acids (Heuzenroeder and Reeves, 1981). The Tsx protein also played a role in the uptake of nucleosides (Hantke, 1976). The protein is thought to function as a porin since neither binding not competition by the nucleosides for the Tsx protein was observed. Such observations would be expected if the Tsx protein were involved in facilitated diffusion. The specificity of this uptake system is thought to be due to the configuration of the walls of the channel.

1.5 Diffusion Through the Pores

The ability of a molecule to diffuse through pores is dependent on several factors. The most important factor, as demonstrated by radioefflux experiments, is the size of the molecule. Molecules that are too large will not be able to pass through the pores. As demonstrated by liposome swelling assays, the size of the molecule is also an important factor in determining the rate of diffusion through the pore. The larger the molecule is, the greater the chances of collision with the rim and wall of the pore, resulting in a slower rate of diffusion. Two molecules of different sizes may be able to diffuse through the same pore

but not necessarily at the same rate of diffusion.

Other factors that influence the ability of a molecule to diffuse through a pore and the rate of its diffusion were demonstrated by the measurement of permeability coefficients of different β-lactam antibiotics across the membrane. The permeability coefficient was calculated by comparing the kinetics of degradation of β-lactams by whole cells that possessed β-lactamases in their periplasm and by sonic extracts of these cells which contained β-lactamases (Zimmerman and Rosselet, 1977). The sizes and molecular weights of β-lactams fall within a narrow range and this emphasized the effect of properties other than size on diffusion through the pores.

β-Lactams possessed high permeability coefficients when several criteria were met. The first criterion is that the molecule be soluble in water. A small but hydrophobic molecule had a lower permeability coefficient than a hydrophilic molecule of similar size. The ability of cephalosporins to penetrate through the pores was enhanced when their hydrophilicity was increased by their conversion into 1-oxa-derivatives (Murakami and Yoshida, 1982). Strongly hydrophobic molecules were able to cross the outer membrane by diffusing through the lipid bilayer itself and thus had no need for the pores (Nikaido, 1976).

A second criterion for unhindered diffusion through these pores is that the molecules be uncharged. At neutral pH, the major pore of E. coli B favoured the permeation of cations over anions as a result of a net negative charge in the pore (Benz, Janko, and Lauger, 1979). This was

demonstrated by the chemical modification of residues that lined the channel (Benz, Tokunaga, and Nakae, 1984; Tokunaga et al., 1981). Acetylation of the amino groups changed the positively charged groups into uncharged moieties. Succinylation of the amino groups changed the positively charged groups into a negatively charged ones. In both cases, the negativity of the surface charge of the pore is augmented but no effect was seen on the rates of permeation of cations. The amidation of carboxyl groups changed the negatively charged group into a positively charged one and resulted in a decrease in the cation selectivity of the pore.

Similarly, at neutral pH, the OmpF and OmpC porins of E. coli K-12 and the F porin of P. aeruginosa formed pores with a net negative surface charge. The diffusion of cations was favoured and the diffusion of anions with one or more negative charges was retarded. (Nikaido et al., 1983; Benz and Hancock, 1981). The repulsion of anions was shown by black-lipid membrane experiments to be influenced also by the size of the molecule: small charged molecules are less affected than larger charged molecules by the surface charge of the channel (Benz et al., 1984).

The pores formed by the PhoE protein of E. coli and the P protein of P. aeruginosa, as discussed in section 1.4.1, favoured the permeation of anions due to the net positive charge lining the channel walls (Benz et al., 1984). Thus, an ionic interaction exists between the permeating solute and the pore walls that influences the penetration of solutes through the pores.

The net diffusion of molecules through the pores was affected by factors other than the physical and chemical properties of the solute and the pore walls. The number of pores present is important in determining the overall permeability of the outer membrane as was concluded from observations made on porin-deficient mutants. The larger the area over which permeation can occur, the faster and greater the amount of diffusion.

The large pore size (2.2 nm diameter) (Benz and Hancock, 1981) and the high molecular weight exclusion limit (3000-9000 daltons) (Hancock and Nikaido, 1978) measured in P. aeruginosa suggested that its outer membrane was a highly porous structure. However, this bacterium possessed a high resistance to antibiotics due to the low permeability of the outer membrane. For example, the rates of diffusion of two hydrophilic cephalosporins, cephaloridine and cephacetrile, across the outer membrane of P. aeruginosa was observed to be 100- to 500-fold less than that observed for E. coli K-12 (Yoshimura and Nikaido, 1982). The low number of functional pores present at any given time would explain how P. aeruginosa could have both a large pore-size and low permeability. The mechanism by which the low number of functional pores is maintained is not clear.

Another factor involved in maintaining the low permeability of the membrane was demonstrated by observations made on a mutant strain of P. aeruginosa called Z61. This mutant has an outer membrane that is 20 to 40 times more permeable to β-lactams than that of the wild type (Angus et al., 1982). As a result of this increased

permeability, this mutant was also sensitive to a variety of other types of antibiotics such as aminoglycosides, rifampin, tetracycline and polymyxins. Measurements of diffusion kinetics of various antibiotics across the outer membrane of this mutant indicated that the area over which molecules can diffuse increased 5 to 10 times over that of the wild type. The mutation did not affect the porin itself. The mutation was found to affect the structure of LPS (Kropinski, Kuzio, Angus, and Hancock, 1982). This observation suggested that LPS played a role in maintaining the low permeability of the outer membrane in the wild-type.

The shape of the pore is also an important factor in influencing diffusion through the channel. The molecular weight exclusion limits of the pores formed by the two principal porins of E. coli K-12 were both about 600-700 daltons and yet there were differences in the ability of various β-lactams to penetrate through the two pores (Harder, Nikaido, and Matsuhashi, 1981). E. coli K-12 is sensitive to cephaloridine, ampicillin, monobasic (eg. cephaloram) and dibasic (eg. carbenicillin) β-lactams. Mutants that lacked OmpF but overproduced OmpC were still sensitive to cephaloridine and ampicillin but were found to be resistant to monobasic and dibasic β-lactams (Harder et al., 1981). Thus, OmpF formed pores with a higher permeability for these antibiotics. The difference in permeability may be due to the slightly larger diameter of OmpF pores as demonstrated by black-lipid membrane experiments. Another possibility is that the configurations of the two pores are different despite their similar pore

sizes. The second possibility also explains the disparity found amongst the permeability of the different major pores of S. typhimurium to various antibiotics despite their identical pore sizes (Kobayashi, Takhashi, and Nakae, 1982).

These differences in permeability amongst the major pores can be employed to control the overall permeability of the outer membrane. The production of the major porins, OmpF and OmpC, of E. coli K-12 can be regulated in response to changes in the osmolarity of the environment (Pugsley, 1983; van Alphen and Lugtenberg, 1977). When the osmolarity increased, the production of OmpF was repressed and a compensating increase in the production of OmpC was observed. The overall permeability of the outer membrane was decreased by this mechanism since OmpC pores are not as permeable as OmpF pores.

In summary, the outer membrane of gram-negative bacteria is a molecular sieve. Porins form water-filled channels of a characteristic size and are the major route by which a large variety of low molecular weight solutes can penetrate across the membrane. Permeation can be affected by the hydrophobicity and charge of the solute or by the charge and configuration of the pore. These factors become important in determining which antibiotics will be effective against gram-negative bacteria.

1.6 Haemophilus influenzae type b

Our laboratory has a continuing interest in the permeability of the outer membrane of Haemophilus influenzae

type b. This bacterium is a small pleiomorphic gram-negative rod which has two nutritional cofactors for growth. These two cofactors are NAD^+ (Factor V) and hemin (Factor X), an iron-containing molecule. The encapsulated members of this species can be serotyped on the basis of their capsule into six different types: a, b, c, d, e, and f. The capsule of H. influenzae type b is made of polyribosyl ribitol phosphate (PRP) (Crisel, Baker, and Dorman, 1975).

H. influenzae type b is the major cause of bacterial meningitis in children under the age of three even though this bacterium is part of the flora of the upper respiratory tract in adults. The seriousness of an infection in infants is due to the inability of the infant to raise an immune response to the bacterium. The use of extracts of the capsule as a vaccine has proven unsuccessful since the extracts did not induce an immune response in children under the age of two (Norden, Michaels, and Melish, 1975; Peltola, Kayhty, Sivonen, and Makela, 1977). There is continuing research in this field involving the linkage of PRP to carriers such as bovine serum albumin and inactivated cells of Bordetella pertussis to improve the immunogencity of this antigen in infants (King, Wynter, Ramlal, Moodie, and Castle, 1981; Schneerson, Barrera, Sutton, and Robbins, 1980).

H. influenzae is extremely sensitive to the β-lactam antibiotic, ampicillin. An infection by H. influenzae will, therefore, be effectively treated by ampicillin. β-Lactamase positive strains of H. influenzae type b

appeared in the early 1970's and chloramphenicol has been used as the alternate drug of choice (Turk, 1982). The permeability coefficients for ampicillin and nine other β-lactams across the outer membrane of H. influenzae have been measured (Coulton, Mason, and Dorrance, 1982). These values were calculated by comparison of the rates of hydrolysis of the β-lactams by whole cells and by sonic extracts of cells. The results showed that the outer membrane of H. influenzae does not act as a barrier to the diffusion of these antibiotics. For example, benzylpenicillin has a permeability coefficient for the outer membrane of H. influenzae that was ten times greater than that for the outer membrane of E. coli. Therefore, the permeability of the outer membrane of H. influenzae is comparatively higher than that of E. coli.

The greater permeability of the outer membrane of H. influenzae type b as compared to that of E. coli may be due to: (1) the presence of pores that are larger in size; (2) the presence of a greater number of pores which are open; (3) the presence of a higher number of porin molecules. To investigate the first of these three possibilities, this thesis describes experiments which measured the molecular weight exclusion limit of the outer membrane of this bacterium using the radiolabel efflux method described in section 1.2.1. The outer membrane was found to contain porins that formed large water-filled channels through which polysaccharides can diffuse.

II. MATERIALS AND METHODS

2.1 Bacterial Strain and Culture Conditions

Lyophils of Haemophilus influenzae type b ATCC 9795 were obtained from the American Type Culture Collection. Bacteria were grown from these lyophils at 37°C in CY*, a medium composed of 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, Michigan) and 1% (w/v) casamino acids (Difco) in 0.1 M sodium phosphate buffer, pH 7.6. The medium was supplemented with 1.0 μg/ml hemin (Sigma Chemical Co., St. Louis, Missouri), 1.0 μg/ml nicotinamide adenosine dinucleotide (NAD^+) (Sigma), 5.0 g/l glucose and 0.5 mM $MgCl_2$. These supplements were prepared separately and added after the medium was autoclaved. Stock solutions of hemin (1 mg/ml) were prepared in 50% ethanol containing 0.2 N NaOH (Lascelles, 1979). Stock solutions of NAD^+ (1 mg/ml), glucose (0.5 g/ml) and $MgCl_2$ (1.0M) were prepared in distilled water and filter sterilized. All solutions were stored at 4°C.

2.2 Extraction of Outer Membrane Proteins

- Outer membrane proteins were prepared as follows

(Coulton and Wan, 1983): Cells were grown from stock culture in CY* medium to an optical density of 1.0-1.5 at 578 nm to obtain cells in the late exponential phase. The cells were harvested by centrifugation at 6,000 xg, 10 min, 4°C with a JA-14 rotor in a Beckman J2-21 centrifuge. The cell pellet was washed three times by resuspension in 10 mM sodium phosphate, pH 7.5, 10 μM phenylmethylsulfonyl fluoride (PMSF) (Sigma), and by centrifugation at 6,000 xg, 10 min, 4°C in a Beckman JA-14 rotor. The cells were resuspended in 30-40 ml of the phosphate buffer and lysed by three passages through a French pressure cell (Aminco, Silver Spring, Maryland) at a pressure of 14,000 lbs/in².

RNase (Sigma), DNase (Sigma), and $MgSO_4$ were added to the lysate to final concentrations of 20.0 μg/ml, 10.0 μg/ml and 1 mM respectively. This mixture was incubated for 45 min at 37°C after which the lysate was centrifuged at 6,000 xg, 10 min, 4°C to remove large cellular debris and unlysed cells. The supernatant was ultracentrifuged at 150,000 xg, 45 min, 4°C in a Ti60 rotor in a Beckman L5-65B centrifuge to pellet the cell envelopes. This pellet was resuspended in phosphate buffer containing 2% (v/v) Triton X-100 (Sigma), a non-ionic detergent which solubilized the cytoplasmic membrane. The suspension was ultracentrifuged at 150,000 xg, 45 min, 4°C. The pellet was re-extracted by suspension in the same phosphate-Triton X-100 buffer followed by ultracentrifugation. The pellet, designated Triton-insoluble (TI) material, contained outer membrane proteins (Coulton and Wan, 1983). The TI material was resuspended in distilled water and the Triton X-100

detergent was removed by absorption onto BioBeads SM-2 (BioRad Laboratory, Mississauga, Ont.) (Holloway, 1973). .2 ml TI material was stirred overnight at 4°C with 0.6 gm of BioBeads. The protein suspension was separated from the BioBeads with a Pasteur pipette and distributed into 25 µl aliquots. The protein concentration was estimated by the dye binding protein assay (Bradford, 1976) using the reagent from BioRad. The TI aliquots were stored at -70°C and thawed just before use in reconstitution experiments.

2.3 SDS-Polyacrylamide Gel Electrophoresis

The proteins present in the TI material were separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide slab gel. The composition of the gel was adopted from the recipe of Lugtenberg, Meijers, Peters, van der Hoek, and van Alphen (1975). The stacking gel contained 3% acrylamide, 0.08% methylene bis-acrylamide, and 0.1% sodium dodecyl sulfate. The running gel was composed of 13% acrylamide, 0.075% methylene bis-acrylamide, and 0.2% sodium dodecyl sulfate (electrophoresis grade, Bio-Rad). Protein samples to be run on the gel were solubilized in a sample buffer consisting of 0.0625 M Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 5% β-mercaptoethanol. The mixture was boiled at 100°C for 5 min and centrifuged at 22,500 xg for 15 min; the supernatant was decanted and was

applied to a SDS-polyacrylamide gel. A 0.75 mm gel was run for 2.5 h at 20 mA. The gel stained with 0.1% Coomassie brilliant blue in 10% acetic acid, 40% methanol for 30 min and destained by repeated washing with 10% acetic acid, 30% methanol. Protein standards from Pharmacia (Uppsala, Sweden) contained a mixture of phosphorylase b (Mr 94,000 daltons), bovine serum albumin (Mr 67,000 daltons), ovalbumin (Mr 43,000 daltons), carbonic anhydrase (Mr 30,000 daltons), soybean trypsin inhibitor (Mr 20,100 daltons) and α-lactalbumin (Mr 14,400 daltons).

2.4 Extraction of Phospholipids

The procedure used to extract phospholipid from H. influenzae type b was that outlined by Kates (1972). Cells were grown and washed in phosphate buffer. The cell pellet was resuspended in distilled water. Methanol and chloroform were added to this aqueous suspension to obtain a final ratio of 2:1:0.8 (v/v) of methanol:chloroform:water. The mixture was allowed to stand at room temperature for 3 h with intermittent shaking. The suspension was then centrifuged at 2,800 xg, 15 min, 4°C with a SS-34 rotor in a Sorvall RC-5B centrifuge to remove insoluble material. The supernatant was decanted and the pellet discarded. A 1:1 (v/v) solution of chloroform and distilled water was added to the supernatant and this mixture was poured into a

separatory funnel. The mixture was allowed to stand overnight at room temperature; the two phases partitioned. The bottom chloroform layer was removed and the volume reduced to 3-5 ml by rotary evaporation. The chloroform suspension was a total lipids extract of the bacterium.

Neutral lipids were removed by chromatography of the chloroform extract on a silicic acid column (Sweeley, 1969). The silicic acid was equilibrated in chloroform and poured into a 25 ml burette. The total lipid extract was layered onto this column and 5 column volumes of chloroform was passed through to elute neutral lipids from the column. The polar lipids (phospholipids) were removed by elution with five columm volumes of methanol and were collected. Methanol was removed by rotary evaporation and the lipid film was resuspended in chloroform. This preparation was the phospholipid extract used in the reconstitution experiments.

2.4.1 Ashing Assay for Phospholipids

Phospholipid concentration was estimated by the ashing technique of Ames and Dubin (1960). 30 µl of magnesium nitrate in 95% ethanol was added to each sample. The samples were dried and ashed by repeatedly passing the tubes through the flame of a bunsen burner. After adding 300 µl 0.5 N HCl the samples were capped and incubated in a boiling water-bath for 15 min. The tubes were cooled and 700 µl of a solution consisting of 1 part 10% ascorbic acid and 6

parts 0.42 ammonium molybdate•$4H_2O$ in 1 N H_2SO_4 was added to each sample. The tubes were incubated for 20 min at 45°C and the absorbance of the sample was measured at 820 nm. A standard curve was constructed using inorganic phosphate, KH_2PO_4.

2.5 Extraction and Purification of Lipopolysaccharide

The procedure of Johnson and Perry (1976) was used for the extraction and purification of lipopolysaccharide (LPS). Cells were grown, harvested and washed as described in section 2.2. The cell pellet was resuspended in buffer containing 50 mM sodium phosphate, pH 7.0, 5 mM EDTA and 0.05% (w/v) sodium azide. 0.1 gm lysozyme (Sigma) was added for every 50 ml of cells and the suspension was incubated with no agitation at 37°C for 90 min. RNase, DNase and $MgCl_2$ were then added to final concentrations of 1 μg/ml, 1 μg/ml and 1 mM respectively and incubated at 45°C for 1 h.

An equal volume of 90% phenol (w/v) preheated to 70°C was added to the cell suspension as it was stirred. The temperature of the mixture was maintained at 70°C and after 20 min, the suspension was cooled rapidly in an ice bath to 15°C. This mixture was centrifuged at 13,200 xg, 15 min, 15°C to separate an upper aqueous layer from a lower phenol layer; a pellet of cellular debris remained at the bottom of the centrifuge bottle. The aqueous layer was removed, the

phenol layer discarded and the pellet resuspended in buffer and re-extracted with an equal volume of hot phenol as described above. The aqueous layers of both extractions were pooled and dialyzed against water to remove phenol dissolved in the aqueous layer. The dialysate was filtered through a Whatman 2 filter-paper and then centrifuged at 2,000 xg, 15 min, 4°C to remove all particulate matter. The pellet was discarded and the supernatant was ultracentrifuged at 175,000 xg, 45 min, 4°C in a T160 rotor in a Beckman L5-65B centrifuge. The supernatant was discarded and the pellet was the crude LPS.

The crude LPS was purified by aqueous extractions to remove remaining sugars and other water-soluble molecules. The pellet of crude LPS was resuspended in double distilled water and ultracentrifuged at 175,000 xg, 45 min, 4°C. The supernatant was discarded and the pellet was resuspended in distilled water and ultracentrifuged. The LPS was washed a minimum of three times. Purified LPS was resuspended in distilled water, freeze-dried and stored in a desiccator at room temperature.

2.5.1 Carbocyanine Dye Assay

Purity of the LPS was demonstrated by the carbocyanine dye assay (Janda and Work, 1971). The carbocyanine dye reagent was prepared as follows: 5 mg 1-ethyl-2-(3-(1-ethylnaphtho(1,2-d)-thiazolin-2-ylidene)-2-methyl-

propenyl)naphtho(1,2-d)thiazolium bromide (carbocyanine dye) (Sigma) was dissolved in 10.0 ml solution of dioxane and 0.03 M sodium acetate, pH 4.0, mixed in a ratio of 1:1 (v/v). To this was added 40 ml 0.03 M sodium acetate, pH 4.0.

To assay for purity of LPS, a small aliquot (20 μl) of the LPS sample was suspended in 1.0 ml of distilled water. 400 μl 0.03 M sodium acetate, pH 7.0, and 600 μl of the carbocyanine dye reagent were added and vortexed. The absorbance of the sample was read by a Bausch and Lomb 2000 spectrophotometer by scanning between the wavelengths of 400-700 nm. The presence of LPS was indicated by a symmetrical peak at 460 nm. The presence of nucleic acids and polysaccharides contributed to the appearance of other peaks between 540 and 600 nm.

2.6 Radiolabels and Fractionation of ^{3}H-Saccharides

Radiolabels used were (^{3}H)-dextran (New England Nuclear Canada, Laval, Quebec; specific activity: 175.4 mCi/g), (^{3}H)-inulin (NEN, spec. act.: 138.8 mCi/g), (^{14}C)-sucrose (NEN, spec. act.: 10.5 mCi/g), (^{3}H)-stachyose and (^{3}H)-raffinose.

High molecular weight (^{3}H)-saccharides of defined size classes were obtained by fractionation of the (^{3}H)-dextran and (^{3}H)-inulin by chromatography on a BioGel P-10 column (1

x 48 cm) (BioRad) (Hancock et al., 1979). The column buffer and elution buffer were 0.5 M ammonium bicarbonate.

The column was calibrated with (^{14}C)-sucrose (Mr 342 daltons, NEN), raffinose (Mr 550 daltons, Sigma), stachyose (Mr 666 daltons, Sigma), 1.3 K dextran (Mr 1.3 Kdaltons, provided by Dr. B.E. Holbein), and Dextran T-10 (Mr 10 Kdaltons, Pharmacia). The unlabelled sugars were detected by the phenol-sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, and Smith, 1956).

The crude (^{3}H)-dextran was chromatographed on the column and 100 x 1 ml fractions were collected (Microfraction Collector, Gilson Medical Electronics, Inc., Middleton, Wisconsin). Three to five fractions with an average Kav value corresponding to a desired molecular weight were pooled and the volume reduced to 1 ml by rotary evaporation. This sample was rechromatographed on the column. Three fractions taken from the peak were pooled and the volume reduced to 1 ml by rotary evaporation. The radioactivity of the samples was measured and the sugar was stored at -20°C. (^{3}H)-Dextrans with average molecular weights of 1,500, 2,500, 3,800, 6,600 and 9,400 daltons were all isolated in this manner. (^{3}H)-Inulin with an average molecular weight of 1,400 daltons was also isolated by this method.

Radiolabelled raffinose and stachyose were prepared by the procedure outlined by Decad and Nikaido (1976). 50 mg of the unlabelled sugar was incubated with galactose oxidase (Pharmacia) and 0.07 ml toluene overnight at 37°C. This reaction was stopped by adjusting the pH to 10 with NaOH.

2.25 mg potassium boro-(^{3}H)-hydride (NEN) was added to this mixture and incubated for 6 h at room temperature. 20 mg of unlabelled potassium borohydride (Fisher) was added to the reaction mixture and incubated for 3 h. 10 ml glacial acetic acid was added to stop the reaction. This mixture was chromatographed on a BioGel P-2 (BioRad) column to separate the radiolabelled sugar from degradation products of the reaction. The chromatography was conducted at 65°C by using a water-jacketted column. The elevated temperature for chromatography facilitated the separation of the sugar from the degradation products (Decad and Nikaido, 1976). Solutes were eluted with distilled water preheated to 65°C. Radiolabelled and unlabelled sugars both showed the same Kav on the Biogel P-2 column.

2.7 Reconstitution of Outer Membrane Vesicles of Haemophilus influenzae type b

The protocol for reconstitution of membrane components was modified from Hancock and Nikaido (1978). Liposomes contained LPS and phospholipids and were formed as follows: 0.5 μmoles of phospholipids in chloroform were dried to a film in a borosilicate test tube using a stream of N_2. The tubes were placed in an evacuated desiccator for 30 min at room temperature. 0.7 mg LPS in aqueous suspension was added to the phopholipid film. For each experiment, control liposomes were always prepared. Vesicles contained LPS,

phospholipids and TI material were prepared as follows: 0.5 μmoles of phospholipids in chloroform was dried to a film in a borosilicate test tube using a stream of N_2. The tubes were placed in an evacuated desiccator for 30 min at room temperature. 10 μg TI material and 0.7 mg LPS in aqueous suspension were added to the phospholipid film. 100 mM NaCl, 1 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), pH 7.2, buffer was added to each tube to obtain a final volume of 200 μl. The mixture was sonicated for 1 min in a water-bath sonicator (DiSONtegrator, Ultrasonic Industries, Inc., Hicksville, N.Y.).

Each suspension was dried to a fine film at 45°C with a stream of N_2. The tubes were then placed in an evacuated desiccator for 30 min at room temperature. The film was resuspended in 200 μl of buffer containing $5x10^5$ cpm of a (^3H)-saccharide and $5x10^5$ cpm of (^{14}C)-sucrose. The mixture was sonicated for 1 min in a water-bath sonicator. The suspension was incubated in a 45°C water-bath for 30 min and allowed to cool to room temperature over 2 h.

The reconstitution mixture was chromatographed on a Sepharose-4B column (1 x 28 cm) and eluted with the same buffer used in the reconstitution. 40 x 1 ml fractions were collected (Microfraction collector, Gilson). The radioactivity in each of the fractions was measured by a Beckman LS8000 liquid scintillation counter. The optical density of each of the fractions was measured at a wavelength of 280 nm to detect the vesicles.

2.8 Negative Staining of Vesicles for Electron Microscopy

Copper grids (300 mesh) (J.B. EM Services Inc., Pointe Claire, Quebec) were precoated with parlodion and carbon (J.B. EM Services). An aliqout (3 μl) of 10 mg/ml bovine serum albumin (BSA) (Sigma) was placed on the grid. After 1 min the BSA drop was removed and a sample of vesicles was applied. This was kept for 10 min in a humid environment after which the drop was removed and the grid was dried. The grid was rinsed with distilled water and dried by blotting with a filter paper. A drop of 1% phosphotungstic acid (PTA) (J.B. EM Services Inc.) was applied onto the grid with a flame-sterilized inoculation loop. The grid was dried and viewed with a Philips 300 electron microscope.

III. RESULTS

3.1 SDS-Polyacrylamide Gel Electrophoresis of Triton-Insoluble (TI) Material

An extract of the outer membrane proteins of H. influenzae type b was required for reconstitution into vesicles. The outer membrane fraction of H. influenzae type b was purified by the use of the non-ionic detergent, Triton X-100. The detergent preferentially solubilized the cytoplasmic membrane, leaving the outer membrane unsolubilized. The protein composition of the TI extracts of the cell envelope of H. influenzae was detected by the use of SDS-PAGE (Fig. 1). The protein profile of the TI material is identical to that observed by Coulton and Wan (1983). A yield of 50 mg of TI material was obtained from 10 g wet weight of cells.

3.2 Preparation of LPS and the Criteria for Purity

The purity of the LPS was monitored by the carbocyanine dye assay. The carbocyanine dye alone shows a peak absorbance at 510 nm (Fig. 2). When the dye binds to nucleic acids and polysaccharides, the absorbance shifts to

a higher wavelength. Nucleic acids absorb in the 540 to 580 nm range while polysaccharides absorb in the 600 to 660 nm range. Only when the dye binds to LPS does the absorbance shift downwards to a peak absorbance at a wavelength of 460 nm. LPS is considered pure when no absorbance above 510 nm is recorded, leaving a single symmetrical peak at 460 nm.

The purified LPS was freeze-dried from distilled water. A yield of 420 mg of LPS was obtained from 147 gm wet weight of cells. As shown in Fig. 2, the water-washed LPS which was prepared and used for reconstitution experiments showed a single symmetrical peak at 460 nm.

3.3 Chromatography of Dextran and Inulin Radiolabels

Dextrans of defined size classes were needed to measure the molecular weight exclusion limit of the outer membrane of H. influenzae type b. To isolate dextrans of defined size classes, the radiolabelled dextrans provided by New England Nuclear were fractionated by gel-filtration chromatography. The first chromatography of (^{3}H)-dextrans revealed a heterogenous mixture of dextrans with a wide range of molecular weights (Fig. 3). Three to five fractions with an average Kav value corresponding to a particular molecular weight were pooled and rechromatographed on the same column. For each pooled fraction, a sharp symmetrical peak with an identical Kav value was obtained (Fig. 3). Three fractions taken from the

peak of the second chromatography were pooled and the volume was reduced to 1 ml by rotary evaporation. These samples were dextrans of a defined size class.

The initial chromatography of (^{3}H)-inulin revealed a single but broad peak (Fig. 4). Fractions were pooled and the volume reduced to 1 ml by rotary evaporation. This was rechromatographed and a single symmetrical peak was observed. The peak fractions were pooled and the volume reduced to 1 ml by rotary evaporation. The preparation obtained was a defined size class of radiolabelled inulin with a molecular weight of 1.4 Kdaltons.

3.4 Reconstitution of Outer Membrane Vesicles of Haemophilus influenzae type b

Optimum conditions were determined for the reconstitution system. The use of Mg^{2+} was initially considered necessary for the insertion of the proteins into the membrane of the vesicle (Nakae, 1975). We observed that vesicles formed in the presence of Mg^{2+} retained less radiolabel than vesicles formed in the absence of Mg^{2+}. The use of Mg^{2+} resulted either in the formation of leaky vesicles or interfered with the formation of vesicles. Mg^{2+} was thus eliminated from the reconstitution system. This effect of Mg^{2+} was observed also by Nikaido (1983).

The use of high amounts of TI material interfered with formation of vesicles. Initially 200 μg of protein was used

as described in the literature by other laboratories (Nakae, 1976a, b; Hancock et al., 1979). This amount was compatible with vesicle formation in E. coli, S. typhimurium and P. aeruginosa. After testing varying amounts of protein, 10 μg of protein was found to be compatible with reconstitution of outer membrane vesicles of H. influenzae.

Uncharged polysaccharides were used to eliminate the effects of charge and hydrophobicity on the diffusion of molecules through the pore. The presence of such factors would affect the estimate of the exclusion limit. Any charge that might be present on the dextrans would be masked by the 0.1 M NaCl present in the reconstitution buffer and column buffer.

(^{14}C)-Sucrose, a low molecular weight saccharide, was used to test for the leakiness of the vesicles. Reference to retention by the control liposomes was very important. An equal retention of (^{14}C)-sucrose and (^{3}H)-saccharide was expected for control liposomes since liposomes are impermeable to solutes. If the retention of sucrose was low in the liposomes, the experiment was discontinued.

3.5 Chromatography of the Reconstitution Mixture

The separation of vesicles from the free labels was accomplished by chromatography on a Sepharose-4B column (Fig. 5). This procedure also allowed for the efflux of any labels from the liposomes and vesicles. Vesicles were not

retarded by the column matrix and were eluted in the void volume (fraction 9). Free (^{3}H)-saccharides and (^{14}C)-sucrose labels were retarded and eluted from the column in later fractions (fractions 17 to 35). The presence of vesicles was detected by reading the absorbance of the fractions at 280 nm. A small secondary 280 nm peak that eluted from the column (fractions 20 to 25) was sometimes observed and may have been due to vesicles which were small enough to be retarded by the column.

A sample of the vesicles that eluted from the column was observed by electron microscopy. Round and closed structures with average diameters of 0.06 μm were seen (Fig. 6).

3.6 The Determination of the Molecular Weight Exclusion Limit of the Outer Membrane of Haemophilus influenzae type b

Radiolabel efflux experiments were performed for each of the different sugars: raffinose (Mr 550), stachyose (Mr 666), inulin (Mr 1,400), and dextrans (Mr 1,500, 2,500, 3,800, 6,600, 9,400). For each experiment a percent retention of the sugar was calculated using the following equation:

$$\text{percent retention in vesicles} = \frac{\text{CPM retained in vesicles}}{\text{total CPM used}} \times 100. \qquad (1)$$

This was done separately for (^{14}C)-sucrose and for the

(^{3}H)-saccharide. The "total CPM" was the sum of CPM associated with the liposomes or vesicles plus CPM of untrapped labels that eluted from the column (Fig. 5, second peak). As an example for control liposomes, given:

CPM in LIPOSOMES:

(^{3}H)-saccharide = A

(^{14}C)-sucrose = B

TOTAL CPM ADDED:

(^{3}H)-saccharide = C

(^{14}C)-sucrose = D

The percent retentions of the radiolabels by liposomes are:

$$\text{percent retention of } (^3\text{H})\text{-saccharide in liposomes} = \frac{A}{C} \times 100 = E$$

$$\text{percent retention of } (^{14}\text{C})\text{-sucrose in liposomes} = \frac{B}{D} \times 100 = F$$

Similarly, for the same (^{3}H)-saccharide we can calculate the percent retention by vesicles given:

TOTAL CPM in VESICLES:

(^{3}H)-saccharide = M

(^{14}C)-sucrose = N

TOTAL CPM ADDED

(^{3}H)-saccharide = O

(^{14}C)-sucrose = P

The percent retentions of the radiolabels by vesicles are:

$$\text{percent retention of } (^3\text{H})\text{-saccharide in vesicles} = \frac{M}{O} \times 100 = R$$

$$\text{percent retention of } (^{14}C)\text{-sucrose in vesicles} = \frac{N}{P} \times 100 = S$$

The retention of the sugar by vesicles was compared to the retention of the sugar by the control liposomes to give a relative percent retention of the sugar. This was accomplished by the following equation:

$$\text{percent retention of sugar} = \frac{\text{retention by vesicles}}{\text{retention by liposomes}} \times 100. \qquad (2)$$

Continuing the example started above, the relative percent retentions are:

$$\text{relative percent retention of the } (^{3}H)\text{-saccharide} = \frac{E}{R} \times 100 = X$$

$$\text{relative percent retention of } (^{14}C)\text{-sucrose} = \frac{F}{S} \times 100 = Y$$

In a "typical" experiment using a high molecular weight saccharide such as 9,400 dalton (^{3}H)-dextran, the following results for retention by liposomes were obtained:

CPM in LIPOSOMES:

(^{3}H)-dextran = 2,200

(^{14}C)-sucrose = 2,500

TOTAL CPM ADDED:

(^{3}H)-dextran = 200,000

(^{14}C)-sucrose = 200,000

The percent retention of the radiolabels by liposomes was:

$$\text{percent retention of } (^3H)\text{-dextran in liposomes} = \frac{2{,}200}{200{,}000} \times 100 = 1.1\%$$

$$\text{percent retention of } (^{14}C)\text{-sucrose in liposomes} = \frac{2{,}500}{200{,}000} \times 100 = 1.25\%$$

For retention of the same saccharide in vesicles, the following data were obtained:

CPM in VESICLES:

(^3H)-dextran = 2,000

(^{14}C)-sucrose = 400

TOTAL CPM ADDED

(^3H)-dextran = 200,000

(^{14}C)-sucrose = 200,000

The percent retention of the radiolabels by the vesicles would be:

$$\text{percent retention of } (^3H)\text{-dextran in vesicles} = \frac{2{,}000}{200{,}000} \times 100 = 1.0\%$$

$$\text{percent retention of } (^{14}C)\text{-sucrose in vesicles} = \frac{400}{200{,}000} \times 100 = 0.2\%$$

The relative percent retentions of the radiolabels in the vesicles would be:

$$\text{relative percent retention of the } (^3H)\text{-dextran} = \frac{1.0}{1.1} \times 100 = 91\%$$

$$\text{relative percent retention of the } (^{14}C)\text{-sucrose} = \frac{0.2}{1.25} \times 100 = 16\%$$

The relative percent retentions of each radiolabelled sugar used in reconstitution experiments were calculated in this manner.

The relative percent retentions of sucrose from 42

experiments, of raffinose from 6 experiments, and of stachyose from 9 experiments were averaged (Table 1) and plotted (Fig. 7). We observed that the percent retentions of sucrose (29%), of raffinose (22%), and of stachyose (29%) were low. These three solutes were therefore capable of passing through the pores.

The relative percent retentions of 1,500 dalton dextran from 6 experiments were averaged (Table 1) and plotted (Fig. 7). The retention of the dextran of Mr 1,500 was significantly higher (82%) than those of sucrose, raffinose and stachyose. Similarly, the dextrans of Mr 2,500, 3,800, 6,600 and 9,400 were retained by the complete reconstitution mixture containing LPS, phospholipids and TI material.

The relative percent retentions of 1,400 dalton inulin from 3 experiments were averaged (Table 1) and plotted (Fig. 7). The retention of inulin (62%) showed a value intermediate to that of the high molecular weight dextrans and the low molecular weight saccharides. Inulin of Mr 1,400 was therefore capable of diffusing through the pore but with difficulty. From this data the exclusion limit of the outer membrane of H. influenzae type b was estimated to be about 1,400 daltons.

IV. DISCUSSION

Several reports in the literature have indicated that the unusual permeability of the outer membrane of H. influenzae type b may be related to the susceptibility of the bacteria to ampicillin. To explore this possibility, we attempted to characterize the outer membrane of H. influenzae type b by determining its molecular weight exclusion limit to water-soluble solutes. The radiolabel efflux method was used to measure the exclusion limit in vesicles reconstituted from outer membrane proteins, phospholipids and lipopolysaccharides extracted from the bacterium.

The molecular weight exclusion limit of the outer membrane of H. influenzae type b was determined to be 1.4 Kdaltons. The accuracy of the measurement of the exclusion limit depended upon: (1) the formation of control liposomes that were not permeable to saccharides; (2) the formation of vesicles with outer membrane proteins reconstituted in them; (3) the specificity of the defined size classes of saccharides tested.

Control liposomes formed in the presence of Mg^{2+} had a lower percentage retention of radiolabelled sugars than control liposomes formed in the absence of Mg^{2+}. This suggested that Mg^{2+} caused the vesicles to be leaky. The interaction of Mg^{2+} with the LPS was thought to interfere

with the insertion of LPS into the phospholipid bilayer (Nikaido, 1983).

The use of (^{14}C)-sucrose permitted us to determine whether the liposomes formed were leaky: sucrose should be readily trapped by the liposomes. To account for any leakiness of the vesicles, the percent retention of radiolabels by porin-containing vesicles was always compared with that of the control liposomes (equation 2). For confirmation of the formation of vesicles, electron micrographs of samples taken from the void volume of a chromatography were made (Fig. 6). The micrographs demonstrated the presence of vesicles which were apparently closed.

The composition of the different size classes of ^{3}H-dextrans and ^{3}H-inulins was greatly improved by a second chromatography of the different fractions as can be seen in Figures 3 and 4.

The baseline retention (22-29 %) of low molecular weight sugars can be explained by the presence of vesicles which have no reconstituted porins. Such vesicles might be impermeable to sugars and would account for the background retention of the low molecular weight sugars. Another possible explanation is that the orientation of the porins in the bilayer is an important determinant of function. The reconstitution of porins into the membrane is a random event and sufficient numbers of porins have to be reconstituted in the correct orientation for their exclusion limit to be measured. The porin needs to be oriented in such a way as to act as a channel that leads from one side of the vesicle

membrane to the other.

The exclusion limit of 1,400 daltons may explain the higher permeability of the outer membrane of H. influenzae by comparison with that of E. coli. Since the outer membrane of P. aeruginosa is known to be less permeable than that of E. coli, by extension, we can conclude also that the outer membrane of H. influenzae is more permeable than that of P. aeruginosa. However, the exclusion limit of P. aeruginosa (3,000-9,000 daltons) is considerably larger than that measured for H. influenzae. The lower permeability of the outer membrane of P. aeruginosa may be attributed to the low number of functional pores. This observation suggests that the outer membrane of H. influenzae, unlike that of P. aeruginosa, lacks a mechanism by which the pore-forming ability of its porins is controlled.

The conditions under which the exclusion limit of the outer membrane of H. influenzae type b was measured were artificial. For example, Mg^{2+} was omitted from the experimental protocol despite the fact that Mg^{2+} is a cation found in the outer membrane. The proteins used were subjected to rigorous treatment: reconstitution into vesicles by sonication. The exclusion limit that was measured may have been influenced by the artificial and rigorous conditions to which the outer membrane proteins were subjected.

We propose that the measurement of the exclusion limit reflects the physiological conditions for the bacterium for the following reason. The major porin of H. influenzae has been recently identified as the 40K protein. This protein

was extracted by the use of a zwitterionic detergent, cetyl trimethyl ammonium bromide, and reconstituted into vesicles. The exclusion limit measured for the 40K protein was also about 1400 daltons (Vachon, Lyew and Coulton, 1985). Other outer membrane proteins of H. influenzae that were tested did not demonstrate pore-forming activity. Thus the experimetnal model allowed us to obtain a reasonable estimate of the exclusion limit of the outer membrane of H. influenzae.

The radius of the pore can be approximated from the molecular weight exclusion limit since a relationship exists between the radius of a molecule (r) and its molecular weight (Mr). The molecular weight is proportional to the to the cube of the radius of the molecule and the following equation has been proposed (Yoshimura et al., 1983):

$$\frac{(Mr1)}{(Mr2)} = \frac{(r1)^3}{(r2)^3}$$

Using the known exclusion limit (Mr1 = 600 daltons) and pore size (r1 = 0.7 nm radius) of E. coli and our measurement of the exclusion limit (Mr2 = 1400 daltons) of H. influenzae, the radius of the pore of H. influenzae was estimated as being about 0.9 nm. This calculation was based on the assumption that the molecules used to measure the exclusion limit were spherical in shape. Since the molecules used in radioefflux experiments were only very roughly spherical, the radius obtained in these calculations is an approximation. The use of the 40K porin in black-lipid membrane experiments described in the introduction is an area of continuing research and should give us a more

accurate measurement of the pore size.

It will be interesting to determine if there are outer membrane proteins involved specifically in the uptake of the nutritional requirement of H. influenzae and whether or not these proteins are also porins. An outer membrane protein of molecular weight of 43 Kdaltons was induced when the cells were starved for hemin but the role of this protein in hemin uptake has yet to be elucidated (Coulton and Pang, 1983). The pores formed by the principal porin are, however, sufficiently large for hemin (Mr 652 daltons) and NAD^+ (Mr 663 daltons) to penetrate into the periplasm without a requirement for facilitated diffusion. Hemin is a hydrophobic molecule and NAD^+ is a charged molecule; such properties might have an adverse effect on their diffusion through the pores of H. influenzae. A receptor or specific porin might, therefore, be needed when hemin and NAD^+ are present in limited amounts in the environment.

The overall picture which emerges of the outer membrane of H. influenzae type b is that it is a relatively porous structure by comparison with E. coli, S. typhimurium and P. aeruginosa. The high permeability coefficients for ampicillin and various β-lactam antibiotics which were calculated by Coulton et al. (1983) for this bacterium, attest to this permeability. One principal porin, the 40K protein, has already been identified and it appears to be the only porin in H. influenzae type b (Vachon, Lyew, and Coulton, 1985). This does not preclude the possibility of secondary porins which may be induced by the presence or absence of specific substrates in the medium.

The studies reported in this thesis have contributed towards a better understanding of the properties of the permeability of the outer membrane of H. influenzae type b. This information may be valuable in predicting the capacity of various solutes to cross this membrane. Such knowledge will ultimately be useful in the design of antibiotics that can cross more efficiently the outer membrane of H. influenzae type b.

V. FIGURES and TABLES

Figure 1. Sodium dodecylsulphate-polyacrylamide gel electrophoresis of TI material extracted from the cell envelope of Haemophilus influenzae type b. The molecular weights listed on the margin indicate the position of protein standards used in the electrophoresis. The standards used were phosphorylase b (Mr 94,000 daltons), bovine serum albumin (Mr 67,000 daltons), ovalbumin (Mr 43,000 daltons), carbonic anhydrase (Mr 30,000 daltons), soybean trypsin inhibitor (Mr 20,100 daltons), and α-lactalbumin (Mr 14,400 daltons). The pattern of proteins of the TI material was identical to that described by Coulton and Wan (1983): 6 major proteins were stained. Their Mr were: 16,000, 27,000, 30,000, 36,000, 40,000, and 46,000.

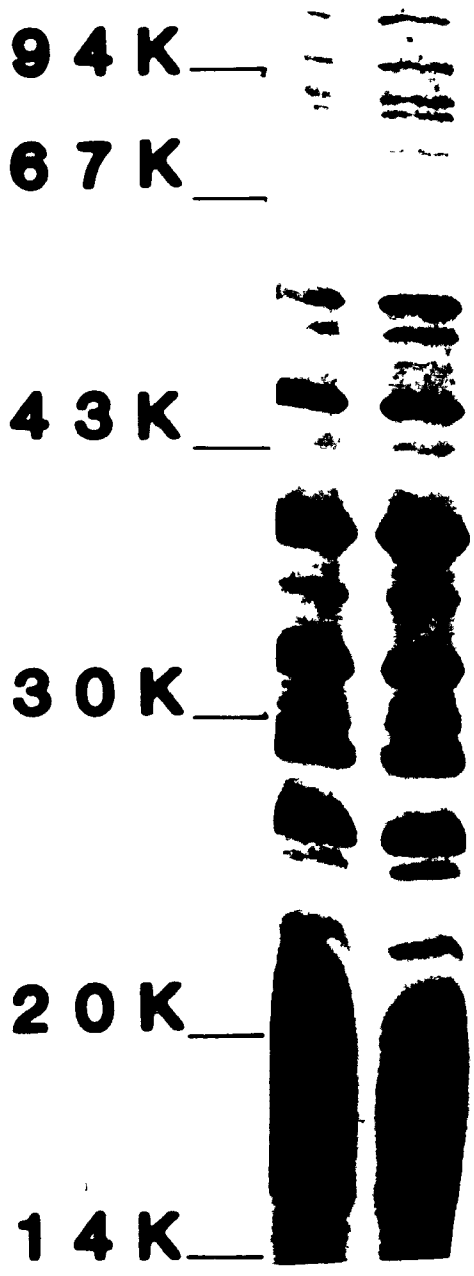

94K
67K
43K
30K
20K
14K

Figure 2. Spectrophotometric scan of the carbocyanine dye assay for LPS. The assay mixture containing LPS was scanned from 400 to 700 nm to monitor the purity of the LPS sample. The solid line (——) is a scan of a sample of crude LPS; the broken line (- - - -) is a scan of a sample of LPS after purification by three aqueous extractions.

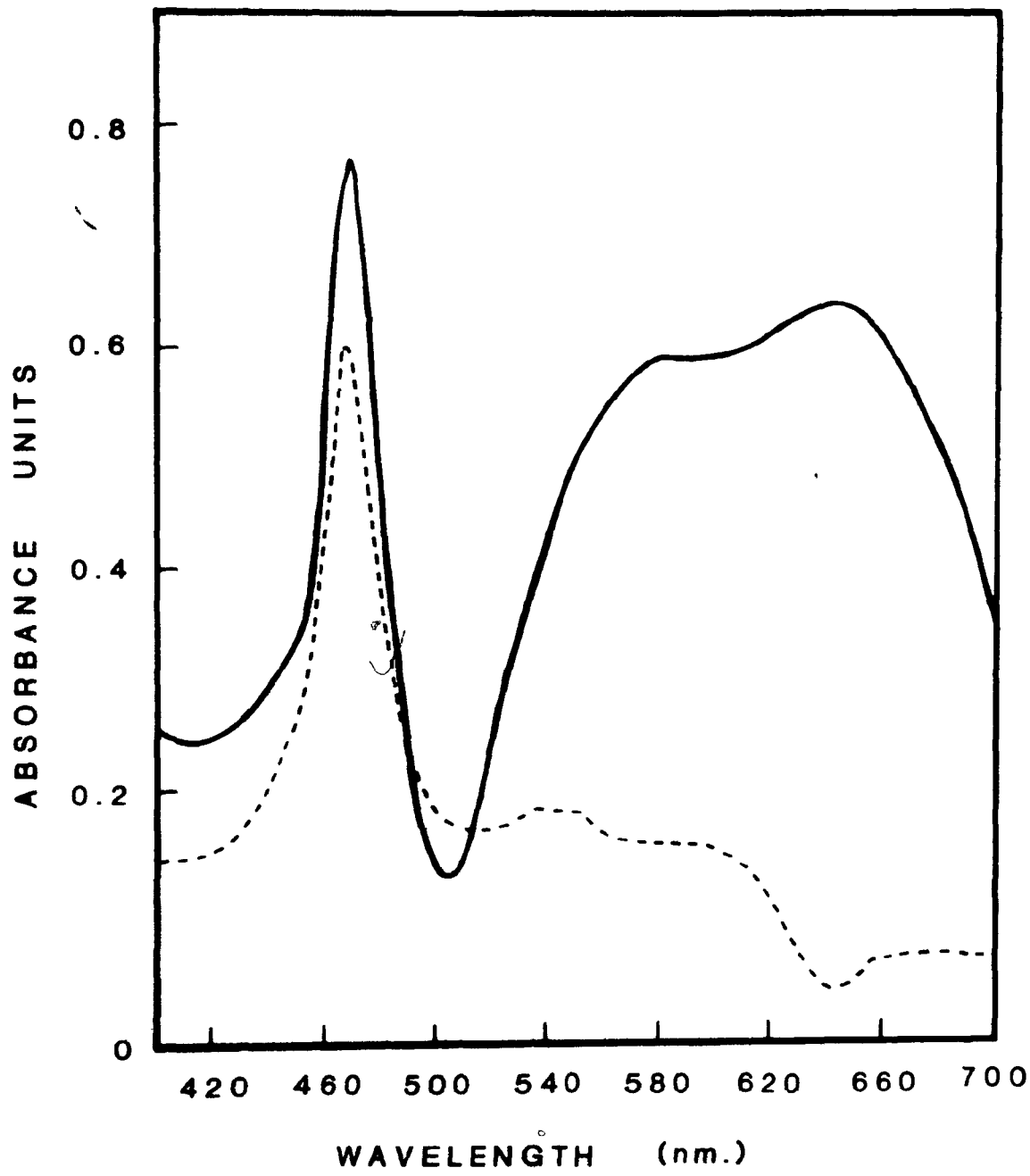

0.8
0.6
0.4
0.2
0
ABSORBANCE UNITS
420
460
500
540
580
620
660
700
WAVELENGTH (nm.)

Figure 3. Chromatography of (^{3}H)-dextran. (^{3}H)-dextran was chromatographed on a Biogel P-10 column (1 x 48 cm) to obtain populations of (^{3}H)-dextrans with different average molecular weights. 1 ml fractions were collected. The void volume of the column was 35 ml as determined with dextran blue. The solid line (——) was the first chromatography of (^{3}H)-dextran obtained from New England Nuclear. The bars (⊢——⊣) indicate the fractions of this chromatography which were pooled and rechromatographed to obtain populations of (^{3}H)-dextrans with average molecular weights of 9,400 (A), 6,600 (B), 3,800 (C), 2,500 (D), and 1,500 (E) daltons. The elution profile of the second chromatography are indicated by the broken lines (----).

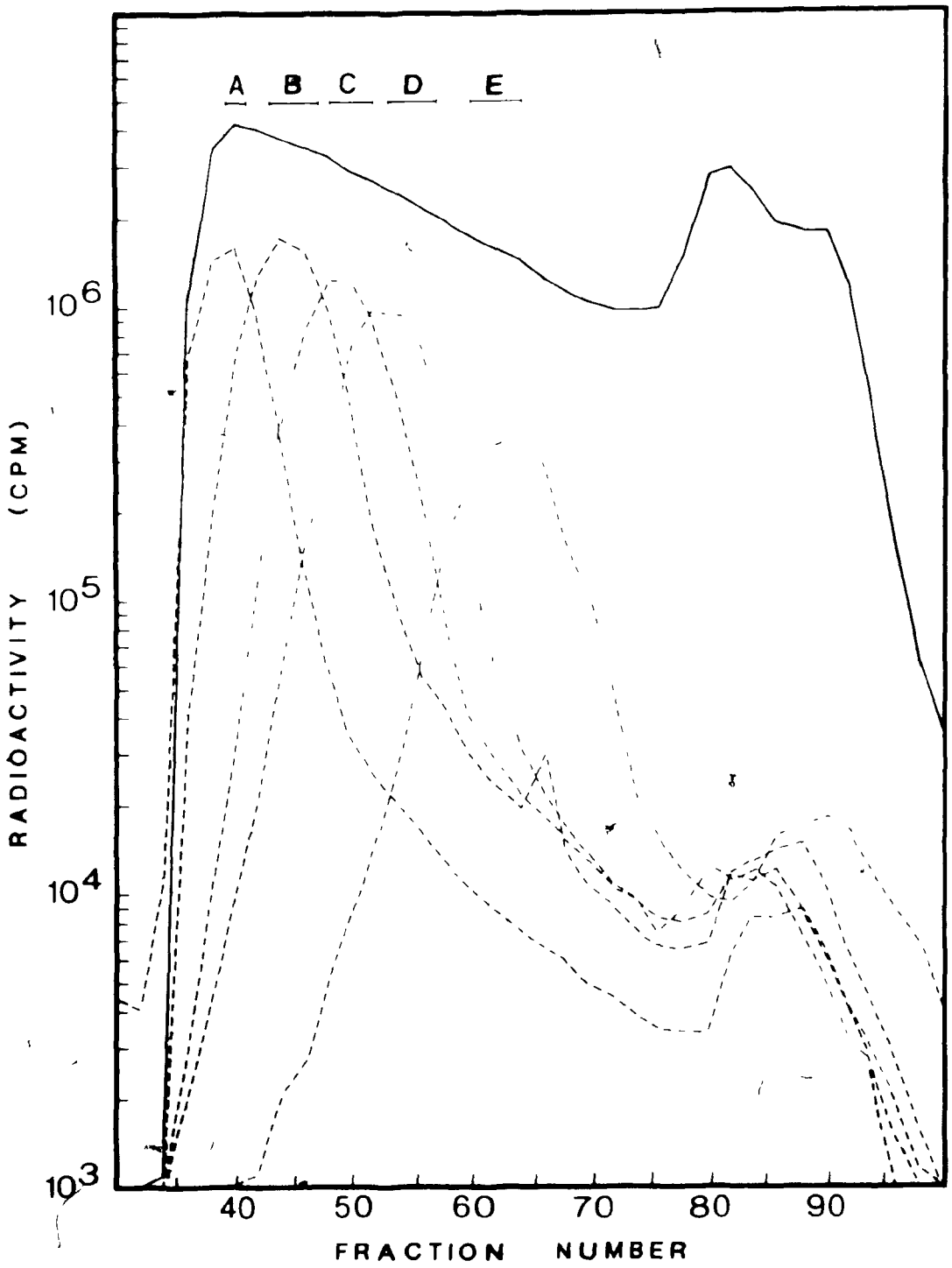

A
B
C
D
E
10^6
10^5
10^4
10^3
RADIOACTIVITY (CPM)
40
50
60
70
80
90
FRACTION NUMBER

Figure 4. Chromatography of (^{3}H)-inulin. (^{3}H)-inulin was chromatographed on a Biogel P-10 column (1 x 48 cm). 1 ml fractions were collected. The void volume was 35 ml as determined with dextran blue. The solid line (——) is the initial chromatography of (^{3}H)-inulin obtained from New England Nuclear. The fractions from this first chromatographic step are indicated by the bar (⟝——⟞). They were pooled and rechromatographed. The result of the second chromatography is indicated by the broken line (- - - -).

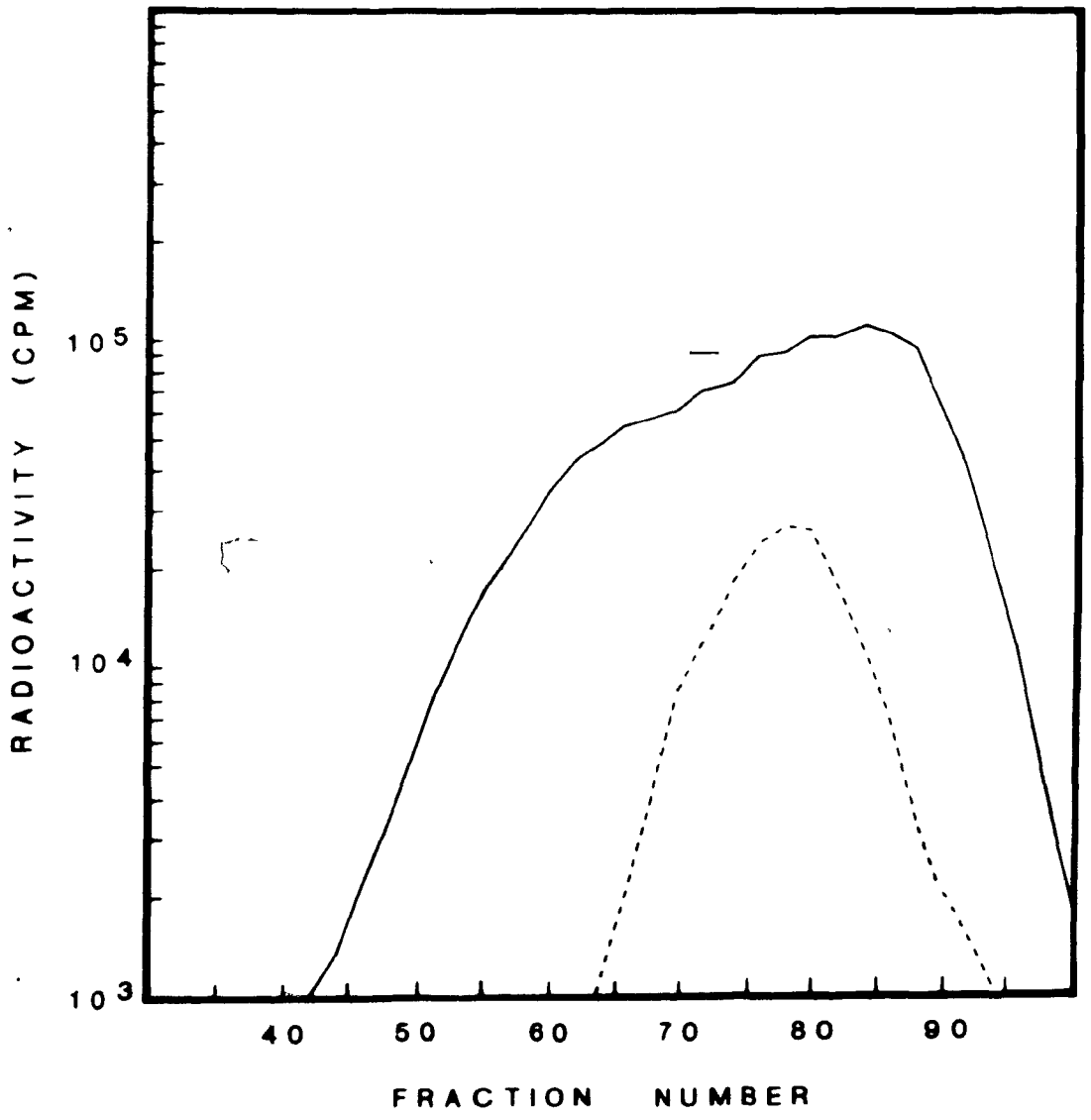
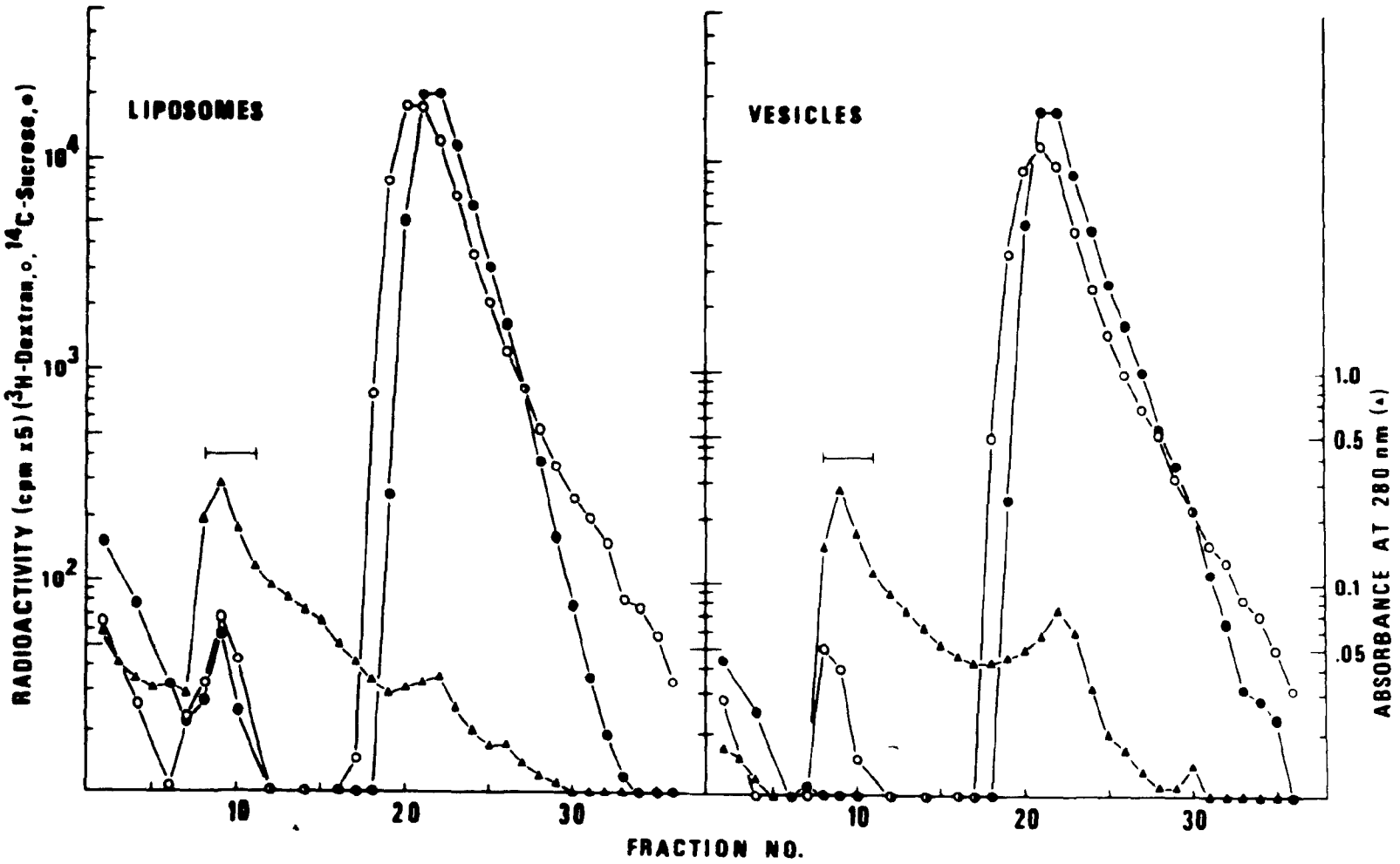
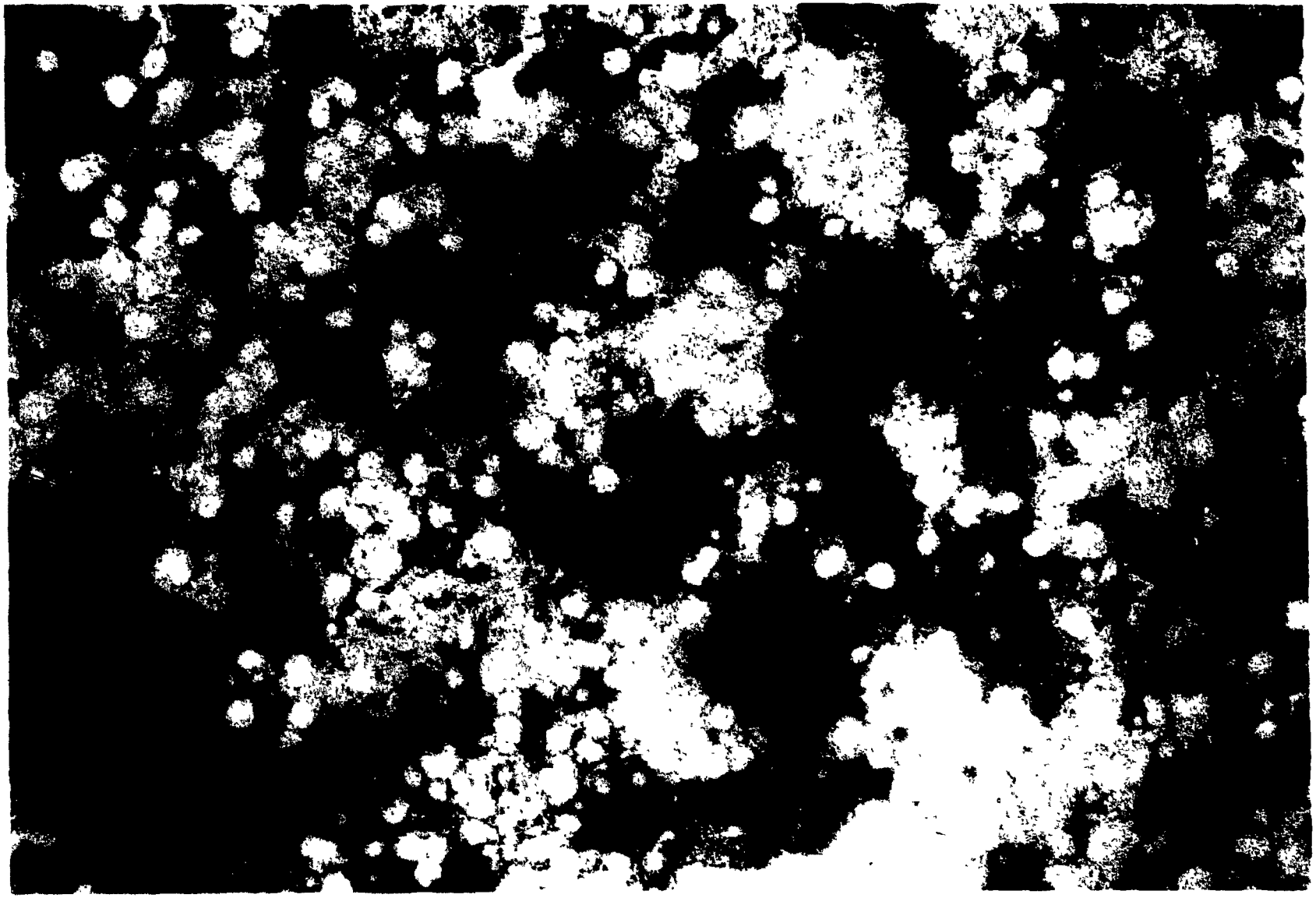

RADIOACTIVITY (CPM)
10 5
10 4
10 3
40
50
60
70
80
90
FRACTION NUMBER

Figure 5. Chromatography of the reconstitution mixture to separate vesicles from untrapped labels. The left panel is the elution profile after the chromatography of a reconstitution mixture containing control liposomes that have no proteins present. The right panel is the elution profile after the chromatography of vesicles with TI material reconstituted into them. These reconstitution mixtures were chromatographed separately on a Sepharose 4B column (1 x 28 cm) and 1 ml fractions were collected. The void volume was 9 ml as determined with dextran blue. The bar (⊢——⊣) indicates the fractions in which the vesicles eluted from the column. The key to the symbols used is: (○) for dextrans with average molecular weights of 9,400 daltons or greater; (●) for sucrose and (▲) for absorbance at 280 nm.

Figure 6. Electron micrograph of reconstituted vesicles of LPS, phospholipids and TI material extracted from H. influenzae type b. These vesicles were eluted from the Sepharose 4B column and negatively stained with 1% phosphotungstic acid. The bar is 1 μm in length.

Figure 7. Determination of the exclusion limit of the outer membrane of H. influenzae type b. The retention of each saccharide is expressed relative to the value for control vesicles. The numbers in the brackets are the total number of experiments performed for each saccharide. Each point is the average value. The saccharides were sucrose (Mr 342), raffinose (Mr 550), stachyose (Mr 666), inulin (Mr 1,400), and dextran fractions (Mr 1,500, 2,500, 3,800, 6,600, and 9,400).


LIPOSOMES
VESICLES
RADIOACTIVITY (cpm x5) (^{3}H-Dextran,○, ^{14}C-Sucrose,●)
10^4
10^3
10^2
ABSORBANCE AT 280 nm (▲)
1.0
0.5
0.1
.05
10
20
30
FRACTION NO.



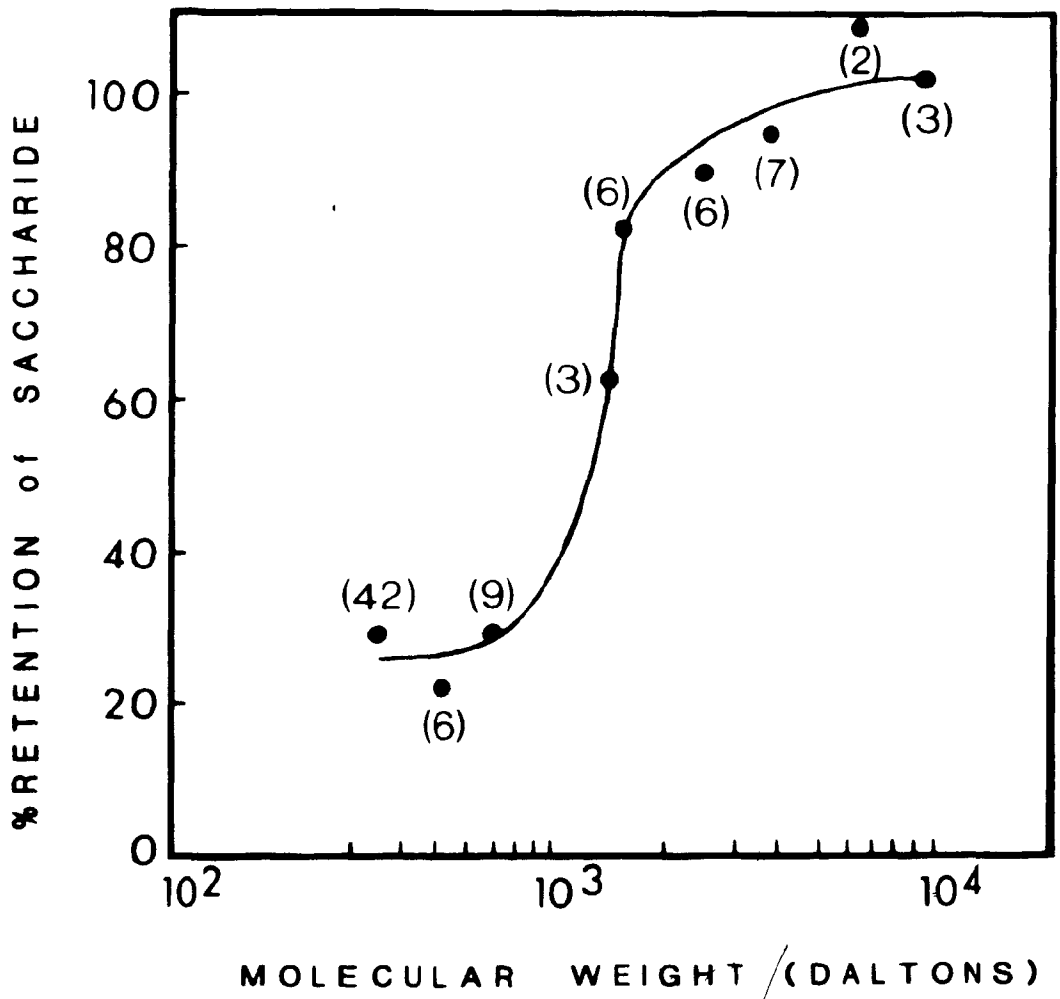

%RETENTION of SACCHARIDE
100
80
60
40
20
0
(2)
(3)
(7)
(6)
(6)
(3)
(42)
(9)
(6)
10^2
10^3
10^4
MOLECULAR WEIGHT (DALTONS)

Table 1. Relative percent retention of saccharides.

Saccharide	Number of experiments	%Retention of saccharide ±standard deviation
Sucrose	42	29 ± 10
Raffinose	6	22 ± 4
Stachyose	9	29 ± 9
1,400 Inulin	3	62 ± 22
1,500 Dextran	6	82 ± 38
2,500 Dextran	6	89 ± 16
3,800 Dextran	7	94 ± 24
6,600 Dextran	2	109 ± 16
9,400 Dextran	3	102 ± 7

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